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Old animal anthrax outbreaks discovered through the analysis of soil

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Summary - Anthrax represents a danger to humans especially when it occurs in areas considered to be free and in atypical seasons and climatic conditions. The underestimation of disease may lead health workers to misdiagnosis and, consequently, to an inappropriate management of affected carcasses with a consequent and inevitable increase in the risk of human infection. The analysis of soil and water samples collected in the Mugello Natural Park (Tuscany - Italy), considered an anthrax free area, showed that *Bacillus anthracis* was isolated from 4 of 6 investigated sites where in the past were registered several deaths of animals. This result was obtained thanks to a new investigation approach including the analysis of some environmental factors that tend to favour the isolation of *B. anthracis* from soil.

Key words: Anthrax, *Bacillus anthracis*, Tuscany, Italy

INTRODUCTION

Bacillus anthracis is known for its virulence deriving from rapid proliferation and dissemination in receptive hosts. However still very little is known about its ability to replicate outside the animal. It was shown that *B. anthracis* can survive as a saprophyte outside the host (Saile and Koehler, 2006) but these data were obtained in experimental conditions and it is not known if this actually occurs in nature. But it is clear that in the external environment, where conditions are less favorable to the survival of vegetative forms, *B. anthracis* spores are among the most resistant forms observed in nature. In the Kruger National Park (Africa) *B. anthracis* spores have been isolated from animal bones estimated to be about 200 years old (Smith *et al.*, 2000). The persistence of spores in the ground is essential but not of sufficient to give rise to new anthrax outbreaks. The studies about the epidemiology highlighted that the outbreaks of anthrax occur mainly during the dry months that follow a prolonged period of rain (Hugh-Jones and Blackburn, 2009). These climatic aspects and the fact that the spores are characterized by a high floating capacity suggest that water plays an important role in the ecology of the bacterium. Rainwater, having washed away the surrounding ground, tends to stagnate in the low

lying parts favoring the concentration of spores. This sequence of events encourages the adhesion and the distribution of spores on soil humus, so the chances of infecting herbivores tend to increase. However time and special natural events are fundamental to create sites of concentrations of spores which can cause new infections in grazing animals (Van Ness, 1971). In conclusion most of the existence of *B. anthracis* is held in the ground as spores until the ideal conditions are created for its reproductive cycle that occurs in a different habitat, primarily domestic and wild ruminants. Nature provides few opportunities to the bacterium for its replicative cycle, and the development of an exceptional pathogenicity is the effective strategy aiming to significantly increase the probability of success against the host's immune mechanisms. Rapid intense multiplication by the vegetative cells take the host to death quickly. Although many of the new generations of bacteria will be neutralized by putrefactive processes, a good part of them survives and spreads into the surrounding soil as spores, ensuring the standard of environmental density of the bacteria that is an essential condition for the continuation of the species. In conclusion, the few cases of anthrax that occur each year are merely the result of a natural ecological balance that seeks through these

extraordinary events simply to promote the maintenance of a bacterial species that otherwise would have been extinguished some time ago (Fasanella *et al.*, 2010).

In Italy where anthrax is still a problem, the disease is sporadic and occurs, with a few exceptions, in the central and southern regions and on the main islands, where it almost exclusively affects animals at pasture (Fasanella *et al.*, 2005). Generally it tends to occur where, in the past, infected animals were buried or leather industry waste was collected. Recently outbreaks were reported following the introduction of contaminated feed (personal data). Despite the fact that thanks to vaccination campaigns the Italian anthrax epidemic situation has improved from the late 1950s onwards, during the last 14 years two main outbreaks involving domestic animals and humans developed in areas inside natural parks. In the summer of 1997 an anthrax outbreak was reported in the Sicilian Madonie Natural Park and it progressed into an epidemic killing cattle, sheep and horses (Di Marco *et al.*, 2005) while during the second part of the summer of 2004, anthrax broke out in the Pollino Natural Park, south west of Basilicata in southern Italy, where in 41 days 81 cattle died, as well as 15 sheep, 9 goats, 11 horses and 8 deer. Two suspected human cases were also registered (Fasanella *et al.*, 2007). This event seems to indicate that *B. anthracis* has found its natural habitat in wild areas like natural parks or natural reserves where the human control is not always efficacious and often the carcasses of dead animals are left untouched by scavengers.

We can define “rural area” the zone located between agricultural and wild areas where generally human activity is limited to the exploitation of pastures. It represents the contact point between the wild and the agricultural world, the habitat where domestic and wild animals share the same space and where the ecology systems tend to influence each other in turn. The proximity to the sources of production of anthrax spores, that are located in the wild area, guarantees the standard level of contamination of soil, favouring the realization of the events that cause the disease in the domestic animals that pasture in this area (personal hypothesis) (Fig 1).

During the end of August and September 2008, in Mugello Natural Park in the region of Tuscany, nine cattle died in ten days. In this last case, since clostridiosis was considered the main cause of death of cattle reared in that area and the preagonic symptoms were suggestive of enterotoxaemia, no laboratory tests were done. When finally, a blood sample of the seventh sudden death which had occurred among cattle at pasture was examined, a fully virulent *B. anthracis* was isolated. This event encouraged investigation in the area to verify the contamination level of *B. anthracis* since the suspect that anthrax had been the cause of the death of the other animals was very high.

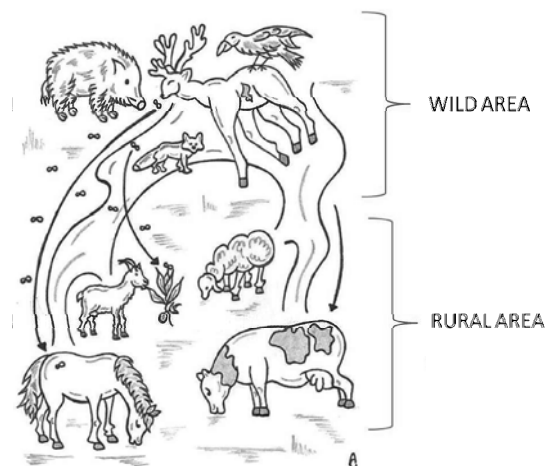


Figure 1 - The “rural area” represents the zone located between agricultural and wild areas where generally human activity is limited to the exploitation of pastures. It represents the contact point between the wild and the agricultural worlds, the habitat where domestic and wild animals share the same space and where the ecology systems tend to influence each other in turn. The proximity to the sources of production of anthrax spores, that are located in the wild area, guarantees the standard level of contamination of soil, favouring the realization of the events that cause the disease in the domestic animals that pasture in this area.

MATERIAL AND METHODS

Collecting samples

Samples of water and soils were collected from pastures associated with the confirmed case and pastures where during the last five years additional deaths were reported with a presumptive diagnosis of enterotoxaemia. In total 6 sites were examined.

a) Soil samples. For this collection were involved the owners because they knew the exact points where had been found the dead animals. For each point five samples of earth of about 50 grams each were collected.

b) Water samples. Several pastures were visited and were investigated only the pastures that presented a low slope because the puddles tend to collect the rain that washes a large surface of the contaminated pasture. For this sampling has been necessary to wait an abundant rain. The choice of puddles has been casual. Then were collected five samples of 50 ml each from selected puddles. The investigation was conducted in autumn and repeated in spring. In both occasion, the sampling was done in the same points.

Isolation

The G.A.B.R.I. (Ground Anthrax Bacillus Refined Isolation) method was used to recover *B. anthracis* organisms from the soil samples. This test, devel-

oped in the laboratories of the Anthrax Reference Institute of Italy in Foggia, is able to culture *B. anthracis* in contaminated soil which contains some 200 spores in 7.5 grams of soil (data not shown) or at a threshold value of 27 spores per gram of soil. Briefly 7.5 grams of soil were added to 22.5 ml washing buffer and shaken for 30 minutes. The solution was then centrifuged at 2.000 rpm for 5 minutes. The supernatant was incubated at 56°C for 20 minutes. After this incubation 3 ml of the supernatant was mixed with 3 ml of Phosphomycin Tryptose Soya Broth (PTSB), and one ml of the supernatant/PTSB was sown onto a plate of Trimethoprim, Sulfamethoxazole, Polymyxine 5% sheep Blood (TSMP) agar. The plates were incubated aerobically at 37°C for 24 h. Suspect colonies on the TSMP plates were identified by colony morphology and Gram staining.

In order to analyze the water a different method was used, 25 ml of water were directly incubated at 56°C for 20 minutes. Then the procedure was the same as previously described.

DNA preparation and PCR

Each *B. anthracis* suspect colony was streaked onto 5% sheep blood agar plates and then incubated at 37°C for 24 hours. After heat inactivation (98°C for 20 min.), microbial DNA was extracted using the DNAeasy Blood and Tissue kits (Qiagen), following the protocol for Gram positive bacteria. Specific PCR assays were used to confirm *B. anthracis*. (Fasanella *et al.*, 2001)

15-loci MLVA and SNR analyses

We utilized 5' fluorescent-labeled oligos, deprotected and desalted, specifically selected for the VNTRs and SNRs used. The 15 specific primer pairs for the MLVA were selected as described by Van Ert *et al.* (Van Ert *et al.*, 2007). The four specific primer pairs for SNR reactions were selected following Garofolo *et al.* (Garofalo *et al.*, 2010). MLVA PCRs were performed in two multiplex reactions in a final volume of 15 µl. The reaction mixture contained: 1×PCR reaction buffer (Qiagen), 1 U of HotStarTaq Plus DNA polymerase (Qiagen); dNTPs (0.2 mM each); 3mM MgCl₂ and appropriate concentrations of each primer (multiplex 1: vntr12 0.37µM, vntr35 0.37 µM, vntr23 0.2 µM, vntr16 0.2 µM, vntr17 0.2µM, vrrB2 0.2µM, vrrC1 0.1 µM, vntr32 0.1 µM; multiplex 2: vntr19 0.2 µM, CG3 0.2 µM, vrrB1 0.2 µM, pX01 0.2 µM, pX02 0.2 µM, vrrA 0.1 µM, vrrC2 0.1); and 1 ng of template DNA. The thermocycling conditions were as follows: 95°C for 3 min; 35 cycles at 94°C for 30 s, at 60°C for 45 s, and at 72°C for 1 min; and finally, 72°C for 5 min.

The SNR PCR was performed in a multiplex reaction in a final volume of 15 µl containing 1×PCR reaction buffer (Qiagen); 1 U of HotStarTaq Plus DNA polymerase (Qiagen); dNTPs (0.2 mM each);

3,5 mM MgCl₂, dNTPs; and appropriate concentrations of forward and reverse primers (HM1 0.2 µM, HM2 0.2 µM, HM6 0.2 µM, HM13 0.1 µM); and 1 ng of template DNA. The thermocycling conditions were as follows: 95°C for 5 min; 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and finally, 72°C for 5 min.

Automated genotype analysis

MLVA PCR products were diluted 1:80 and subjected to capillary electrophoresis on ABI Prism 3130 genetic analyzer (Applied Biosystems) with 0.25 µl of GeneScan 1200 and sized by GeneMapper 4.0 (Applied Biosystems Inc.) Amplified SNR PCR products were diluted 1:80 and subjected to capillary electrophoresis on ABI Prism 3130 genetic analyzer (Applied Biosystems) with 0.25 µl of GeneScan 120 Liz, and sized by GeneMapper 4.0 (Applied Biosystems Inc.). In all the analyses the samples were processed in triplicate to allow the correct sizing of the fragments.

RESULTS

Bacillus anthracis was isolated only from the samples collected in autumn. On 6 examined sites *B. anthracis* has been isolated from 4 of them. In these sites *B. anthracis* was always isolated from soil samples while in only two of them it was isolated also from water coming from puddles. *B. anthracis* was isolated from three sites that were involved in the outbreaks of the summer 2008 while the last one was isolated in a site where a cow was found died two years before. All the isolated strains belonged to the genotype 1 cluster A1a. The SNR showed the presence of two sub genotypes. SGt/Mug 1 was found in the sites involved in the outbreaks of 2008 while SGt/Mug 2 was found in the sites involved in the preview outbreak.

DISCUSSION

Mugello Natural Park represents the typical area where anthrax finds its habitat and shows its presence when the disease occurs in domestic animals. The analysis of soil demonstrates that anthrax has been probably the cause of death of the animals. Several aspects contributed to the success of the investigation:

- Test. The development of a sensitive test, like GABRI test, that permits the isolation of anthrax from soil characterized by low level on contamination.

- Sample. Study of the area to individuate the points where to carry out the water samples. An important factor has been the collaboration with farmers that are essential in the individuation of the points that were found the dead animals.

- Meteorology. It is very useful to pick ground of water samples after abundant rain because rainwater, having washed away the surrounding ground, tends to stagnate in the low lying parts favoring the

concentration of spores.

-Season. We cannot still overlook the importance of the season. Autumn and winter seem to be the periods of the year that permit the individuation of the slope of the examined area much better. In fact in these periods of the year the vegetation is very scarce and it permits the individuation of the puddles that collected water much better. The grass that grows during spring or summer obstructs the observation and consequently may influence the good quality of the sampling. This has been confirmed by the results that evidenced the presence of anthrax only in the samples collected during autumn.

Up to now the programs to prevent the disease are based on the vaccination in domestic animals that live in areas where the disease is manifest. These programs often last from five to ten years. It is frequent for new anthrax outbreaks to develop after a few years following the last vaccination (personal observation). The described experience could be useful to think of a new form for the prevention of anthrax. The analysis of environmental contamination level of anthrax based on a good test to isolate anthrax and a efficacious sampling could be the new frontier in the control of the disease. The investigation of a suspected area can show the contamination level of the soil, indicate the risk of new outbreaks and suggest the introduction of vaccination programs involving receptive domestic animals. Besides, further advantages consist in a greater knowledge for veterinarians and physicians of the disease so as to reduce the misdiagnosed suspect cases and, consequently, the subsequent inappropriate management of infected carcasses that leads to an inevitable increase in the risk of infection in humans and other livestock (Kreidl *et al.*, 2006). Finally the isolation of new strains and consequently the knowledge of the genotypes of *Bacillus anthracis* circulating is essential to understand the natural or deliberate release origin of the outbreak.

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Public health response after the “Pakistan Flood 2010”: a case study.

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Summary - The Authors describe an integrated complex of Public Health actions implemented, with the economic support of the International Community, in some districts of the North-West Frontier Province (NWFP) of the Islamic Republic of Pakistan, as a result of the monsoon flood in July 2010. Some health facilities are rehabilitated and empowered to ensure basic health and social services to affected people. The actions are consistent with the principles of *Linking relief, rehabilitation and development* (LRRD).

Key words: KPK, Malakand, Flood, Disaster, LRRD.

Over the course of the 2010 monsoon season, Pakistan experienced the worst floods in its history. Heavy rainfall, flash floods and riverine floods combined to create a moving body of water equal in dimension to the land mass of the United Kingdom. The floods have affected 84 districts out of a total of 121 districts in Pakistan, and more than 20 million people - one-tenth of Pakistan's population - devastating villages from the Himalayas to the Arabian Sea. More than 1,700 men, women and children have lost their lives, and at least 1.8 million homes have been damaged or destroyed.
(UNOCHA, 2010)

INTRODUCTION

This communication deals with the description of some emergency/development Public Health actions implemented, with the economic support of the International Community, in some districts of the North-West Frontier Province (NWFP) of the Islamic Republic of Pakistan, as a result of the monsoon flood in July 2010.

Malakand Division* is the mostly northern area of Khyber Pukhton Khwa (KPK) and located close to the afghan border which is stretched from west to north trough out the division boundary.

It consists on Malakand, Lower Dir, Upper Dir, Swat, Shangla, Buner and Chitral districts (Government of Islamic Republic of Pakistan, Ministry of Information & Broadcasting). It has a lengthy border with Afghanistan stretched from west

to north, the people across the border shared same culture, religion, language and social norms, even the tribes are divided in both the countries in Pakistan and Afghanistan. Therefore a bulk of Afghan refugees has been living here since the emergence of crisis in Afghanistan.

Malakand division mostly consists on mountainous ranges and very limited plan area exists here. The majority of the population belongs to rural area largely depends on agriculture, forestry, cattle rearing and other daily manual work. The pace of development and socialization is very slow due to low investment in human capital, the young generation are working in Punjab, Karachi, Middle East or other urban part of KPK on daily wages basis due to non availability of job opportunities in their locality. Although their soil is fertile and capable of producing much more than the present but they need skill and finance for development, which is again bound to education and skill Social and economic infrastructures are very limited due to the scattered population, lack of resources and inaccessibility. So, overall literacy ratios particularly in women are very low. Now the people are migrating to the available too small rural urban areas to avail better life facilities of health, education, services and income generation. Hence, the urban areas are becoming congested day by day and the existed rural urban composition has been deteriorated.

Malakand division as a whole and Lower Dir espe-

(*) In August 2000 the “Divisions” were abolished as part of a plan to restructure local government, followed by elections in 2001. Many of the functions previously handled by the provinces have been transferred to the districts and tehsils. In 2008, the new civilian government restored the former tier of “Divisions”.

cially consist of mountainous ranges due to which small plane area is very limited. Lower Dir is one of the backward districts of Malakand Division with low profile educational and health system.

Health facilities are inadequate and lacking and general utilities are mostly not functioning. District has no technical or skilled education institute or vocational training centre. Due to very low literacy rate and lack of skills the local youth manage to go outside of district or abroad as ordinary labours. The lack of basic socialization phase is huge barrier for development. While on the other hand low investment in the human capital is affecting the chances of benefits from the available resources of the area. Lower Dir is a rural and mountainous area of the northern Pakistani province Khyber Pukhton Khwa with considerable lack of infrastructures and low profile health and education system which is aggravating the rate of migration to urban areas.

Moreover, due to its location close to the country borders, many afghan refugees are also settled here in both the rural and urban areas where migration is taking place intensively. These areas do not have enough capacity to accommodate this huge number of population. This is creating intolerance between local communities and immigrants.

The situation is worsening day by day with the intensity of displacement and can lead to very serious law and order situation. The situation is leading to marginalise the services of Government institutions. The border is located geographically on such position that divides the people of same culture, religion, values, customs, social norms and language. Even in most of the area border is dividing the tribe of same families. Because of all these similarities people across the border have strong social and blood ties. Therefore a considerable number of afghan refugees is living in this area located near border.

The local community is mainly dependant of agriculture, forestry and live stock. The educational and

development. Due to all the above mentioned barriers literacy level is very low especially among women. Economic prosperity is also very unlucky here and that is the major reason why rural population is frequently migrating to urban areas in order to have access to better educational, health and economic facilities. The urban areas are heavily congested and have no more space for new comers. This process is a huge problem in the rural urban composition.

The area has a high rate of contagious diseases due to non hygienic environment, no sterilized surgical, gynaecology and obstetrics equipments and untrained Lady Health Visitors (LHVs) and Traditional Birth Attendant (TBAs) working for Mother and Child health care (MHC). The main aim of the proposed facilities in the target health units in either form will have effects on the Maternal and neonatal mortality rate, prevention of contagious diseases and mother's future infertility. This will also generate a great impact on the economy and livelihood and quality of life. Since the unavailability of the above mentioned facilities is now compelling the community to travel to urban areas which obviously affects the socio economical structure.

MATERIALS AND METHODS

Specific Information on the Population Planning Group

The rural urban areas of the Malakand Division are population planning group having some of the basic infrastructures, livelihood resources and facilities for quality of life. But the available educational and health institutions are deficient in every aspect to serve the community effectively. The potable drinking water and sanitation system in health units are not enough to meet the needs of the overcrowded urban areas.

<i>Demographic Data by Population Planning Group (Current Situation, 2010)</i>						
Age range	Male		Female		Total	
	in numbers	in %	in numbers	in %	in numbers	in %
0-4	13371	16%	11510	15%	24881	15%
5-17	27998	33%	25999	32%	53997	33%
18-59	31178	37%	26457	33%	57635	35%
60 and >	11486	14%	16402	20%	27888	17%
Total:	84033	100%	80368	100%	164401	100%
Major locations:		6 target Union Councils: Chukdara, Badwan, Ouch, Khadag Zai, Talash and Kodigram are located in the Lower Dir District of Malakand Division				

health system is very weak and the inadequate infrastructures and limited accessibility of these rural areas are strong barriers to the social and economic

Actions implemented

Human physical and mental health promotion always play an important role in the development of

a civil society having prosperous, peaceful and harmonious environment around. The provision of all health facilities and promotion services leads to a society in which skilled and efficient members can positively use the available natural resources with the available financial resources. By profiting of their skills and efforts these members also work on the social building of the society which generate peace, harmony, prosperity, tolerance, coexistence and task orientation to the society. Therefore for ideal social structure of human capital is as important as other sectors like economy, empowerment and social integration.

Recent flood that affected the area have seriously worsened the population living conditions, weakening the existing social and sanitary infrastructures as well as fundamental sectors of the local economy such as agriculture. As a result, the current situation has become even more dramatic, making the need for health (medical supplies) and educational services, water supply and sanitation (WATSAN)

neonatal mortality rate is alarming in these areas. Unfortunately Malakand division faced such horrible and devastating flood on 28th and 29th July 2010 generating a greater need of economic and reconstruction efforts. In addition existing infrastructures have been affected not only by the flood but also by aerial bombings.

The present document will address the issues of 6 UCs which were affected by migration, war and recent devastated flood.

Health Units

The following 4 BHUs and 1 RHC was selected through need assessment for constructing model labour rooms and other facilities:

Basic Health Unit of **Chukdara**

Basic Health Unit of **Ouch**

Basic Health Unit of **Khadag Zai**

Basic Health Unit of **Kodi Gram**

Rural Health Centre of **Talash**

	Name of BHU/RHC	Labour room	Reconstruction	Essential Medical supply	Drinking Water	Latrines/ WATSAN	Current status
1	Chukdara	✓	✓	✓	✓	✓	All of the health units are lacking Gynaecology & Obstetrics facilities affecting Maternal and Child Health Care. No availability of fresh drinking water. WATSAN basic infrastructures in these institutions were found during the need assessment survey. The community greatly deserves Gynaecology & Obstetrics facilities
2	Ouch	✓	✓	✓	✓	✓	
3	Khadag Zai	✓	✓	✓	✓	✓	
4	Kodi Gram	✓	✓	✓	✓	✓	
5	Talash	✓	✓	✓	✓	✓	

regarding drinkable water, wells, latrines etc and communication services even more urgent and crucial for the assistance to the local community.

It was conducted a rapid needs base assessment survey of 6 Union Councils (UCs) and found that majority of them are lacking safe water, sanitation,

The following 4 dispensaries will be provided drinking water, latrines and medical kits:

Government Civil Dispensary **Badwan**

Government Civil Dispensary **Khadag Zai**

Government Civil Dispensary **Kodi Gram**

	Name of Dispensary	Reconstruction	Essential Medical Kit/supp	Drinking Water	Latrines/ WATSAN	Current status
1	Badwan	✓	✓	✓	✓	All of these institutions lacking fresh drinking water, sanitation, water closets, health and hygiene facilities and basic infrastructures.
2	Khadagzai	✓	✓	✓	✓	
3	Kodi Gram	✓	✓	✓	✓	

water closets, furniture and lot of other basic required necessities. Basic Health Units (BHUs) and Rural Health Centres (RHCs) have no Gynaecology and Obstetrics facilities due to which maternal and

The project aimed to implement the following major activities in 4 BHUs, 1 RHC and 3 Dispensaries:

- Construction of Water closets in all 8 target health units

- Water and sanitation facilities in all 8 target health units
- Construction of model Labour Rooms in 4 BHUs and 1 RHC
- Procurement and proper allocation of Medical Supplies for target health units
- Procurement and distribution of Kits for Lady Health Visitors (LHV) and Traditional Birth Attendants (TBAs)
- Procurement and proper allocation of Medical tools and other required necessary Equipments
- Other essential furniture for the Health Units
- Training for LHVs and TBAs for better health care of Mother and Child

Thanks to the proposed intervention 60.047 individuals (direct beneficiaries) will directly benefit from the provided facilities while more than 180.143 individuals (general beneficiaries) will also benefit from the availability of improved and increased health and sanitation services in the target areas.

The project will also bring the community and the stakeholders forward to strengthen the Community Based Organisations (CBOs), BHUs. These working groups will ensure the sustainability, authenticity, transparency, monitoring and security of the ongoing project activities. The project performed activities, future planning and ongoing activities progress will be shared and reported periodically with the donor institution, relevant stakeholders and other concerned authorities.

Implementation Arrangements

Lower Dir is one of the backward districts of Malakand division where the development process is very slow and there is an urgent need for strengthening the institutions. The rapid growth of population, the settlement of afghan refugees and urbanization is worsening the situation. In addition, the previous phase of militancy and ultra conservatism and operations by state against such activities has affected the pace of institutional and social development. The institutional and social structure was formerly very poor but with this extra stress even the former structure has collapsed.

Due that collapse the institutions are unable to provide quality health care and education to the local community. The former structure was also lacking basic facilities like safe water, sanitation, electricity, equipments and furniture which were needed. After the stated plights the structure collapse and community have no support at all. In these circumstances local community feel that Government is not capable to overcome the prevailing situation. Due to the presence of the afghan refugees the local community social cohesion is also at risk since people consider refugees as an additional burden on their poor social and economic structure. Severe Law and order problems are very luckily to occur due to this intolerance.

The proposed project is an effort for the facilitation

of health care system of the target area to minimise the reservation and unsecure feelings of local community which will obviously lead to harmonious and prosperous co-existence in the target area. The project will facilitate the collapsed system in the provision of safe drinking water, the construction of water closets, of labour rooms etc in target area 4 BHUs, 1 RHC and 3 Dispensaries. The impact of the rehabilitation of the non functioning structures of the target area will help building trust of local community and will change their attitude about refugees. The resulting cohesion of host and guest refugees communities in the target areas will prove a valid contribution to the achievement of “*Greater social Cohesion through community development*” and “*Restoration of social services and infrastructure*” outcomes.

The concept of this intervention is the result of the baseline survey in the area and the gathering of needs information about these institutions gaps in the system. According to the survey the target health care institutions are lacking basic facilities like drinking water, water closets, sanitation system and labour rooms for gynaecology and obstetrics facilities which are necessary for Maternal & Child Health Policy (MCH). The provision of all these facilities will strengthen these institutions, enhance the quality of life and improve health and hygiene conditions of the target community which will further contribute to the outcome “*Restoration of social services and infrastructure*”.

The Project will provide facilities in the mentioned 8 target health units according to proposed project as these areas needs these facilities to be established. The main focus will be on the provision of water and sanitation facilities, the construction of water closets, maintenance and rehabilitation of the basic infrastructure, Water Sanitation and Hygiene (WASH) facilities and health and hygiene promotion education in the target health units. The construction of 5 model labour rooms with essential medical equipments, construction of water closets in 8 health units and provision of fresh drinking water in 8 health units is intended. Specialised trainings on MCH and gynaecology and obstetrics will be given to LHVs of the target health units. In account of WATSAN facilities health and hygiene will be the integral part as well.

The activities will be accomplished by the active and effective participation of CBOs, BHU and concerned Government of Pakistan (GOP) departments to establish cooperation between public and private bodies. Liaison with community and between community and HUs will help to assess the needs of institutions and will also monitor the ongoing activities as partner organisation. Liaison GOP concern departments will also lead to effective implementation of project goals and objectives.

The proposed project will be self sustainable due to common interest of the community, institutions and

committees. All the developed and provided facilities will be handed over to concern GOP departments in a legal way to ensure their future operational status. All the activities will be carried out with the prior consultation and consensus of GOP concerned officials by which project will be able to get surety of optimum utilization and durability of the provided facilities and services.

RESULTS

Related Inputs and Projects

The Activities were implemented in order to achieve the project goals and objectives as follows:

1. Recruitment of human Resources and setting up of logistic and technical organisation for the implementation of the project activities.
2. Liaisons with stakeholders and concerned departments of the target areas for effective coordination of action.
3. Establishment of cooperation with Health Committees (HCs) of the selected Health units within the target CBO for mobilizing and organizing them effectively.
4. Provision of drinking water from available natural resources, construction and rehabilitation of water closets and sanitation system in the aforementioned health units.
5. Construction of a new model labour rooms in 4 BHUs and 1 RHC.
6. Recovery of the damaged and inadequate infrastructures in 4 BHUs, 1 RHC and 3 dispensaries.
7. Procurement of required necessary medical and other related equipments for target Health Units.
8. Procurement and proper allocation of medical supplies and distribution of delivery kits to LHV's & TBAs of target area Health Units.
9. Specialized trainings on health & hygiene for LHV's, TBAs.
10. Production of visibility material for the project results achieved and health & hygiene promotion.
11. Monitoring and Evaluation of ongoing activities and project goals and objectives.
12. Intermediate and Final progress reports of the activities and financial statements.

Project Implementation Strategy

The implementation strategy was intended to be adopted as follows:

Water Supply and sanitation in Health units

The implementation Team during survey identified 8 health units of the area consist of 4 BHUs, 1 RHC and 3 Dispensaries with the help of local community and concerned officials. Small tube wells (hand pumps) along with water closets and sanitation system will be provided to target BHUs, RHCs and Dispensaries of the target areas.

The local community and concerned Departments

will also provide support regarding improvement works, construction, Skilled labours, availability of fresh drinking water resources and security of the Project's staff, material and equipments at projects sites.

Construction of a model labour room

The project constructed 5 model labour rooms in 4 BHUs and 1 RHC of the target area identified in need assessment survey in the areas of Chakdara, Badwan, Ouch, Khadagzai, Talash and Kodi Gram to provide quality health care at their door step.

Reconstruction and Maintenance of basic infrastructure

The Project also proposes to reconstruct basic infrastructure like Medical Office (MO), Outpatient Department (OPD), LHV in 8 selected health units identified in need assessment survey in the areas of Chakdara, Badwan, Ouch, Khadagzai, Talash and Kodi Gram in order to provide accessible, increased and quality health care to the local community.

Provision of Medical Supplies

The Project intended to provide essential medical supply of required equipments for 4 BHUs and 1 RHC while first Aid and Medical tools and medical supplies like sphygmomanometer, oxygen cylinder, bed and couches, Drip stand, DNC (“dilation and curettage”) sets etc. for 3 dispensaries. Provision of such facilities and supplies will play an important role in the improvement of quality of life and health care services.

Provision of LHV Training

A specialized training course of 3 days on Mother & Child Health care and gynaecology and obstetrics will be given to LHV's of the target health units 4 BHUs, 1 RHC and 3 dispensaries to handle the cases effectively and serve the community efficiently. The action will play an important role in the control of maternal and infant mortality rate.

Provision of Health & Hygiene training

Through health hygiene education and awareness sessions target beneficiaries will be sensitized for proper Health and hygiene Practices. HCs of the target area will receive appropriate training. Women will be the focus group for health and hygiene education because through this target group greater results can be expected.

Community Participation

As fundamental goal the Project will arrange special awareness and education sessions for the community to ensure their motivation and their help in all intervention of rehabilitation and other matters in the target areas. HCs will be strengthened and if needed new HCs will be formed to encourage community participation and mobilization. These sessions will include orientation of health committee on project

objectives, community development and their active participation on current and future Health interventions. The local community will also be involved in construction of model labour rooms and distribution of medical tools in health units.

DISCUSSION

The overall response (Asian Development Bank, The World Bank, One-United Nations Development Group, Global Facility for Disaster Reduction and Recovery, 2010; Government of Khyber Pakhtunkhwa, 2010; NDMA, 2011; Pakistan Council of Scientific and Industrial Research - Laboratories Complex, 2010; The World Bank, 2010a; The World Bank, 2010b) of local authorities and the international community has made it possible to limit the damage caused the disaster and to promote a progressive rehabilitation of basic infrastructures. The complex of actions described is recognized in the logic that relates to methods of LRRD (Buchanan-Smith and Maxwell, 1994; Commission of the European Communities, 2001). Linking relief, rehabilitation and development. The basic justification for LRRD is simple, sensible and still valid: disasters are costly in both human life and resources, they disrupt economic and social development and lead to separate bureaucratic structures and procedures which do not systematically take into account long term development issues. Development policy, at the same time, is not enough prepared to cope with drought, conflicts and the need to protect vulnerable households by helping them to develop coping strategies. LRRD expresses the view that if relief and development can be appropriately linked, these deficiencies can be reduced. Better development can reduce the need for emergency relief, better relief can contribute to development, and better rehabilitation can ease the transition between the two.

Therefore in the context of disaster management, social protection strategies should help the affected populations, and in particular poor and very poor households, cope with the immediate after-effects of the disaster and in the medium- to longer-term build systems that mitigate the effects of future shocks.

It is particularly important to try to limit family and community risk-taking through informal arrangements (negative coping strategies) that are likely exhaust their asset base and have damaging social and economic consequences, especially in terms of human development. Counterproductive strategies with lifecycle effects can be particularly damaging: eating less, women eating less than men, reducing spending on health care, withdrawing children from school, and so on.

The actual formal social protection strategies that are applied for disasters or emergencies may be similar to those that address chronic poverty and structural unemployment. They would be designed in a way that recognizes that, although the problems are similar - poverty and unemployment, the cause

and duration of the contingency may be different. As a result, social protection programs that focus on immediate relief from the effects of the disaster may assign higher weight to redistributive goals as opposed to programs that address chronic poverty and unemployment, and focus on lifecycle contingencies.

Finally this report highlights and testifies a strong need that international aid - in emergency situations such as that caused by "Flood 2010" in Pakistan - recognizing few concepts that Authors take as "*lessons learnt*":

- greater investment needs to be made in national and local capacity development for reasons that are both appropriate and practical: appropriate because not to do so would be to undermine the responsibilities of sovereign states; practical because the international community will not have the capacity to assist in light of the growing number of crises around the world;
- one of the persistent problems that still haunts humanitarian response is poor coordination, in truth, those who could take significant steps to deal with this continue to fail to do so;
- too often, needs assessments, while technically sound, fail to take into consideration cultural, gender and social concerns of the affected population; they are not undertaken with sufficient reference to the individuals who have clear views on priority needs, i.e., *those in need*.

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International trade and public health in Africa: the example of Swaziland

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Summary - International trade is an important source of potential growth for African economies, through the exploitation of export opportunities both at regional and international level. The WTO SPS Agreement allows for better harmonization of measures intended to protect animal and plant health and food safety. The European Commission, and other donors, are supporting Swaziland's efforts for improving its compliance with the Agreement and enhancing the level of protection guaranteed to all its population. The results of the study show that Swaziland is facing some problems common to most of the Region, affecting the ability to participate in the global market. Nevertheless, its commitment to comply with international rules, if appropriately supported, could constitute a positive example also for other countries in the Region.

Key words: International trade, public health, SPS Agreement, Africa

International trade in Africa: an overview

International trade is an important source of potential growth for African economies, through the exploitation of export opportunities both at regional and international level. Mutenyo (Mutenyo, 2011) reports that the process of market liberalization in the 1980s, increased Africa's export from 22% of gross domestic product (GDP) in 1983 to an average of 32% in the past twenty years. Nevertheless, the share of Africa's in respect of the total world export decreased, representing about 2.4% in 2009, 30% of which is attributed to South Africa. The same author lists the most important problems faced by Africa: lack of diversification of product intended for export, with a predominance of primary commodities, more vulnerable to changes in world prices; lack of diversification of importing markets, mostly represented by the European Union and the United States. The author recommends that Africa countries should increase intra-regional trade through the expansion of regional trade blocs and the elimination of regional trade barriers. Despite the possible difficulties and constraints, Page (Page, 2011) suggests that well-functioning regional institutions need to be prioritized, but notes that aid agencies continue to prefer dealing with national rather than regional authorities in Africa.

World Trade Organization and Public Health

Trade agreements have implications for health and the health sector, whether they are multilateral, bilateral, linked to the World Trade Organization (WTO), or linked to regional trading systems, such as the Southern African Customs Union (SACU) and the Southern African Development Community (SADC) in Africa. All SACU and/or SADC member states are WTO members, except Seychelles.

According to the World Health Organization (WHO) (WHO, 2005a), four of the multilateral trade agreements of WTO may affect public health: the General Agreement on Trade in Services (GATS), the Agreement on Application of Sanitary and Phytosanitary Measures (SPS), the Agreement on Technical Barriers to Trade (TBT), and the Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS).

As reported in the Statement of WHO on International Trade and Health (WHO, 2005b), the liberalization of trade promoted by WTO can affect health in multiple ways. Sometimes the impact is direct, as when a disease, for instance a zoonoses, crosses border together with the importation of animals or animal products. Other times the effects are more indirect, for example, in the field of food safety, Codex Alimentarius standards, guidelines

and recommendations can affect the possibility to export and trade foodstuffs.

According to Loewenson (Loewenson *et al.*, 2007), the health problems in east and southern Africa arise largely from social and economic poverty and inequality. Trade policies can contribute to overcoming diseases arising from food insecurity, lack of access to basic safe water, sanitation, energy, transport and shelter and poverty. Trade rules and agreements, including those at WTO, should not, in principle, conflict with actions to meet public health. In a conflict between trade agreements and public health obligations, the state must respect and honor public health obligations. To do this, however, governments, parliaments and civil society need to be informed of and able to scrutinize proposed trade measures and agreements, to identify and deal with any possible areas of conflict with a country's public health obligations or with regional and international health protocols and conventions. WHO suggests to identify a focal point for trade in health-related services and to establish contacts and systematic interactions with trade and other key ministries and with representatives from private industry and civil society.

The SPS Agreement in Southern Africa

The SPS Agreement, entered into force with the establishment of the WTO on 1 January 1995, concerns the application of food safety and animal and plant health regulations. The WTO in its Trade policy review of the SACU five member states – Botswana, Lesotho, Namibia, South Africa, and Swaziland – found that these countries continue to show substantial differences in levels of economic development (WTO, 2009a). Botswana and South Africa are classified as upper middle-income countries, while Namibia and Swaziland are considered lower middle-income countries, and Lesotho is a least developed country. Nonetheless, SACU countries face common challenges, notably unemployment, income inequality, poverty, HIV/AIDS and other health issues.

The new SACU Agreement, signed in 2002 and entered into force in 2004, provides for further harmonization in a number of areas, including SPS measures. According to Articles 28 and 33 of the 2002 SACU Agreement, there should be harmonization of technical regulations and product standards, and sanitary and phytosanitary measures. However, SACU countries do not have a common policy on these issues. Lesotho and Namibia use South African standards, while Botswana established its own Bureau of Standards in 1995, and Swaziland enacted its Standards and Quality Act in 2001. Article 30 of the 2002 SACU Agreement indicates that members reserve the right to apply SPS measures in accordance with their national SPS laws and international standards, but should consult with each other to ensure the free flow of goods without

endangering animal, and plant health. Members may impose import restrictions to safeguard human, animal and plant health. Goods traded within the customs union are free of custom duties but must be declared at each border post for checking compliance with sanitary and phytosanitary requirements of each SACU member state.

The SADC Treaty was signed in 1992 with the objective of creating a development community that would achieve economic integration in the whole of Southern Africa, including through increased intra-regional trade. It provides for several protocols in specific areas, including trade and health. Fifteen countries, namely Angola, Botswana, Democratic Republic of Congo, Lesotho, Madagascar, Malawi, Mauritius, Mozambique, Namibia, Seychelles, South Africa, Swaziland, United Republic of Tanzania, Zambia and Zimbabwe are members of SADC, that attained the Free Trade Area Status in January 2008 and whose main objective is to facilitate the free flow of goods among its member states. The SPS Annex to the SADC Protocol on Trade constitutes the framework for regional cooperation in SPS matters in SADC against the background of each country's obligations with respect to the WTO SPS Agreement.

International Trade and Public Health in Swaziland

Swaziland is a small, landlocked country, challenged by surplus labor force, high poverty incidence, and food insecurity. According to the last available data (WHO, 2009) the percentage of people living below the poverty line has increased over time, rising from 66% in 1995 to 69% in 2001. Unemployment also depicts an upward trend increasing from 22% in 1995 to 29% in 2002. This has resulted in a large part of the population being dependant on food aid. The country is experiencing an increasing trend of conditions related to nutritional deficiencies, such as interrelated effects of HIV/AIDS, high poverty levels and recurrent droughts. Some 29% of children under five years of age are stunted, an indication of high levels of chronic malnutrition. According to data available for the period 2005-2007, the percentage of population below the minimum level of dietary energy consumption is around 18%. Malnutrition is especially dangerous in women of childbearing age, where is associated with many risk factors for maternal and perinatal mortality. The HIV/AIDS epidemic, which affects about one quarter of the population, continues to pose a major threat to the Swazi nation and its impact is already felt in all sectors. Tuberculosis is the leading cause of morbidity and mortality among adults in Swaziland, the country with the highest TB notification rates in the world. The national health budget increased over the last years, reaching 10.2% in 2009, but is still far from the Abuja Declaration commitment of 15%. Of

the total health budget, only 16.6% is allocated to public health services.

As reported in the WHO Country Cooperation Strategy (WHO, 2009), all health-related activities fall under the Constitution of the Kingdom of Swaziland and should be aligned with national development policies and strategic frameworks, such as the Poverty Reduction Strategy Action Plan and the Swaziland Programme on Economic Empowerment and Development.

As stated in the WTO Trade Policy Review (WTO, 2009b) and shown in table 1, Swaziland is a net food importing developing economy, where trade

market access. Its success, nevertheless, depends mostly on continued technical assistance from the WTO and the rest of the international community.

The mission of the Ministry of Agriculture (MOA) is “to transform Swaziland’s agricultural production system from its prevailing subsistence mode to more commercially oriented production system through commercialisation and the diversification of small and medium holder...(omissis)...within the next four to five years”.

Among the strategies pursued by the MOA, the following two are particularly related with international trade:

Table 1 - International trade in Swaziland, 2009

<i>MERCHANDISE TRADE</i>	<i>Value</i>		
Merchandise exports, f.o.b. (million US\$)	1 450		
Merchandise imports, c.i.f. (million US\$)	1 600		
<i>Share in world total exports</i>	0.01	<i>Share in world total imports</i>	0.01
<i>Breakdown in economy's total exports</i>		<i>Breakdown in economy's total imports</i>	
<i>By main commodity group (ITS)</i>		<i>By main commodity group (ITS)</i>	
Agricultural products	28.3	Agricultural products	21.
Fuels and mining products	1.8	Fuels and mining products	15.2
Manufactures	69.7	Manufactures	62.2
<i>By main destination</i>		<i>By main origin</i>	
1. South Africa	79.8	1. South Africa	81.4
2. European Union (27)	13.9	2. China	4.0
3. Namibia	2.8	3. European Union (27)	2.7
4. Mozambique	1.8	4. Japan	2.4
5. United Arab Emirates	0.5	5. Chinese Taipei	1.9

(Source: WTO)

competitiveness can only be achieved with continued assistance and cooperation from regional and international trading partners. In particular, food security and agricultural productivity are promoted through the diversification and commercialization of activities, while ensuring community participation and sustainable development of its natural resources. Manufacturing remains largely based on sugar and related products (confectionery and soft drinks), which benefit from preferential access to the EC and the United States.

The trade environment is subject to minimum restrictions on imports in Swaziland and import control are carried out on a limited number of goods for social and environment, health, sanitary and phytosanitary and security concerns. The import permits are issued by the Ministry of Finance in consultation with relevant ministries such as Agriculture, Health and Commerce, Industry and Trade. According to WTO (WTO, 2009b) Swaziland’s Government is very committed to address the challenges linked with international trade and socio-economic problems and to overcome its inability to maximise the benefits of existing

✓ MEDIUM TERM: pursue development of national minimum standards and international standards (by commodity) to ensure access to national and international markets.

✓ LONG TERM: venture into sustainable commodity-based agricultural production and processing with a view to directly access lucrative international markets via the Sikhuphe airport.

MATERIALS AND METHODS

The Competitiveness and Trade Support Programme for Swaziland, financed by the European Union, has the overall objective, to contribute to GDP growth and poverty alleviation and to increase pro-poor economic integration and participation by Swaziland in the international trading system through the provision of technical support to the Government and the private sector for export development.

The programme includes two main components. The first component is related to trade policy formulation and implementation, as well as trade negotiations. The second component provide assistance for market development and product development. It includes, among others, technical assistance for

standards setting, and risk management.

Strengthen of SPS compliance is considered one of the priority to enhance international trade in Swaziland. A complete assessment of Swaziland's compliance with the SPS rules was performed as a component of the European Union programme, using the approach described below.

1. Collection of relevant legislation and documentation.
2. Meetings and discussions with relevant partners (MOA - Department of Veterinary and Livestock Services, Department of Agriculture and Extension and Malkerns Research Station¹; Ministry of Health; Customs and Excise Department²; Swaziland Standards Authority (SWASA) to assess how SPS provisions are implemented, shortcomings and perspectives.
3. Meetings with major stakeholders to be acquainted of their knowledge, participation and vision on SPS Agreement (Dairy Board; Namboard; Cotton Board; Swaziland National Agricultural Union; Swaziland Meat Industries; Eswatini kitchen; etc.).
4. Visits to Border Inspection Posts to assess the control and inspection procedures applied on imported animals, animal products, plants and plants products, including selected products subject to special provisions.

RESULTS

Results are presented examining the most relevant article and annexes of the SPS Agreement.

Article 3. Harmonisation: *“To harmonise sanitary and phytosanitary measures on as wide a basis as possible, members shall base their sanitary or phytosanitary measures on international standards, guidelines or recommendations (omissis)”*.

The SPS Agreement explicitly recognizes the right of governments to take measures to protect human, animal and plant health, as long as these are based on science, are necessary for the protection of health, and do not unjustifiably discriminate among foreign sources of supply.

Likewise, governments will continue to determine the food safety levels and animal and plant health protection in their countries.

The SPS Agreement does, however, encourage governments to “harmonize” or base their national measures on the international standards, guidelines and recommendations developed by the three Standard-Setting Bodies recognised by the SPS Agreement: World Organization for Animal Health (OIE), Codex Alimentarius Commission (CAC), International Plant Protection Convention (IPPC). Swaziland is a Member of WTO as well as of all these Organizations.

According to SADC report, most Member States have some legislation in place, but most outdated and/or under review. These policies and legislation are usually difficult to obtain by importers and exporters, unless they are able to contact the relevant government official who can provide the information.

According to OIE, there is an urgent need to review, complement and harmonise the existing veterinary legislation, in consultation with relevant representatives of stakeholders and in accordance with international regulations.

The most relevant national legislation under SPS Agreement is reported in table 2.

In addition to the above mentioned legal provisions, during the Senior Official Meeting held in Johannesburg on 14-15 April 2011, SADC adopted Regional Guidelines on:

- ✓ *Management of SPS matters in SADC and SADC Member States* - These guidelines will ensure that SPS management will not differ significantly from one Member State to another to the extent of impeding movement of food within the region or internationally. In addition, they will ensure that all the institutions in the Member States having mandates related to SPS management work together and collaborate with stakeholders.
- ✓ *Food Safety Regulation* - These guidelines are intended to provide a reference for Member States when developing and operating regional and national food safety management systems.
- ✓ *Plant Protection Products* - The objective of these guidelines is to give advice on the legislative framework for plant protection, including pesticide management in the framework of a sustainable agricultural development. In addition, the guidelines will foster the collaboration, cooperation and exchange of information between various government and non government organizations.
- ✓ *Registration of Veterinary Drugs in SADC Member States* - These guidelines are intended for providing a general scientific framework, including basic methodology, technical requirement, ethical principles and regulatory aspects to register veterinary drugs in member States.

These guidelines are under approval. They will constitute a relevant step towards regional integration and improvement of international trade.

Article 4. Equivalence: *Members shall accept the sanitary or phytosanitary measures of other Members as equivalent, even if these measures differ from their own or from those used by other Members trading in the same product, if the exporting Member objectively demonstrate to the importing Member that its measures achieve the importing*

¹ Malkerns Research Station is a branch of the Ministry of Agriculture, with its own laboratories, deputed to research in plant health, quarantine and import-export issues related to plants and plant products.

² At the time of the study, competencies of the Department of Customs and Excise were passing over to the Swaziland Revenue Authority, a parastatal organization also under the Ministry of Finance

Table 2 - Swaziland main legal provisions for animal health, food safety and plant health

<i>Sector</i>	<i>Legal provisions</i>	<i>State</i>
Animal health	Animal Disease Act No. 7, 1965	In force
	Stock Disease Regulation, 1933	In force
	Animal Disease (Amendment) Act 1999 (modify section 2 definitions)	In force
	Animal Disease (Amendment) Act 2000 (modify section 2 definitions)	In force
	Stock Diseases (Amendment) Regulations, 1999 (modify definitions and regulations on specific diseases)	In force
	Milk production and processing hygiene and inspection regulation, 2001	In force
	Livestock Identification Act No. 13, 2001	In force
	Animal Disease (Regulation and Control of Veterinary Drugs and Medicinal Substances No.1) Regulations, 2010	In force
	Veterinary Public Health Bill, 2007	Waiting for Parliament's approval
	The Public Health Act No. 5, 1969	In force. A new text is in preparation to be in line with Codex standards
Food safety	The Public Health Regulations, 1974	In force
	The Urban Government Act, 1969. The Sanitation in Food Premises bye-laws	In force
	Memorandum of Understanding entered into between Swaziland Standards Authority and Ministry of Agriculture Kingdom of Swaziland	To be signed
	The Plant Control Act, 1981	In force. To be repealed by the Plant Health Protection Bill, waiting for Parliament's approval
	Pesticide Bill	In Cabinet

Member's appropriate level of sanitary or phytosanitary protection (omissis).

According to OIE, Swaziland has implemented equivalence and other types of sanitary agreements with trading partners on selected animals, animal products and processes. Agreements on procedures and certifications are conducted mainly with the European Union and South Africa.

Mutual recognition and equivalence is also envisaged by SADC Regional guidelines.

Article 5. Assessment of Risk and Determination of the Appropriate Level of Sanitary or Phytosanitary Protection: *Members shall ensure that their sanitary or phytosanitary measures are based on an assessment, as appropriate to the circumstances, of the risk to human, animal or plant life or health, taking into account risk assessment techniques developed by the relevant international organizations (omissis).*

In animal health, the Department of Veterinary and Livestock Services compile and maintain data but do not systematically conduct risk assessment. Risk management is implemented for some diseases, such as Foot and Mouth Disease (FMD) and Avian Influenza (AI), but still need to extend to other potential risk sources, such as wildlife fauna. No reference to risk assessment is present in the Animal Disease Act.

With respect to food safety, the preamble of the Veterinary Public Health Bill makes reference to the assessment of public and animal health risks for establishing the nature and intensity of the official controls.

In the field of plant health, the Plant Health Protection Bill includes the concept of pest risk analysis in order to determine whether a pest should be regulated and the strength of any phytosanitary measures to be taken against it.

Training on pest and disease management was developed by the MOA - Department of Agriculture and Extension for tobacco and grain legumes growers.

All SADC Regional guidelines, except those dealing with SPS management, include the concept of risk assessment. The adoption of SADC guidelines and drafted legislation will provide a legal basis for the introduction of risk assessment procedures in all fields covered by SPS Agreement.

Article 6. Adaptation to Regional Conditions, Including Pest- or Diseases-Free Areas and Areas of Low Pest or Disease Prevalence: *Members shall ensure that their sanitary or phytosanitary measures are adapted to the sanitary or phytosanitary characteristics of the area (omissis) from which the product originated and to which the product is destined (omissis). Members shall, in particular, recognise*

the concept of pest- or disease-free areas and areas of low pest or disease prevalence (omissis).

In respect to animal health, the Department of Veterinary and Livestock Services implemented bio-security measures that enable to establish and maintain a disease free zone for selected animals and animal products, as necessary. Swaziland is surrounded by a perimeter, patrolled fenced and in this regard would be considered to be zoned from neighbouring countries, Mozambique and South Africa. Within the country, a separate zone was established to assist eradication of Rinderpest in 1956 and East Coast Fever in 1960. This zone, separating Swaziland from Mozambique, has been maintained, it lies within Swaziland, it is double fenced and patrolled and was utilized in the control of FMD. Compartmentalization could be established in appropriate areas where risk of disease introduction is possible (i.e. northern parks).

According to information collected, the present animal health surveillance system is based on passive surveillance of domestic animals for diseases that are clinically recognisable at the dip tank. There is no connection with health services in respect of zoonoses surveillance.

Food safety surveillance system is lacking. The coordination and exchange of data by relevant Authorities seems to be poor.

With respect to plant health, the surveillance system includes a regular monitoring of some diseases affecting sugar cane and fruit trees. For pine trees, a monitoring system for *Syrex* is managed by forestry industry, due to the recent introduction of the disease in Swaziland. Other plant diseases are put under surveillance in case of outbreaks occurrence. The new Plant Health Protection Bill under approval, includes specific provision for the declaration of pest free areas and areas of low pest prevalence.

Article 7 and Annex B. Transparency: *Members shall notify changes in their sanitary or phytosanitary measures and shall provide information on their sanitary or phytosanitary measures in accordance with the provisions of Annex B.*

Swaziland has notified the Secretariat (1) an enquiry point as provided in paragraph 3 of Annex B of the SPS Agreement and (2) its national notification authority as provided in paragraph 10 of Annex B of the Agreement. The National Notification Authority is the Principal Secretary of the MOA, while the enquiry point, staffed only with part-time persons, is split among three Administrations: MOA – Department of Veterinary and Livestock Services and Malkerns Research Station - and Ministry of Health.

Notifications received through the Secretariat are sent to major stakeholders for comments. Diffusion of notification on new measures proposed by other SPS Member seems to be very limited, without

using any bulletin or newsletter for such transmission.

According to information received by stakeholders, the Enquiry Point is not used for gathering information on SPS measures in place in other countries. In general, awareness on SPS Agreement between stakeholders seems to be very low.

During a Workshop convened by the Interim SPS Committee, participants agreed on a proposed SPS structure for Swaziland, reviewing the model suggested in SADC guidelines. According to this proposal, the National SPS Committee should have a secretariat/unit, in charge of:

- ✓ Implement decisions of the SPS Committee.
- ✓ Information, coordination and dissemination.
- ✓ Administration (manage finances, convene meetings for stakeholders, compiling reports and minutes, identify training needs).
- ✓ Notify trading partners in case of disease outbreaks.
- ✓ Implement SPS programme.
- ✓ Develop a food safety monitoring system (for food safety and quality).
- ✓ Aggressive public awareness creation for consumers.
- ✓ Develop a programme to improve the existing SPS structure (including data collection and storage).

The dissemination of information is done through the Information Office under the MOA, using two local newspapers, TV, radio (dedicated slots for MOA), pamphlets, leaflets, etc. The Information Office is also coordinating the upgrading of the MOA page in the Government website, using a service oriented model. A proposal for SPS issues has already been prepared.

Information on Swaziland's measures and procedures for other SPS Members is difficult to gather. In general, exporters and importers need to contact and visit a number of Administrations to have all documents and permits requested, wasting time, due to the different location of such bodies.

According to the SADC report, this problem is widespread in the region, in fact *"complete, up to date and relevant WTO/SPS information is not easily available within SADC Member States or on a regional basis. There is currently no database available containing information on SPS requirements, which would ensure transparency and dissemination of information to SADC Member States – even though some countries have established their own enquiry points. Current procedures applied for import of agricultural products, for veterinary medicines and plant quarantine that regulate the quality control measures pose problems as importers have to produce a number of certificates including quarantine certificates and veterinary certificates through a range of measures which differ substantially from the international standards and are not scientifically based and supported by*

risk assessment. In addition, the information on SPS is not centralized in only one institution – SPS related responsibilities are commonly scattered over several ministries”. SADC is engaged in providing a design of a SPS/Food Safety database system which will facilitate the management and sharing of SPS information between Member States and other trading partners.

Article 8 and Annex C. Control, Inspection and Approval Procedures: *Members shall observe the provisions of Annex C in the operation of control, inspection and approval procedures (omissis).*

Swaziland has a fairly open border with respect to the movement of animals, vegetables, and their products, provided that the point of origin is an approved establishment. Any person or organisation engaged in the trade business of import/export of good or commodities of any nature require a licence. A Trade and Business Facilitation Bill, aimed at simplifying procedures for trade licensing, is currently going through the Parliament. Importer and exporter must be registered with the Custom and Excise Department under the Ministry of Finance. Declaration is through the Single Administrative Document (SAD) introduced in 2006, which applies to all imports and exports regardless of their origin or destination.

Import prohibitions are maintained for various reasons, including health and safety considerations. They cover, *inter alia*, plants, seeds, bulbs, raw cotton, uncooked poultry meat, honey, beeswax, bees and their larvae or eggs, and used beehive appliances.

Under the Import Control Order of 1976 and Legal Notice No. 60 of 2000, 15 broad categories of products are subject to import permits, including specified agricultural products, among which dairy products, maize and rice. Permits are issued by the Ministry of Finance and are valid for one year (renewable for another year).

According to the NAMBoard Act No. 13 of 1985, one of the major functions of the National Agricultural Marketing Board (NAMBOARD) is the registration of distributors and import carriers of scheduled agricultural products and the issue of permits for those same products. In addition, it may limit imports of agricultural products by defining quantities that may be imported and by raising import levies. Levies and quotas are set on the basis of the balance of local production, or of seasonal production (e.g. bananas); in relation to national disasters; or in relation to infant industry protection under which the quantity or quality of imports is monitored. Dairy Board is entitled to issue permits for import of dairy products. South Africa has a list of approved establishments for export to Swaziland. Both NAMBOARD and Dairy Board have their own inspectors at the BIPs that work in close collaboration with veterinary inspectors. Only documental

and fiscal checks are performed.

In the field of animal health, the Animal Disease Act laid down the conditions which are required for import. The Minister of Agriculture may restrict the import or export of animals, animal products, live virus and other pathogens or biological or chemical products for the treatment of animals. The Minister may also fix maximum and minimum wholesale and retail prices for animals or animal products sold in Swaziland.

The Animal Disease Act specifies various import requirements in terms of veterinary certification, and a quarantine period of 30 days for animals intended for breeding purposes. Animals intended for slaughter are not subject to quarantine, but must be slaughtered within 48 hours of arrival. Livestock from approved regional suppliers (on condition that they are tested prior to export) may be imported without restrictions. For live animals, the import permits are issued at regional level by a veterinary officer. This function cannot be delegated. Regional Veterinary Offices are equipped with some computers, but the connection between all regions and headquarter is still lacking. Animals should be accompanied by health certificate issued by the Official Authorities of the sending country and can only be rejected at the border, before being allowed into Swaziland.

The Custom and Excise Act regulates the import of hides and skins, meat and eggs. Enforcement issues are joint responsibility of the Department of Custom and Excise (now Swaziland Revenue Authority) and the MOA.

Phytosanitary conditions for the import of plants and seeds are basically regulated by the Plant Control Act and the Seeds and Plants Varieties Act of 2000. Phytosanitary certificates are required from the MOA to import citrus fruits or trees. For cotton, the Cotton Board is empowered to issue certificates. The Sugar Act of 1967 empowers the sugar industry itself to determine the varieties of sugar cane that may be imported, and the conditions for import and for testing varieties of sugar. Phytosanitary standards are set by notice in the Government Gazette and are updated as new diseases are identified.

In general, all plants and plant products require an import permit, although some are not normally restricted if they bear proof that they are from SACU; these goods include fruit and vegetables other than citrus, flowers, and other materials regularly sold by florists and greengrocers; seed potatoes; and a range of other plants or vegetables including certain subtropical fruit and crops, vegetables, legumes, ornamentals and others. Purchases by individuals of plants from state-registered nurseries, fruit and vegetables (including not more than 20 kg of citrus per person), and cut flowers, wreaths and herbaceous pot plants bought in South Africa, for immediate use or consumption and not for sale, may be imported without a permit.

The importation of seeds produced under certifica-

tion by the International Seed Test Association is not restricted. Under the Seeds and Plants Varieties Act, all imports must be tested by a phytopatologist based in Swaziland and/or issued with a clearance certificate. Swaziland has a closed list of seed and plant varieties, applied to important, certified crops, including maize, beans, sorghum, groundnuts, tobacco, cotton, and cowpeas. Seeds produced by regional producers of good repute, such as some companies in South Africa and Zimbabwe, are allowed entry without certification. Indigenous plants imported from the region require a permit issued by the National Trust Commission on the basis of the Convention on International Trade and Endangered Species of Wild Fauna and Flora (CITES). This body also protects local plant species and maintains a list of protected and specially protected species for which an export permit would be required.

In respect of genetically modified organisms, draft legislation is currently awaiting Parliament approval.

There are three designated Border Inspection Posts (BIPs) for the imports of live animals and animal products between Swaziland and South Africa: Ngwenya, Mahamba, and Lavumisa. In these posts, veterinary staff with different qualifications is on duty, who checks the consignments, particularly the documental controls. Data are registered manually. A separate register is kept for imported and rejected animals and animal products. Guidelines prepared by the Department of Veterinary and Livestock Services are available in all BIPs.

Most of the times, veterinary staff performs also control over plant and plant products.

When an outbreak of disease is notified by the exporting country to the Headquarter of the importing country, the Veterinary Services at BIPs are immediately informed, and the Order issued for restriction or banning of imports is made available. So far, this system has been very effective.

The MOH has its own inspectors at the border posts, generally nurses for checking human health, using mobile units, while an equipped office is not available.

In some BIPs, condition improved, but still are very poor, lacking electricity, water and sanitation, communication means, and computerised system, means for carrying out disinfections, or disposal of dangerous materials. No facilities for storing products, taking samples and handling them are available.

The Official Veterinary Quarantine Services are in very poor conditions in respect of infrastructures and equipment. All animals are clinically checked on a daily basis. Due to the fact that animals are certified for brucellosis, tuberculosis and FMD, no testing is done for these diseases.

In the past, private quarantine was allowed under approval by the Veterinary and Livestock Regional

Service, and subject to periodical visits by the assistant health inspector.

Article 9. Technical Assistance: *Members agree to facilitate the provision of technical assistance to other Members, (omissis), either bilaterally or through the appropriate international organizations (omissis). Where substantial investments are required in order for an exporting developing country Member to fulfil the sanitary or phytosanitary requirements of an importing Member, the latter shall consider providing such technical assistance as will permit the developing country Member to maintain and expand its market access opportunities for the product involved.*

Swaziland is receiving assistance from several partners, including the European Union, the Food and Agriculture Organization (FAO), the Norwegian Independent Meat and Poultry Association (KLF). Swaziland, as Member of SADC, is also receiving assistance under the Food Safety – Capacity Building on Residue Control project. One of the outputs of this project is the enhancement of coordination and communication between national SADC regional authorities involved in the regulatory framework on SPS relevant issues. To pursue this objective it is foreseen the establishment of a SPS/Food Safety database and communication network for efficient information exchange.

In synthesis, recommendations to strengthen SPS compliance in Swaziland, are summarized in table 3.

DISCUSSION

The assessment of SPS system implementation in Swaziland shows that the country is facing some problems common to most of the Region, affecting the ability to participate in the global market. Food safety and food security were chosen as a priority area for improving international trade and extend their benefits also to products destined to the internal market.

The strategy developed to overcome these limits should take into account:

- ✓ the recognition of the importance of promoting agriculture and of allocating an appropriate share of official development assistance (ODA) to the field of agriculture, including assistance for farmers on access to markets;
- ✓ that equal access to adequate food is a universal human right and that agricultural development must be based on the right to be able to feed and to produce food realizing food security (both quantitative and qualitative) and the right to be, as far as possible, self-sufficient;
- ✓ the importance of food security, defined as the ability of a country or a region to democratically implement its policies, priorities and strategies in agriculture and food through a sustainable agricultural model;
- ✓ the importance of an approach to governance in

terms of food safety that includes an overall framework for the prioritization of a food policy that goes beyond food aid, cooperation among donors and between donors, and that aids recipients to work in close partnership with local communities.

Addressing them in the right direction, the SPS interim committee play an important role in coordinating activities and promoting better management of SPS issue within the country and inside the region. Its work should be supported at all levels and enough resources in terms of personnel, equipment and financing should be assured to make it fully operational.

The SADC guidelines and, more in general, SADC technical assistance, are useful tools for promoting better participation of Member States in interna-

tional trade, and for an integrated development of the region.

Further technical assistance should be considered, especially for improving BIPs and quarantine conditions. Training of inspectors is another important issue, and should be complemented using national experts for further training of regional and local staff.

As recalled during the last General Assembly of the United Nations (UN, 2011), North-South, South-South and triangular cooperation is a pillar for improving health worldwide, and all developed countries should fulfill their official development assistance-related commitments, including the commitments to achieve the target of 0.7 per cent of gross national income for official development assistance by 2015.

Table 3 - Swaziland. Main recommendations to strengthen SPS compliance

SPS provisions	Recommendations
<i>Article 3 - Harmonization</i>	<p>Appoint a working group within the Department of Veterinary and Livestock Services to prepare amendments to the Animal Disease Act and prepare new regulations for specific animal diseases in line with OIE standards</p> <p>Request technical assistance for drafting new regulations concerning animal welfare</p> <p>Complete work on animal identification database and connect all regions and sub-regions for its implementation</p>
<i>Article 4 - Equivalence</i>	<p>Include equivalence principles in the training program for inspectors</p>
<i>Article 5 - Assessment of Risk and Determination of the Appropriate Level of SPS protection</i>	<p>Include risk analysis in the training program for inspectors</p> <p>Support further training for regional and sub-regional staff, using national experts already trained in risk analysis</p> <p>Develop further seminars and training activities on risk analysis in the extension programme for farmers</p> <p>Establish a technical committee on risk analysis under the National SPS committee and ensure follow-up</p>
<i>Article 6 - Adaptation to Regional Conditions, Including Pest- or Disease-Free Areas and Areas of Low Pest or Disease Prevalence</i>	<p>Improve surveillance system for animal and plant diseases in accordance with laboratories capabilities</p> <p>Request technical assistance to establish an alert and early warning system</p> <p>Request technical assistance to establish a surveillance databases for animal health, plant health and food safety</p> <p>Ensure better coordination between relevant partners in food safety issues, establishing a technical committee under the National SPS committee</p>
<i>Article 7 - Transparency</i>	<p>Appoint dedicated staff to the SPS unit and ensure follow up</p> <p>Organize training on operation of Enquiry Points and National Notification Authority using national staff and the WTO procedural manual</p> <p>Prepare a newsletter/bulletin to disseminate notification to targeted stakeholders</p> <p>Make a revision and collect relevant documents from different administrations to be made available to other Member</p> <p>Request technical assistance to prepare the SPS webpage</p> <p>Plan and design an awareness campaign for stakeholders in collaboration with the Information Office of MOA</p> <p>Collaborate with MOA Department of Agriculture and Extension to include dissemination of information on SPS issues to medium and small farmers</p> <p>Establish a technical committee on information, communication and capacity building under the national SPS committee</p>
<i>Article 8 - Control, Inspection and Approval Procedures</i>	<p>Prepare a list of prohibited goods from all Administrations involved in SPS issues, and make it available for Custom officers</p> <p>Prepare an assessment of laboratory capability in term of tests for animal and plant diseases and food safety</p> <p>Prepare comprehensive multi-disciplinary guidelines for border posts controls, including testing</p>

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One health in central Asia. A situational analysis informing the future

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Summary - Under the One Health initiative in Central Asia, financed by the World Bank, public health and veterinary services gap assessments were undertaken in four countries - Kazakhstan, Kyrgyzstan, Tajikistan and Uzbekistan - to develop a strategy and action plans, which could build capacity to detect, diagnose and prevent or control zoonotic diseases. The assessments support some stereotypes of governance in the four countries but also document refreshing changing attitudes. There are opportunities to leverage regional collaboration as a result of their common socio-economic history, training and language. Intersectoral collaboration among the public health services, veterinary services and civil society groups are established at district and community levels, often to higher degrees than in Western countries. The lack of some modern skills and lack of opportunities for refresher education were common complaints across all four countries. The paper presents the results of these assessments, underlying common features and differences between the four countries, and calls for further enhancement of an intersectoral collaboration approach.

Key words: Intersectoral collaboration, Central Asia, zoonoses

INTRODUCTION

Whether living in urban or rural settings, human are part of a biological continuum that also covers animals and the environment as a whole. In the 1970s, Professor Adriano Mantovani was pioneering the One Health approach, promoting intersectoral collaboration in zoonoses prevention and control, and providing joint training opportunities for physicians and veterinarians at the *WHO-FAO Collaborating Centre for Research and Training in Veterinary Public Health* in Rome. The term 'One World One Health', used for the first time in 2004, emerged from a meeting at Rockefeller University in New York City with a set of principles that recognizes the link between human, animal, and wildlife health and a need for holistic approaches to disease surveillance for the prevention and control of emerging infectious diseases (Gibbs and Anderson, 2009). While accurate estimates are difficult to make, 61% of human infections are estimated to occur from pathogens that cross animal/human boundaries (Ceveland *et al.*, 2001) and over the past three decades 75% of recent emerging diseases are zoonotic (Taylor *et al.*, 2001). News health challenges exist in the 21st century, among which are the

emergence of new pathogens such as bovine spongiform encephalopathy (BSE), new strains of influenza, and the spread of zoonotic diseases in new habitat due to environmental changes, increasing international trade and travel. As yet unidentified or poorly investigated influences, such as the effect of climate change, exist.

Thus, the concept of One Health stresses the advantages of working closely together to prevent and control zoonotic diseases, whether affecting animals or humans (Gibbs and Anderson, 2009). In 2008, the most important international development organizations produced a 'Strategic framework for reducing risks of infectious diseases at the animal-human-ecosystem interface' (FAO *et al.*, 2008). This document, as well as the most recent Tripartite Concept Note (FAO *et al.*, 2010) and The 'People, Pathogens and Our Planet' document (WORLD BANK, 2010), call for strengthening of joint efforts to diminish the risk and global impact of epidemics and pandemics by enhancing disease surveillance and emergency response systems at national, regional and international levels, by supporting them through strong and stable public health and veterinary services.

WHO believes (WHO, 2011a) the so called

neglected zoonotic diseases, which include anthrax, bovine tuberculosis, brucellosis, echinococcosis/hydatidosis, neurocysticercosis, rabies and others, receive little international support. These diseases affect more poor rural populations who keep livestock and their effective control must be pursued through an intersectoral approach. Accordingly and in conjunction with the desires of Central Asia countries, these diseases were targeted in the World Bank approach.

The World Bank, together with the Eurasian Economic Community, sponsored Central Asia AIDS Project, adopted the One World One Health approach to perform a situational analysis in Central Asia (CAOH) in recognition that the conventional approach to controlling zoonotic diseases in low- and middle-income countries is inadequate. Its goal was to strengthen national and regional programs to address food safety and zoonotic disease, while simultaneously strengthening veterinary and medical systems, and provide an economic assessment of major zoonoses at a regional level. The common history and close socio-economic integration of these countries strongly suggest the need for close cross-border and intersectoral collaboration. Thus the CAOH project focused both on national and regional needs for reducing the effects of zoonotic diseases. The objective of the project was to identify and contribute to the harmonization of national and regional action plans to address zoonotic diseases. It included the implementation of both Public Health and a Veterinary Gap Analysis in each country - Kazakhstan, Kyrgyzstan, Tajikistan and Uzbekistan - as a starting point for strategy planning, needs assessment and identifying activities for a better prevention and management of zoonoses in Central Asia.

Public Health Services in Central Asia

Following dissolution of the Soviet Union in 1991, the Central Asia countries inherited a health system organized according to the Semashko model of Soviet health care, whose main principle was that health services should be free and accessible to everybody (Ahmedov *et al.*, 2007; Khodjamurodov, Rechel, 2010; Kulzhanov and Rechel, 2007; Meimanaliev *et al.*, 2005). In the Soviet period, the health system was oriented towards the control of communicable diseases and the development of a rural primary health care infrastructure. Initially, each citizen was assigned to a feldsher obstetrical post (FOP) in rural areas, and to a polyclinic in urban areas, according to the place of residence. The emphasis changed between 1950 and 1970 to specialist and hospital care, when many hospitals and polyclinics were built, reducing the resources available for primary health care. In the 1980s, the system began to deteriorate. In recent years, some reforms were initiated in each country, mostly directed to decentralization and strengthening of

primary health care. The introduction of private medical services and insurance schemes has created two-tiered systems as governments struggle to define ways to best deliver health care with a basic package of services for all in a marketplace changing from a state-run socialized system to one with significant private insurance schemes. These changes are occurring in the relatively poor countries of Central Asia, which range from 96th place (Kazakhstan; \$444) to 158th place (Tajikistan; \$95) among 193 WHO member countries (194, including Taiwan) in 2008 per capita health care spending, adjusted by purchasing power parity. Nevertheless, even the seemingly low figure of \$95 for Tajikistan represents a significant proportion of GDP spent on health care, 5.0%, with other countries ranging from 1.9% (Turkmenistan) to 5.7% (Kyrgyzstan) (WHO, 2011b).

In this changing market place, all countries have retained centralized Sanitary-Epidemiological Services for infectious diseases (including zoonoses), which provide public health epidemiologic and laboratory services, and Centres for Healthy Lifestyles, which provide health promotion services, with branches at regional and local levels. Due to lack of resources, however, today the public health services suffer from inadequate human resources in terms of number of staff and training, and obsolete equipment which is poorly maintained.

Veterinary Services and farming systems reforms

During the decades within the Soviet Union, veterinary services in the Central Asian countries followed the patterns established in all Soviet republics. Animal disease control programs were centrally planned and directed to state and collective farms. Individual animal disease diagnosis, treatment and breeding were carried out principally on intensive and large state or collective enterprises. Privately owned livestock, which were comparatively few compared to those raised in state-owned enterprises, and pets, were generally managed as a secondary responsibility by these same institutional veterinarians. Veterinary staff in the industrial livestock enterprises also managed a large cadre of livestock technicians (i.e. artificial insemination technicians) such that there were on average one-third to one-fifth the number of livestock units for each food animal veterinarian compared to the West (Schillhorn Van Veen, 2004). Thus in most countries animal health labor was and still is over abundant.

The veterinary system in the former Soviet Union was generally able to effectively control animal diseases, reduce prevalence of zoonotic diseases to low levels and obtain relatively high levels of productivity through better health and improved feeding and breeding. The system relied on a large and theoretically trained cadre of specialists and was well organized with standard methods based on

Soviet science understanding. This system rather rapidly deteriorated after only a few years beginning at independence as farming became dispersed to small units, services and inputs were not consistently available, livestock numbers contracted and demand for livestock products diminished (Schillhorn Van Veen, 2004).

The 1990s were very difficult economic times for former Soviet Union citizens, their livestock and agriculture workers who served the livestock industry. Agriculture output decreased in absolute terms by up to 40 percent and subsistence farms largely replaced intensive agriculture (Vares, 2003). Following independence, the elaborate interdependent Soviet Union-wide livestock husbandry system collapsed and food animal populations in some countries fell to less than half of 1990 numbers (Schillhorn Van Veen, 2004). Soviet-trained veterinarians in the Central Asian republics had been more isolated than those in Eastern Europe and had little or no knowledge about service delivery to now widely dispersed livestock, where families in villages raised a few food animals, where remedies and vaccines (and their quality assurance) were in short supply and the new small-scale livestock owners had little money nor experience with paying for services or remedies. Under these conditions, livestock and public health deteriorated as the prevalence of zoonotic diseases such as brucellosis rose (Pappas *et al.*, 2006) and livestock production fell.

In the late 1990s - early 2000s, numerous international donors and the Food and Agriculture Organization (FAO) began implementing village-based, fee-for-service veterinary (and artificial insemination) delivery systems with the intention that they become financially self-sustainable. Most notable success in establishing these sustainable delivery systems, that focus on smallholder agriculturists who own the majority of livestock, has been in Afghanistan (Schreuder and Ward, 2004), Tajikistan and most recently in Kyrgyzstan and in Orissa state in India (Ahuja *et al.*, 2003).

Governments had to recognize an urgent need to facilitate private delivery of veterinary services and to not stifle the private sector. Similar lessons were learned in many Sub-Saharan Africa countries in the 1980s and 1990s (Cheneau *et al.*, 2004) but African country experiences are generally dismissed as invalid examples by authorities in former Soviet Union countries. So far, reorganization of Government vet services has lagged behind that of formal or informal private delivery of clinical services and artificial insemination to livestock owners.

MATERIALS AND METHODS

The veterinary and human health systems were assessed using different approaches. For the veterinary gap analysis, the well-tested World Organization for Animal Health (OIE) Performance

of Veterinary Services Pathway (PVS) tool was used, while for the public health gap analysis, a recently devised World Bank-sponsored tool was used.

Public Health Gap Analysis

One approach to evaluating public health services is based on examining the existence and functioning of a defined set of “essential public health functions,” (WHO, 2009). Formulating these functions was an integral component in the elaboration of the WHO’ Health for All policy (WHO, 1998).

Our principal assessment tool was an adaptation of the *Zoonosis and Public Health Systems Gap Analysis Tool*, based on a tool that arose out of the *Public Health in the Americas* regional initiative. This initiative, developed by the Pan American Health Organization, CDC and the Latin American Center for Health Systems Research, sought to measure the performance of essential public health functions within Latin America. The *Zoonosis and Public Health Systems Gap Analysis Tool* narrowed the focus of the initiative’s tool to zoonoses and made extensive revision of indicators, variables, measures and submeasures. The tool evaluates 11 Essential Public Health Functions pertaining to zoonoses, called Essential Zoonosis Functions (EZFs), and is completed by interviewing key informants (Tab. 1).

We modified the tool and the approach to its use. We believed that the tool was too complex and duplicative. Thus, we streamlined it, eliminating some questions and collapsing others. We also believed the original tool would not generate the information necessary for quantification of actions necessary to deliver better zoonoses management. To address this perceived weakness, questions to capture additional information on resource availability (funding and staffing levels) and obstacles to the use of resources were added.

The existing tool was meant to be used at a national level, completed in group work by national experts. While this approach simplified completion of the tool, we believed that it would result in insufficient understanding of local capability. Thus, we altered the approach to data collection, using interviews both at national and sub-national levels, with some questions used only at national level.

To achieve consistency of interviewing and, thus, data, we wrote *Methodologic Guidelines*. These guidelines oriented interviewers to the Gap Analysis Tool, guided interviewers to their job, answered likely questions interviewers would have, and provided scripting and prompting for asking questions and adding commentary.

Each EZF was evaluated through indicators and variables, using a binary score. The analysis of the score permitted a quantitative evaluation that was conducted using a spreadsheet in Excel format. Data were coded according to a binary system of

“yes/present” or “no/absent.” “Don’t know” and “Not applicable” answers were allowed and treated as invalid data for the calculation of parameters. Means and ranges were calculated for each variable. Because a qualitative evaluation should permit a better understanding of the situation, a strong effort was made by interviewers to record commentary during the interviews. Problems and suggestions were specifically requested as part of the commentary, and materials (legislation and orders, guidelines, planning and activity reports, forms, standards, health education materials, training programs, epidemiologic data, etc.) were collected in each area. All comments collected during interviews were summarized for each question. When appropriate, comments received from national level were taken into consideration separately from those collected at sub-national level, and an indication on frequency of comments was included. If available, examples were also reported.

All interviews were administered 10 May-10 June 2011 by one or two teams per country, composed of two persons each, one for questioning and stimulating discussion, the other for recording answers and comments. Teams were comprised of country nationals and interviews were conducted using Russian or the national language of the country. Daily supervision and monitoring of work of the teams were performed, and included in-country visits to national and sub-national public health services, to FOPs and public health laboratories by us.

We were limited in the time available to complete the study.

To have a vision of the public health services dealing with zoonoses, we devised a sampling strategy applied in each country. We identified three oblasts (regions, or States), two with the highest incidence of zoonoses and one with the lowest and, within each, randomly chose two rayons (districts), yielding nine administrative levels for interview. We also interviewed individuals at national level yielding, in total, interviews from ten levels.

Personnel to be interviewed varied somewhat by country, but attempts were made to consistently interview at oblast- and rayon-level one or more persons at Sanitary-Epidemiological Services, Healthy Lifestyles, the chief doctor in infectious diseases and the chief epidemiologist. Frequently, additional persons, e.g., laboratory specialists, were consulted and interviewed. All completed interview forms were sent periodically, both electronically and via mail, to the project central office in Almaty for analysis.

The rating of each EZF score is calculated as the average value of indicators reflecting the capacity, infrastructure, processes or outcomes related to the particular function. The value is expressed in percentage: the minimum value of the indicator is 0

(the function is not performed), the maximum 100% (function entirely performed).

Veterinary Gap Analysis

The methodology was based on the World Organization for Animal Health (OIE) Performance of Veterinary Services Pathway (PVS) (OIE, 2010). All four countries had completed the PVS Evaluation, which is a tool for the ‘diagnosis’ of where weaknesses in state veterinary services may exist. The next step in the ‘pathway’, the PVS Gap Analysis, is the ‘prescription’ for addressing the weaknesses identified and for raising the overall, system-wide functional capacity of the national veterinary service. Within the study countries, the PVS Gap Analysis had only been completed in the Kyrgyz Republic at the commencement of the CAOH project.

The original proposal to work in direct collaboration with the OIE certified auditors proved to be unrealistic due to scheduling limitations. In response, a new methodology was devised under the study to undertake a limited assessment of the veterinary services in each of the four project countries. Thirteen criteria drawn from the 46 critical competencies contained in the OIE PVS Pathway were selected for evaluation because of their essential relevance to detecting, controlling or eradicating zoonotic diseases and for mounting a rapid response. Among the critical competencies not reviewed by the study, include veterinary laboratory diagnosis and quality assurance. Competent laboratories are essential for diagnosing zoonotic as well as non-zoonotic and even non-infectious diseases and, are thus, not specific to zoonotic diseases. Additionally, over the last 5 years, the diagnostic capacity of laboratories in the four Central Asian countries has been significantly upgraded due to assistance received under programs for control of highly pathogenic avian influenza.

We selected the 13 criteria below for evaluation under the four main categories from the OIE PVS Evaluation manual (2005 version) as currently being essential for detecting, controlling or eradicating and responding to zoonotic diseases, either those that are endemic or epidemic.

- I. Human, Physical and Financial Resources:* Continuing education and Emergency funding.
- II. Technical Authority and Capability:* Risk analysis, Quarantine and border security, Epidemiological surveillance, Early detection and emergency response, Disease prevention, control and eradication and Identification and traceability.
- III. Interaction with Stakeholders:* Consultation with stakeholders and Participation of producers & other stakeholders in joint programs.

IV. Access to Markets: International harmonization, Transparency and Zooning.

Precise definitions and criteria for evaluating these 13 critical competencies, and indeed all 46, are found in the OIE PVS manual and official evaluators are trained in using this methodology. Additionally, countries provide prior to evaluation missions copious documentation as prescribed in the manual. It is important to recognize that this limited assessment of 13 criteria is not a substitute for a full PVS Gap Analysis, which is a systematic and holistic analysis, intended to strengthen the national veterinary service in its entirety. It is a fundamental aspect of the OIE PVS Evaluation and Gap Analysis that a system-wide approach be taken when considering the performance of veterinary services and focusing exclusively on zoonotic diseases can only provide a partial understanding of the interventions necessary for overall service strengthening.

Participants in strategy planning workshops were solicited from the veterinary, public health and environment/wildlife services of their respective Ministries of each country. Other pertinent stakeholders were invited, including representatives of livestock producer groups, hunter groups and consumers. Fifteen to twenty persons participated in the workshops. Workshops were conducted over three half-days, with participants discussing and debating in their common language. For each criterion, a discussion took place to arrive at a consensus over a level of attainment of the OIE standards (1 lower - 5 higher) that fitted with national priorities, was reachable over five years, and addressed identi-

fied problems or deficiencies. With the level to be achieved in mind, participants further debated a strategy that would take the veterinary service to the agreed level. During discussions a culture of intersectoral collaboration was impressed upon participants in order that human, animal and environmental health concerns for best zoonotic diseases control were addressed. Specific activities, inputs or investments for reaching the desired level and for leveraging intersectoral collaboration were mentioned for later refinement and budgeting.

RESULTS

Public Health Gap Analysis

The qualitative and quantitative analysis of the 11 EZFs shows that interviewees generally rated the overall public health service for zoonoses highly in all countries (Tab. 1, Fig. 1). In particular, at regional level, the function dealing with monitoring of the health status and the function dealing with laws and regulations gained the highest scores. The lowest scores were given to quality assurance/monitoring and evaluation, and equitable access to health services, mostly because questions related to this last function were generally considered not applicable to the national health system currently in place in these countries.

In all countries, a multidisciplinary approach for the prevention and control of zoonoses has been implemented, to some extent, both at national and sub-national levels. In particular, in Kyrgyzstan, a collaboration exists between public health, veteri-

Table 1 - Assessment of Public Health Essential Zoonoses Functions in Central Asia countries (EZFs evaluated only at national level are colored in grey).

Essential Zoonoses Functions	Kazakhstan	Kyrgyzstan	Tajikistan	Uzbekistan
1. Monitoring, Evaluating and Analyzing Health Status including Zoonoses-related Data	74%	78%	77%	100%
2. Public Health Surveillance of Zoonoses Risks and Threats to Public Health	88%	86%	67%	68%
3. Health Promotion in Zoonoses Control, Prevention and Eradication	54%	69%	45%	92%
4. Social Participation in Zoonoses Control and Prevention	76%	80%	84%	53%
5. Development of Policies and Institutional Capacity for Planning and Management of Zoonoses related activities	77%	74%	57%	57%
6. Strengthening of Institutional Capacity for Regulating and Enforcing Laws that Protect Public Health from Zoonoses	92%	74%	100%	94%
7. Promotion of Equitable Access to Necessary Health Services, Including Priority Zoonoses-related Services	77%	35%	49%	63%
8. Human Resource Development and Capacity Building in Zoonoses and Public Health	88%	68%	45%	69%
9. Ensuring the Quality of Personal and Population-based Health Services, Including Priority Zoonoses-related Services	63%	13%	45%	40%
10. Strategic Research on Zoonoses and Public Health	100%	20%	100%	80%
11. Controlling and Preventing Zoonoses in Emergency and Disaster Situations	81%	68%	15%	100%

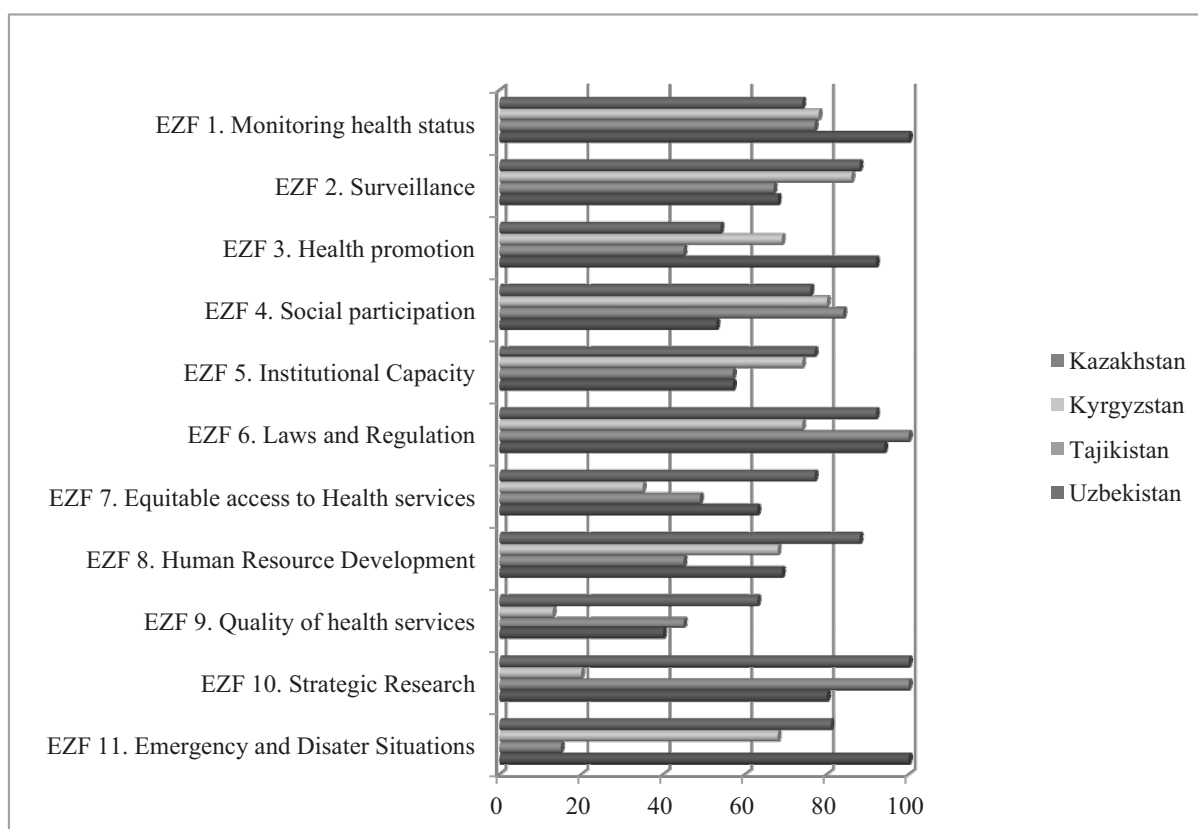


Figure 1 - Assessment of Public Health Essential Zoonoses Functions in Central Asia countries

nary services and the Agency for Environmental Protection; the same in Uzbekistan, where the inter-sectoral collaboration involves public health, veterinary services and the Committee for Nature Protection and Emergencies.

A comparative analysis of the most important features for each EZF is reported below.

EZF 1 - Monitoring, Evaluating and Analyzing Health Status, including Zoonoses-related Data.

This EZF is evaluated through four indicators, dealing with monitoring cases of zoonoses, expertise, multidisciplinary approach and technical assistance. In all countries, *ad hoc* legislation for monitoring cases of zoonoses is available. Expertise in epidemiology and statistics seems to be generally lacking, especially at sub-national level, where working conditions are poorer. Two main problems were noted in respect with human resources: shortage of staff and lack of training. An exception is represented by Uzbekistan, where these problems were not reported.

An integrated multidisciplinary approach towards detection and management of zoonoses has been implemented in all countries. In Tajikistan, for example, a joint work plan was issued by the Ministry of Health with the Ministry of Agriculture for prevention and control of anthrax, rabies, brucellosis and tuberculosis in the years 2005-2010.

In all countries, technical assistance is provided by the Ministry of Health to sub-national services.

EZF 2 - Surveillance of Zoonotic Risks and Threats to Public Health.

The EZF is measured by assessing the surveillance system; expertise; laboratory capability; response to zoonoses; technical assistance; and compliance with International Health Regulations (2005).

A surveillance system on zoonoses is in place in all countries, linked with that of veterinary services, while connection with international surveillance systems is often lacking. No formal mechanism to coordinate activities with one or more international laboratories of recognized excellence was reported. With the exception of Tajikistan, the capacity and experience for the surveillance of zoonotic diseases was evaluated positively. Public health laboratories are very widespread, but in all countries, except Kazakhstan, where modernization is on-going, they urgently need maintenance, including sanitation and proper waste disposal and more modern equipment. The capacity to respond in a timely and effective manner and the availability of technical support were evaluated positively by all countries. In Tajikistan it was noted that, even if there are legal documents that regulate this activity, the real capacity is limited due to outdated instructions, poor working conditions and lack of human and financial

resources. All countries started working on implementation of IHR (2005) and have appointed a focal point.

EZF 3 - Health Promotion in Zoonoses Control, Prevention and Reduction.

Five indicators were used to assess this EZF: develop and implement health promotion activities; national planning and coordination; integrated multidisciplinary approach; technical assistance; and reorientation of health services towards health promotion.

In all countries, a national program for healthy lifestyles is in place but zoonoses are included only in Kyrgyzstan and Tajikistan, where interventions were developed at local level and educational activities carried out among the population for the prevention of brucellosis, anthrax, echinococcosis/hydatidosis, rabies and botulism. In these efforts, a wide range of means, e.g. radio, television, websites, newspapers, brochures, telephone hotlines, and mobile teams, were used to convey health education messages. International organizations (FAO, European Union) and non-governmental organizations (NGOs) played an important role in supporting health education campaigns against zoonoses. The National Centre for Health Promotion in Kyrgyzstan, or its equivalent in other countries, is the body assuring coordination of health education programs. Such coordination was reported to be weak in Kazakhstan, where zoonoses are not included into its agenda. An integrated multidisciplinary approach is pursued in all countries with a number of partners such as Ministry of Education, veterinary services, youth committee, and local authorities. Support from central level was reported to be available in all countries, while the re-orientation of the health services towards health promotion scored high only in Uzbekistan.

EZF 4 - Social Participation in Zoonoses Control and Prevention.

This EZF is evaluated by means of three indicators: empowerment of civil society; strengthening social participation; and technical assistance.

Tajikistan and Kyrgyzstan are the two countries where social participation is most developed, while it was reported to be very weak in Uzbekistan.

In Kyrgyzstan, civil society is actively consulted on zoonoses issues through the Centre for Health Promotion and its branches, health promotion committees, village health committees, village women's councils, and primary health services. In Tajikistan, self-governing bodies (jamoats) and neighborhood committees (councils) are involved in social mobilization. These bodies are authorized to represent the interests of the population and can go directly to local and central government authorities. The population takes part in determining its health priority goals, but not in decision making concern-

ing the administration of health services. Lack of financial and technical resources were mentioned as limiting factors for social participation.

EZF 5 - Development of Policies and Institutional Capacity for Planning and Management of Zoonoses-related Activities.

Six indicators were used to evaluate this EZF: definition and development of policies and objectives; monitoring and evaluation of policies; institutional capacity; integrated multidisciplinary approach; international cooperation; and technical assistance.

Kazakhstan and Kyrgyzstan gave relatively high scores to this function, which received lower values in Tajikistan and Uzbekistan due to its almost complete centralization, and consequent low scores reported at sub-national level. The definition and development of national and sub-national policies is one of the strategic objectives of the Ministries of Health of all countries, sometimes hampered by the lack of sufficient financial resources as is the case for Kyrgyzstan. Shortage of specialists, especially at sub-national level and trained on development of evidence-based public health policies, health impact assessment and other issues were recorded in all countries, except Uzbekistan.

Monitoring and evaluation of public health policies via indicators is periodically performed in all countries, but the system is not fully automated. Data are transmitted to higher levels in aggregate form, where at central level key details are often lost. Changes in policies and plans for prevention and control of zoonoses are made depending on the epidemiological situation, but the ability of the current system to adequately assess the situation was questioned. The evaluation of institutional capacity varied from completely adequate in terms of availability of qualified and skilled personnel and adequate information system (Uzbekistan) to insufficient due to lack of training opportunities, qualified staff and financial resources (Kazakhstan, Kyrgyzstan, Tajikistan). A multidisciplinary approach to prevent and manage zoonoses exists in all countries, as well as collaboration with international organizations, in particular WHO, USAID, CDC, FAO, OIE and UNICEF. The Ministry of Health provides technical assistance via training opportunities and information.

EZF 6 - Strengthening of Institutional Capacity for Regulating and Enforcing Laws that Protect the Public Health from Zoonoses.

This function is the responsibility of the national level. Therefore, the questions under the four indicators – periodic monitoring of regulations; enforcement of laws; technical assistance; and integrated multidisciplinary approach – were answered only by representatives of the Ministry of Health.

This function is completely centralized in Kazakhstan and Uzbekistan, while it has been

decentralized to some extent in Kyrgyzstan and especially in Tajikistan where local authorities have their own boards which independently develop and approve local regulations. The Ministry of Health in all countries can count on its own legal department whose specialists are involved in the preparation of legal documents. Most zoonoses legislation was recently revised in Kyrgyzstan and Tajikistan. Collaboration with international organizations was often mentioned. Prior to the adoption by the Parliament of new laws, a consultative process involving multiple sectors and partners occurs but enforcing laws and regulations seems to be weak. The National Centre for Health Promotion, or its homologous body, plays an important role in informing and educating the population about existing legislation. Websites are another instrument used for ensuring dissemination of information on legislation but their real impact, especially in rural areas, should be assessed.

EZF 7 - Promoting Equitable Access to Necessary Health Services, including Priority Zoonoses-related Services.

The five indicators used for assessing this EZF deal with monitoring and evaluation of access to health services; knowledge to improve access to health services; advocacy; integrated multidisciplinary approach; and technical assistance.

The right to health is stated by the Constitutions of all countries. There is a guaranteed amount of care provided by public hospitals and family medicine centers. In case of some zoonoses (e.g. acute brucellosis in Kyrgyzstan; rabies in Tajikistan) the patient should receive free, or co-payment for, medical services.

In Tajikistan, before the adoption of the National Strategy for Health for 2010-2020, a nationwide assessment was carried out which examined the need for health services and their availability. Nevertheless, because of the limited funding from the State, as well as the low socioeconomic standard of the population, access to medical services is a problem, in particular for rabies post-exposure vaccination in humans. In addition, some groups have limited access to services because of remoteness of their place of residence. The opposite pertains in Uzbekistan, where the system places primary health care within walking distance of homes and no barriers to access of health services were identified, either at national or sub-national level. In Kazakhstan, as well, access by distance is taken into account at sub-national level planning but not at central level. Monitoring and evaluation of access to health services is periodically conducted in all countries in collaboration with other sectors, in particular, veterinary services. Advocacy activities were reported in all countries. The availability of trained staff on community outreach and appropriate methodologies capable of detecting disparities, such

as disaggregated data, sampling and surveys, etc., seem weak in all countries.

EZF 8 - Human Resource Development and Capacity Building in Zoonoses.

This EZF was evaluated through six indicators concerning the public health workforce profile: the improvement of quality of workforce; continuing education; capacity building; integrated multidisciplinary approach; and technical assistance.

The public health workforce profile and assessment of the number of specialists needed are carried out annually. In all countries except Uzbekistan, lack of specialists was reported. In Kyrgyzstan, a special program to encourage young professionals to work in the field is available but results seem limited. Continuing education courses are required every five years in all countries but lack of funding and opportunities for training were mentioned both in Kyrgyzstan and Tajikistan. In the same countries, an integrated multidisciplinary approach has been implemented very effectively for the prevention of zoonoses, and many examples of joint training activities were reported, in particular related to emergency preparedness and brucellosis. A positive example is the development of human capacity under the FAO project to control brucellosis in Tajikistan. Other International organizations are involved in capacity building, such as WHO, USAID, CDC, etc., but these collaborations seem to be scarce in Uzbekistan. Availability of technical assistance was consistently reported. Cultural appropriateness was considered not applicable to the Uzbek and Tajik situations.

EZF 9 - Ensuring the Quality of Personal and Population-based Health Services, including Protection from Priority Zoonoses.

This Function was judged by four indicators: definition of standards; improving user satisfaction; integrated multidisciplinary approach; and technical assistance.

Approaches to ensuring quality of services varied highly among countries. Continuous improvement of the quality of health care is one of the objectives of the Tajik National Strategy for Health for 2010-2020. This document outlines measures to ensure the quality of services, as well as their monitoring and evaluation. The quality assurance system consists of the analysis of reports submitted periodically and on-site inspections aimed at verifying the implementation of existing legal instruments. Tajikistan plans a National System of Accreditation for the Health Sector as an independent external evaluation body within the next decade. Such a body already exists in Kyrgyzstan and Kazakhstan and, in the latter, a semi-independent and decentralized system of quality control has existed since 1996, specifying quality indicators for outpatient and inpatient services and using patient satisfaction surveys.

In Uzbekistan, an accreditation body is lacking and user satisfaction is not used. Technology management and health technology assessment were reported to be centralized functions, except in Tajikistan.

EZF 10 - Strategic Research on Zoonoses.

This EZF was evaluated at central level only by five indicators: inclusion of zoonoses into the research agenda; institutional capacity; conduct of field research; technical assistance; and an integrated multidisciplinary approach.

The EZF received high scores, except in Kyrgyzstan, where it was the second lowest-scoring EZF. In Kyrgyzstan, there are no research plans that include zoonoses. In the other countries, research on zoonoses is included into the public health research agenda coordinated by the Ministry of Health. The Academy of Sciences in Uzbekistan, and the Scientific Centre for Quarantine and Zoonotic Diseases in Kazakhstan, are other important agencies in setting priorities and developing research programs. Only in Kazakhstan, operational research and scientific studies are conducted in collaboration with a number of international partners. Technical assistance is generally provided, and an integrated multidisciplinary approach is implemented, together with the Ministry of Agriculture and the veterinary services.

EZF 11 - Controlling and Preventing Zoonoses in Emergency and Disaster Situations.

Four indicators assessed this EZF: zoonoses control and prevention in emergencies; development of standards and guidelines; technical assistance; and collaboration and co-ordination with other sectors.

Uzbekistan provided no comments. In Kazakhstan and Kyrgyzstan, zoonoses are included in the comprehensive Plan to Combat Emergencies and Natural Disasters and the Plan of Civil Defense for reducing the impact of emergencies and disasters on the population's health, respectively. In Tajikistan, the Plan for Emergency Situations does not include zoonoses with the exception of pandemic influenza. Standard Operating Procedures and instructions concerning the control of zoonoses in emergency situations were developed in Kazakhstan and Kyrgyzstan, while the Ministry of Health of Tajikistan plans to adopt them in the future. Technical assistance is provided through the delivery of medicines and vaccines, the availability of mobile teams and training opportunities. These latter are developed in collaboration with International organizations.

Differences between countries exist. Kazakhstan and Uzbekistan were the countries with the most effective system for human resources development, where opportunities for improving the quality of the work force and education are available both at national and sub-national level. In Kazakhstan, a

coordinating body for capacity building is working together with other health services, veterinary services, city administrations, the National Economic Chamber, business associations, etc. Also in the field of strategic research Kazakhstan received high scores. The Scientific Centre for Quarantine and Zoonotic Diseases, together with Republican Sanitary-Epidemiological Services, are involved in research activities on zoonoses using high technology and in collaboration with several international organizations, such as CDC, WHO, and Liverpool University. There is also an on-going collaboration with other Central Asia countries and with the Bio-safety Association for Central Asia and Caucasus.

In Kyrgyzstan social participation proved to be very high, especially in areas where NGOs – in this case the Swiss and Swedish Red Cross – were working since a long time. Civil society is actively consulted on zoonoses issues, through the Centre for Health Promotion and its branches, health promotion committees, village health committees, village women's councils, and primary health services. Meetings, lectures, discussions, telephone counseling, health education at schools and other activities are regularly carried out. In some regions, local leaders are involved in training activities, as well as are women's councils, action groups, patients recovering from brucellosis, civil society, and elders' councils. This work is done by trained professionals from the offices of health promotion and village health committees.

In Tajikistan social participation was considered well developed. Working with people is carried out mainly through self-governing bodies in towns and villages, through neighborhood committees and through the Centre of Healthy Lifestyles and its branches. In addition, public participation in the activities for prevention and control of zoonoses is provided through collaboration with health professionals and veterinarians, farm foremen, private health care providers, school teachers and mullahs.

Laboratory capacity is, however, a weakness both in Kyrgyzstan and Tajikistan due to outdated facilities and obsolete equipment. Having most health staff at near retirement age constitutes another important constraint in Kyrgyzstan, partially counterbalanced by high levels of motivation and experience.

Veterinary Gap Analysis

Each national veterinary service assessment brings out country-specific needs. There were, however, common themes identified which are summarized under each four main OIE PVS categories below.

Figure 2 indicates that, in 2007 - 2009 when the PVS evaluations were carried out in the various countries, for most of the critical competencies relating directly to zoonotic disease control and response, national veterinary services in Kyrgyzstan,

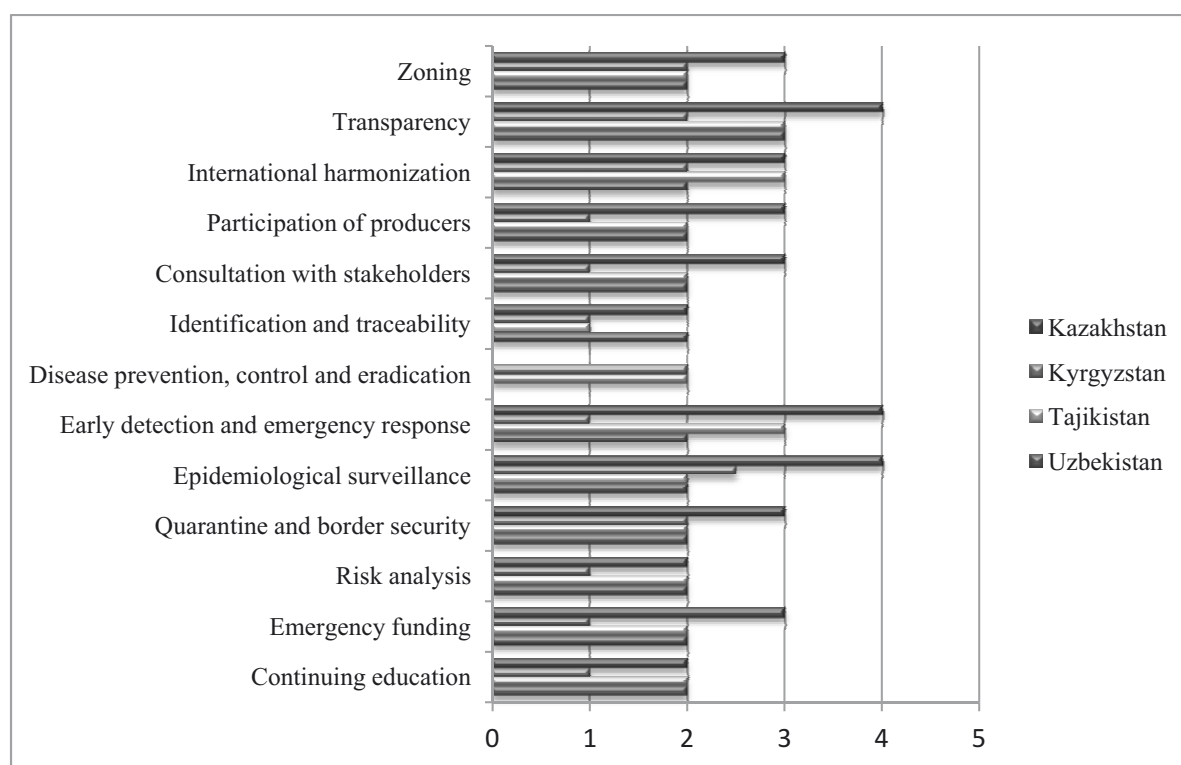


Figure 2 - Current country veterinary service target levels for 13 critical competencies from OIE PVS evaluations in each country, 2007-2009.

Tajikistan and Uzbekistan were evaluated at about level 2. The Kazak veterinary service enjoyed generally a level 3 evaluation for these same 13 critical competencies. The numbers indicate the evaluated level of advancement toward compliance with OIE standards. Level 1 indicates no compliance while level 5 indicates full compliance with standards.

I. Human, Physical and Financial Resources

This category was evaluated through two criteria: continuing education and emergency funding.

There was a need for refresher training in multiple disciplines in all four countries. In several countries, existing institutes were identified, usually in the human health field, where refresher training could be made available provided budgets and management were in place. Interestingly, participants indicated a need for refresher training that meets the current needs of the trainees, not only what was on offer from the trainers and their institutions. In other words, veterinary and public health professionals working in the field were demanding updated and relevant curricula from established academic institutions. Attaining updated and relevant curricula will be a major challenge. The former Soviet Union health curricula did not include principles and practices of modern epidemiology, risk analysis was not a recognized subject, and participatory methods for negotiating with communities were not a feature in the public health repertoire. Where refresher training

institutions exist, participants had no hesitation in combining human and veterinary training in a spirit of intersectoral collaboration. In Kazakhstan, a Steering Committee made up of the three ministries dealing with health and emergency response was proposed to coordinate relevant refresher training.

Emergency funding for public health emergencies was generally forthcoming but often not under the control of the Chief Veterinary Inspector. Likewise, an identified indemnity fund to compensate livestock owners for animals condemned as a control measure for epidemic diseases, e.g. foot and mouth disease, or for endemic diseases, e.g. brucellosis or tuberculosis, was lacking or without clear guidelines for use. Task forces and legal specialists were charged with remedying these deficits. Most Central Asian countries were not anxious to fund compensation for animals slaughtered even if this is an internationally accepted disease control measure, and market price compensation is required for compliance by livestock owners. In Kyrgyzstan, Uzbekistan and Tajikistan, task forces were proposed to draft an 'alternative compensation system' that is socially and economically acceptable to local livestock owners and national veterinary services. The specifics of each alternative compensation system remain to be worked out. But, it is proposed to include replacing slaughtered or removed animals with replacement stock from certified disease-free herds or villages with State authori-

ties rendering the meat safe for consumption and securing a market price for now safe meat.

II. Technical Authority and Capability

Six criteria were assessed under this main category: risk analysis; quarantine and border security; epidemiological surveillance; early detection and emergency response; disease prevention, control and eradication; and identification and traceability.

There was a need in all countries for refresher training in risk analysis and practical, applied epidemiology to use these skills in zoonotic disease control. There was a concerted effort to identify regional 'centers of excellence' where these and other subjects could be taught. Centers of excellence were identified in each of the four countries, with several in Kazakhstan, which is wealthier and more advanced. A private company in Kazakhstan was currently carrying out risk assessment training and indicated their capability for providing biological risk analysis training.

In each country, and in Kazakhstan as a direct instruction from the Ministry of Agriculture, enhancing epidemiological surveillance was a high priority. Retraining and reactivating Disease Registries and Joint Disease Investigation Teams, instituted in the Soviet days but largely in disuse due to lack of funds, were among agreed activities for this criteria. Early detection and emergency response activities were two-pronged. First, collaboration with Finance Ministries was required to agree on the rapid availability of an emergency fund to respond to zoonotic epidemics. Second, the terms of reference for using the funds, compensation agreements for livestock condemned, and obtaining community support were all priority activities. Activities and information for harnessing stakeholders and community group for more rapid identification of diseases were critical and identified. Additionally, various incentive funds and their terms of reference for use were proposed to encourage the earliest reporting of unusual disease events in humans, domestic animals or wildlife. Awards to reporting persons at the village or district levels were in the range of 100\$ – 150\$ for each laboratory-confirmed case.

Brucellosis and echinococcosis/hydatidosis were the two zoonotic diseases mandated for assessment by the World Bank under this regional One Health project. In the veterinary services assessment, activities and budgets focused on awareness raising and capacity preparation at central, province, district and community levels for when specific technical strategies for reducing incidence of these two diseases were implemented. The strategies for controlling these two priority diseases should be epidemiologically based and take into account baseline prevalence surveys in each country. Traceability activities were proposed in select populations, or not at all, in the various countries. Kazakhstan is in the process

of permanently identifying, with a numbered ear tag, all cattle and small ruminants but progress is slower than expected and the intensive commercial livestock sector is covered more fully than the small-holder sector. In Tajikistan, permanent numbered identification was proposed only for the intensive dairy sector and as a component of animal identification for production records. Improving productivity of dairy cattle was foreseen as the driver for individual animal identification. A related 'production driver' was the justification in Uzbekistan where imported cattle will receive individual animal identification and records maintained. In Kyrgyzstan, permanent identification for traceability was considered premature although small ruminants vaccinated against brucellosis are ear notched.

III. Interaction with Stakeholders

The two criteria assessed under this theme – consultation with stakeholders and participation of producers & other stakeholders in joint programs - are related. The degree of interaction with stakeholders was variable and often the interaction seemed to be based on former Soviet Union concepts. In Uzbekistan, intense interaction of field veterinarians with stakeholders at the village level seemed to be a standard operating procedure. Activities in this country centered around an intensive participatory training program, developed by FAO and known as 'PIHAM', which aims to help veterinarians (and others) 'talk with farmers'. That is, talk on an equal footing and deliver messages effectively. In other countries, quarterly Steering Committee meetings at central and province levels were proposed where veterinary, public health and wildlife authorities would rotate the chairmanship and relevant program progress would be reviewed with the view to inform all stakeholders on activities in progress and to coordinate activities such as training, simulation exercises, etc. Additionally in all four countries, existing community or producer committees (e.g. supervising livestock grazing access) would receive technical support for media awareness messages and would be contracted to these disseminate messages. They would also be contracted to assist with zoonotic disease control programs, such as with vaccinations or dog deworming. Selected grass-roots committees have been strengthened as essential outreach partners of veterinary and public health programs and field activities.

IV. Access to Markets

Three key criteria were assessed under this main category: international harmonization; transparency; and zoning.

As marketing is often a strong driver for exports of livestock or livestock products, these commodities should meet international health standards as prescribed under the World Trade Organization. Therefore activities and funds were committed for

1) updating and harmonizing zoo-phytosanitary laws and 2) empowering participation in inter-governmental meetings and standard setting committees. Transparency of the animal disease situation in a country is also a key for facilitating cross-border trade and thus for developing a comprehensive veterinary service that can achieve acceptable levels of livestock health. Transparency was fostered in all four countries through financing exchange visits to laboratories and serosurveys to verify disease prevalence in neighboring countries. Likewise, promoting transparency was considered a fundamental goal of any Central Asian Regional Platform or Regional Body. 'Zoning' is a practice, with standards defined by the OIE, for controlling animal diseases to facilitate livestock trade. In several countries, particularly Kyrgyzstan and Kazakhstan, internationally recognized zones could facilitate trade if funding was allotted to designing such zones. In Tajikistan and Uzbekistan, there was no commercial driver for zones.

During the country workshops, the rapporteurs noted when ideas or options for regional collaboration were proposed and these were further discussed to give a regional dimension to zoonosis control and mitigation. Trust and transparency among the four country veterinary services were two consistent issues with a regional dimension. The Kazak, Kyrgyz and Tajik State veterinary services were generally transparent in maintaining up to date reporting to the OIE. Enhancing trust and transparency in disease reporting were viewed as regional issues where discussions and peer pressure could lead to improvements.

The Ministries of Agriculture and State veterinary services in Kazakhstan and Kyrgyzstan have a need for regional collaboration as each Government is pursuing opportunities for regional trade in livestock and livestock products. Several regional bodies, i.e. the Economic Cooperation Organization, Eurasian Economic Community and the Customs Union of Belarus, Kazakhstan and Russia, aim to form a wide regional 'single economic space' with freer trade in goods and services and common customs tariffs. As inter-regional trade in livestock and their products is envisaged, national veterinary services will need to agree to minimum sanitary standards. Countries (or zones) with surplus livestock or their products and more favorable sanitary status will need to protect this status. The Kazak and Kyrgyz veterinary services in particular will take an active role in setting requirements for movement of livestock and their products within each economic trade region.

DISCUSSION

Our evaluations identified technical, policy and infrastructure gaps which hinder zoonotic disease control in all four countries. And a strategy, action plan and indicative budgets were elaborated for addressing these gaps in each country. Improving

national capacity must be the major focus as the first line of defense in controlling zoonotic (and other) diseases in humans and livestock. It is upon these strengthened national capacities that region-specific technical and policy issues can be addressed. Thus, strategies and actions plans were prepared for each country's public health and veterinary service. Plus a separate strategy and action plan was prepared for tackling relevant region-specific issues.

In particular, the national strategies and action plans included the following key points.

- Joint research programs for zoonoses prevention, control and eradication should be supported as well as further training in applied epidemiology, expanding existing initiatives and using also distance-learning methods.
- Regional Centers of Excellence were identified where refresher education and training could be provided on surveillance and monitoring of zoonoses, on health promotion and social participation, on multidisciplinary training on zoonoses, and designation of a regional reference laboratory for emerging and neglected zoonoses.
- A One Health Regional Platform and Technical Secretariat are required to coordinate zoonotic disease control programs. Agreement must be reached on regional policies on: cross-border animal movements, transparency of disease reporting, compliance with IHR (2005), periodic review of prevalence and incidence data to demonstrate control progress (or lack thereof) and encourage countries to adopt better strategies, support of regional consultation for evaluation of vaccines, laboratory tests, science-based measures, and collection and diffusion of information.

Pilot experiences, conducted at local level with the support of NGOs, have proven to be very effective in promoting the empowerment of civil society in better addressing their health problems. They also have been a very powerful tool for increasing inter-sectoral collaboration and setting up coordination mechanisms between all interested stakeholders. The high level of social participation recorded in Kyrgyzstan and Tajikistan is the best proof of their effectiveness, less hampered by budget constraints than Government institutions. These examples should be collected and used, after appropriate adaptation, as best practices in all four countries. For that purpose, it was suggested to implement a document center in each country and to support their coordination through the organization of regional conferences, meeting and similar events, in which scientific foundations could play a relevant role.

Additionally, all four Governments have a vested interest in regional cooperation to reduce the incidence of zoonotic diseases and diseases of trade to reduce the pressure of re-introducing these diseases from neighboring countries. Of particular concern are foot and mouth disease (FMD), anthrax and

brucellosis which can be reintroduced via live animal movements or trade in livestock products. The Central Asian countries might usefully be considered as one large ecological zone for zoonotic and other disease control purposes.

A proposed One Health Regional Platform and Technical Secretariat could be a forum for animal, human and environmental health issues in the region relating to control, prevention and reaction to zoonotic diseases. The comparative advantage of a One Health Regional Platform is to focus on wider regional issues identified during both the public health and veterinary assessments of the four countries. These wider issues are of a policy nature, technical and institutional within each country and across borders. Several examples are given above but also include evaluation of national disease control programs via periodic serosurveys, cross-border movements of livestock for grazing and applied research into cross-border issues such as periodic high mortality in Saiga antelope (*Siaga tatarica*) in Kazakhstan and Uzbekistan. These wider animal health issues are also fully supportive for control of non-zoonotic transboundary animal diseases such as FMD. Likewise, the public health issues under any One Health Regional Platform will of necessity be consistent with and supportive of WHO's regional and global initiatives such as disease reporting obligations under the IHR. In time, the three other countries included in the Central Asian cluster (Afghanistan, Pakistan and Turkmenistan) may join a Central Asia One Health Regional Platform and Technical Secretariat.

Knowledge on risk factors affecting zoonoses transmission in the region is still scarce and studies are required to better target prevention and control measures. A review of existing knowledge could be useful as well. The knowledge, attitudes and practices (KAP) method could be an useful tool to expand understanding of zoonoses epidemiology in Central Asia, as shown by the One Health Network project which successfully implemented this methodology to study the perception of cysticercosis in eastern Zambia and echinococcosis/hydatidosis in Morocco and identify sociologically acceptable control methods. Ramlawi (Ramlawi, 2009) makes a definitive review of KAP studies on brucellosis in Eurasia and the Middle East.

To foster intersectoral collaboration, a horizontal approach should be preferred as many prevention and control measure will have a positive impact on more than one infection. In addition, according to the Health in All Policy (HiAP) approach promoted by WHO, measures affecting zoonoses epidemiology are not limited to the health (in a broad meaning) sector. As reported by Stahl (Stahl *et al.*, 2006), major zoonoses – both “neglected” and emerging – share many risk factors, facilitating the implementation of systematic responses. Instead of seeing zoonoses as a challenge to the health sector only,

HiAP highlights the fact that the risk factors, or the determinants of health, are modified by measures that are often managed by other Government sectors as well as by other actors in society. Broader societal health determinants – above all, education, employment and the environment – influence the distribution of risk factors among population groups, thereby resulting in health inequalities.

As reported by WHO (WHO, 2011a), neglected zoonoses, in particular, are strictly linked with wealth distribution, primarily affecting poor rural populations who keep livestock. The burden of neglected zoonotic diseases, measured through the disability-adjusted life year (DALY), has been poorly quantified. Nevertheless, the health burden is only one component of the impact of these neglected diseases. The increased mortality and reduced productivity of livestock that are associated with zoonotic diseases compromise food security and adversely affect opportunities for income generation.

Even if our study focused on neglected zoonoses, the concept of zoonoses is very wide. According to WHO (WHO, 2011b), it includes a diverse range of conditions caused by infectious agents (viruses, bacteria and parasites) that can be transmitted from animals (mammalian and avian) to humans either directly, or indirectly by fomites or by insect vectors. Since the 1980s, new zoonotic diseases such as BSE, avian influenza, severe acute respiratory syndrome (SARS) and influenza A(H5N1), have emerged and caused global outbreaks. The cost to national economies of these outbreaks via reductions in international trade and tourism is billions of dollars. The potential impact on global public health and the possibility of further financial losses has led to large-scale international support for the development of strategies for early detection, control and mitigation of the impact of these diseases.

Zoonotic diseases have no national borders. Migrations of wild animals can carry pathogenic agents to other countries. Even when rules against animal importation are in place, advertent and inadvertent transgressions of such rules commonly occur. This means that the effective management of zoonoses can be a difficult and expensive undertaking for a single country.

Trade, technology and travel are assisting in the expansion of zoonotic diseases into new locations and with higher frequencies. The resulting negative health and socio-economic impact are increasingly being experienced by many countries, particularly developing countries. These countries, with fragile economies, cannot afford to be impacted by outbreaks of zoonotic infections. The recent outbreak of *E. coli* in Europe shows that all countries have a reason to invest and cooperate in the prevention of zoonoses. Yet, in these countries, the establishment and implementation of adequate measures for livestock and consumer health protec-

tion against zoonoses, especially those that are new and emerging, has proven to be very difficult. Thus, zoonotic diseases will continue to burden public health and clinical care systems, as well as impact on animal health and productivity (WHO, 2005). Nevertheless, we can and should do better.

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The possible role of entomological surveillance in mosquito-borne disease prevention

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Summary - Current GIS technologies and laboratory diagnostic capacities are strongly improving the potentiality of vector monitoring and vector-borne disease surveillance programs. When the entomological/veterinary surveillance activities are organized and implemented with the support of a strong baseline of bio-environmental data set, they are showing to be of high usefulness for the early detection of invasive exotic mosquito species and the definition of the colonized area; the risk assessment of vector borne diseases such as Dengue and CHIK throughout vector density estimation at local scale; the surveillance of arboviruses activity in large areas; and the support to epidemiological understanding of vector borne diseases. The efficiency of the surveillance program may be optimized and related costs reduced, by the progressive introduction of GIS satellite supported technologies, by the progressive understanding of the role played by environmental determinants, and by the introduction of more efficient methods of sampling.

Key words: *Aedes albopictus*, invasive mosquito, dengue, Chikungunya, West Nile

INTRODUCTION

Vector borne diseases are threatening a large part of the world human population and seem to make roots in temperate regions previously considered at low risk. Evidences indicate that factors driving this new phase are currently more related to globalization of human activities than to climate change (even if the two aspects can not be clearly distinguished for certain parts), including movement of people (travelers, workers and refugees), animals, and goods (i.e. used tires and ornamental plants).

Environmental changes related to new agricultural practices and wetland restoration, influence the wildlife species as well as the vector species compositions opening the way to new epidemiological scenarios.

Improvements in diagnostic tests and surveillance capacity play of course a relevant role in evidencing the situation, especially in the case of rare or mild diseases, which were probably much less diagnosed in the past.

Human movements through the globe have initiated

the spread of invasive mosquito species and related vector-borne diseases centuries ago and the ongoing globalization is just accelerating the phenomenon (Reiter, 1998).

Several mosquito species have been introduced recently into Europe, such as *Aedes albopictus*, *Ae. aegypti*, *Ae. japonicus*, *Ae. atropalpus*, *Ae. triseriatus* and *Ae. koreicus*, all sharing the ability to develop in artificial containers and to rely on dry resistant eggs.

Of these species *Ae. albopictus* has proven its capacity to act as a vector in temperate region, being responsible of the Chikungunya virus epidemic in northern Italy in 2007 (Angelini *et al.*, 2007; Bonilauri *et al.*, 2008), and of Dengue virus transmission in France and Croatia in 2010 (La Ruche *et al.*, 2010; Gjenero-Margan *et al.*, 2011).

Ae. albopictus is already established in Albania, Bosnia and Herzegovina, Croatia, France, Greece, Italy, Montenegro, Spain, and Switzerland, with the potential to expand its distribution in Europe. The key of its successful adaptation to temperate

climates is the egg diapausing mechanism enabling the permanent establishment in temperate regions, despite the high mortality during the winter period. Evidences indicate that international dispersal mainly exploits trade of used tires and of ornamental plants to a less extent, while local dispersal is easily obtained by ground transport vehicles. *Ae. albopictus* mainly exploits man made containers thus showing distribution mostly related to inhabited areas, while very low presence is observed in rural and natural areas. This distribution has important implication on the vector related sanitary risks.

Thus it becomes necessary to develop capacities to promptly detect introduction of new mosquito species and pathogens, as well as to keep under surveillance the sanitary risk related to the known vector-pathogen systems. This should be done at the country/regional level in a comprehensive EU coordinated frame.

The entomological surveillance, when supported by strong baseline data, may offer the capability for early detection of arbovirus circulation in a specific area.

The case of West Nile virus (WNV) is a good example of this possibility. WNV is one of the most extensively distributed flavivirus worldwide. A large number of wild and domestic bird species may serve as reservoirs of WNV, while transmission is mainly performed by *Culex* species. Mammalian species, including humans and horses, are terminal hosts, unsuitable as virus reservoirs because of low viral titers they develop.

Several outbreaks have been registered in Europe, the largest were in southeastern Romania in 1996 and in Central Macedonia (Greece) in 2010.

The epidemiological picture of WNV is complex being influenced by a number of environmental, climatic and faunistic parameters, often obscuring the role of vector density. The recent evidences obtained in Europe confirm the primary vector role of *Cx. pipiens* (a complex including *Cx. pipiens pipiens*, *Cx. pipiens molestus* and intermediated hybrids) in the amplification as well as in the human infection. The virus is able to overwinter in temperate regions developing a certain level of endemicity. Cost benefit analysis of concurrent surveillance methodology/systems may be conducted on case-by-

case basis providing that the optimization of methods and procedures may progressively be achieved. It is of relevance that the European Centre for Disease Prevention and Control (ECDC) has just launched an initiative to produce guidelines for implementing surveillance/monitoring of invasive mosquitoes (ECDC, 2011) in order to assist EU Member States and EEA/EFTA countries.

MATERIALS AND METHODS

Invasive mosquitoes

Thorough assessments are necessary to actively prevent and/or control the introduction and establishment of new mosquito species in previously free territories. One of the key issues to be carefully analyzed is the selection of sites of possible introduction through the creation of a priority check list. This issue requires a thorough knowledge of the region under surveillance to efficiently consider the sites with a high risk of importation from far away regions (i.e. used tire facilities, plant import companies, harbors, airports, freight containers, container terminals) and from neighboring already infested countries/regions (i.e. border crossings, rest areas and petrol stations along traffic paths, and facilities for local transport and trade). An adequate balance/compromise between risk and effort must be progressively developed on the basis of a continuous long term managed effort.

Other key issues to be considered are the collection/trapping methods tailored not only to species biology/behavior but also (Tab. 1) according to analyses of cost efficacy adapted to local conditions; the possible engagement of municipality/local people in the monitoring activities; and the level of information to the public.

An emergency plan should be prepared with clear and complete organization of the surveillance and control activities and attribution of responsibilities, to be adopted in case of detection of invasive species.

In Emilia-Romagna, since 2007, it has been established a working group coordinated by the General Direction for Health and Social Policies composed by physicians, veterinarians and entomologists with the aim of creating a regional surveillance and risk

Table 1 - Scheme of relative usefulness of some mosquito collection methods related to newly introduced species

	CO:trap	Light trap	BG sentinel	Ovitrap	Gravid Trap	Sticky trap	HLC	Larval inspection
<i>Aedes albopictus</i>	+	-	++	++	-	+	+++	++
<i>Aedes aegypti</i>	+	-	++	++	-	++	+++	++
<i>Aedes japonicus</i>	+/-	-	-/+	-/+	+	+	-	++
<i>Aedes atropalpus</i>	+	-	?	+	-	?	++	++
<i>Aedes koreicus</i>	?	?	?	+	-	?	?	++
<i>Aedes triseriatus</i>	++	?	++	+	?	?	+++	++

Legend: - low efficacy/efficiency; + fair efficacy/efficiency in some situation; ++ good efficacy/efficiency; +++ excellent performances; ? not known

assessment system based on multidisciplinary network with the capability of collecting data about both vector populations dynamics and possible presence of pathogens in vectors, men and animals. Referring to the entomological surveillance on invasive mosquitoes the work of the group is dedicated to keep under control sites potentially involved in the introduction of new species, such as Ravenna seaport and companies importing used tyres. Near these sites some traps are located and entomologists conduct periodical inspections to collect larvae in potential breeding sites.

Established mosquitoes

In case of sanitary surveillance focused on already widespread species (autochthonous and exotic) the entomological surveillance may result highly efficient in producing reliable information (Tab. 2).

It may be useful to distinguish between entomological surveillance aimed at estimating the population density distribution for the direct implication it has on the epidemiological risk (i.e. the *Ae. albopictus*-Chikungunya or *Ae. albopictus*-Dengue binomials), and entomological surveillance aimed at collecting mosquito samples to be directly screened for pathogens.

In any case it is important to standardize trapping techniques and to organize a network sensitive enough to detect temporal and geographical variation of population density at the local scale and/or to collect sufficiently significant samples to be screened for pathogens.

The "classic indices" used to evaluate *Stegomyia* population densities such as the House Index (HI: percentage of houses with at least one active breeding site), the Container Index (CI: percentage of containers with larvae), and the Breteau Index (BI: number of active breeding sites per 100 premises), still widely used in tropical countries are of limited value in Europe because of the high requirement of man power and the contribution of important breeding sites in public areas (i.e. road drains) on the productivity per unit area. Other indices such as the PPI (number of pupae/premise), the PHI (number of pupae/hectare), the PDS (Pupal Demographic Survey), and the API (adult productivity index), which defines the mosquito density per unit area, considering both public and private domains, are

well correlated with ovitraps data but more expensive to perform and standardize (Carrieri *et al.*, 2011a). As a consequence in Emilia-Romagna the surveillance of *Ae. albopictus* in urban contexts, aimed to assess the epidemiological risk of Chikungunya and Dengue transmission, is based on the use of ovitraps as a tool for mosquito population density estimation. During the favorable season (May-October), about 2,800 ovitraps are activated in the urban areas of 242 municipalities according to standard criteria and checked bi-weekly.

Referring to the surveillance of mosquitoes in rural contexts, aimed to assess the circulation of viral pathogens, in Emilia-Romagna the WNV surveillance system is based on the regular (bi-weekly) collection of mosquitoes in the period June-October. Mosquito collections is conducted using 90-100 CO₂ baited traps positioned in fixed stations in a grid of 10x10 km, to cover the surveillance area. The collected mosquitoes are managed in a cold chain, pooled (max 200 individuals) by species, date and site of collection and examined by RT-PCR pan-Flavivirus, and in case of positivity to species specific RT-PCR. When virus activity is low a super-pool strategy is applied to reduce the number of analysis (Calzolari *et al.*, 2010).

Geographic information systems

In recent years, the use of the Geographic Information Systems (GIS) is providing important practical contributions to the investigation and understanding of the spatial component of the epidemiology of vector-borne diseases such as malaria, trypanosomiasis, rickettsiasis and a range of arboviral diseases. The collection and thorough management of georeferenced epidemiological data is useful in the investigation of possible environmental/climate explanatory parameters.

Global and local indicators of spatial association like Moran I or Getis-Ord statistics may assist in the measure of data clustering level.

Geostatistical techniques are also used to produce prediction surfaces and level of uncertainty for these surfaces, which provides an indication of how good the predictions are.

The mapping of the vector population density geographic distribution may provide information both on the environmental variables that drive

Table 2 - Scheme of relative usefulness of some mosquito collection methods for established mosquitoes of sanitary importance

	CO ₂ trap	Light trap	BG sentinel	Ovitraps	Gravid Trap	Sticky trap	HLC	Larval inspection
<i>Aedes albopictus</i>	+/-	-	++	++	-	+	+++	++
<i>Culex pipiens</i>	+++	+	+	-	+++	-	-	-
<i>Aedes japonicus</i>	+/-	-	-/+	-/+	+	+	-	++
<i>Anopheles</i>	+	+	?	-	-	?	+	-

Legend: - low efficacy/efficiency; + fair efficacy/efficiency in some situation; ++ good efficacy/efficiency; +++ excellent performances; ? not known

species development, and on the epidemic diseases risk level, which are essential to developing effective disease prevention programs, particularly for Chikungunya and Dengue.

In the Emilia-Romagna *Ae. albopictus* surveillance system, the universal kriging interpolation is used to estimate the seasonal abundance of the species at unsampled locations, while the spatial cluster analysis is used to identify particular areas that had statistically significant high or low mosquito density.

Model parameters obtained by variogram analysis were used in an ArcGIS Geostatistical Analysis to obtain prediction maps, the quality of which may be examined by creating a prediction of standard error. The predicted standard errors quantify the degree of uncertainty for each location on the surface.

The extrapolation and interpolation of data need to be conducted with caution, and the production of computer-generated maps that appear to be more informative than the data upon which they are based, should be avoided. Bearing this in mind, contour smoothed maps obtained from geostatistical analyses and cluster maps obtained from cluster detection can be overlaid on other smoothed informative layers to identify environmental variables such as elevation, rainfall distribution, mean air temperature and relative humidity, that could influence seasonal mosquito population densities in the region. These maps can also be overlaid on epidemiological data to identify health risks.

RESULTS

Invasive mosquitoes

The experience matured in Europe in the case of *Ae. albopictus* invasion (VBORNET vector maps: <http://ecdc.europa.eu>) indicates that, where attempts to early detect and eliminate the invasive species when still confined to a limited area at the beginning of the colonization process have been implemented locally, without a country level of coordination and support (in Italy, Switzerland, Spain) only limited temporarily successes were achieved. Even in Southern France where a more organized plan was deployed the species is rapidly spreading. This is probably because the large number of vehicles coming from infested Italian areas bringing inadvertently mosquitoes on board directly into the towns, where the species is difficult to locate in the initial colonization phase. Huge efforts and investments are deemed necessary to organize a preventative program in the case the invasive species has already achieved a wide distribution range in the continent, with high population density pushing continuously for expansion.

Ae. japonicus has also been introduced, probably via the used tire or the ornamental plants trade (the way of introduction has been hypothesized), from the original Asian area to USA and Central Europe (Austria, Belgium, France-eradicated, Slovenia,

Switzerland and Germany) where it is spreading (Schaffner *et al.*, 2009; Becker *et al.*, 2011; Schneider, 2011). This species has been tested a lab competent vector for several arboviruses (i.e. West Nile and Japanese encephalitis), and found positive in the field (i.e. West Nile in the US), but its real medical importance remains to be clarified.

Looking at the distribution the species has achieved in the US (see at <http://www.rci.rutgers.edu/~insects/ojdist.htm>), where the invasion started about 10 years before than in Europe, it seems that the species has the potential to colonize large part of Europe.

Aedes aegypti originated in Africa has progressively colonized tropical and subtropical areas around the world. It is highly anthropophilic and synanthropic and notorious as the vector of the yellow fever, chikungunya and dengue viruses. In the first half of last century it was present in southern Europe and involved in deleterious dengue epidemics in Athens in the 1927-28 (Theiler *et al.*, 1960), but disappeared afterwards. Nowadays it is spreading along the Black Sea coast (since 2004), was introduced to Madeira (2004) and in the Netherlands (2010). In the Netherlands, *Ae. aegypti* was found at a company that imports used tires and presumably imported by a tire shipment from Miami together with *Ae. albopictus*. It is currently intolerant to cold temperatures (no diapausing eggs) that will limit possible northerly spread in Europe.

Another exotic species that recently showed up in Europe is *Ae. koreicus*, a poorly known species with Asian distribution, recently detected in Belgium during a research study (Modirisk, 2009) and in northeastern Italy (Capelli *et al.*, 2011). The relevance of this species in terms of possible sanitary impact is largely unknown.

The one Nearctic species invasive to Europe, *Ae. atropalpus*, was first observed in Italy in 1996 (Romi *et al.*, 1997), at used tires storage company, and was most probably collaterally eradicated by the treatments against *Ae. albopictus*. Since that, it is observed in France (2003) and Netherlands (2009, 2010). The species is native to Central and North America, up to southeastern Canada, and probably would have high potential for establishing itself in Europe. In United States is mainly considered a nuisance species that readily bites humans. There, it is found positive for WNV in nature but vector status of this species is still unclear.

One more temperate climate, Nearctic species native to North America (Southern Canada and the eastern United States, south to the Florida keys and west to Utah and Idaho) that has potential to invade Europe is *Ae. triseriatus*. This species is the most widely distributed tree hole-breeding mosquito in North America. The larvae develop occasionally in artificial containers such as wooden tubs, barrels, and watering troughs. Females are included in second tier of mosquitoes causing nuisance in the USA (McKnight, 2005). *Ae. triseriatus* is vector of La

Crosse virus and potential vector of West Nile virus in North America. In Europe, it is intercepted only once, in France (2004) in used tyres imported from USA, but is potentially hazardous species having the diapause in egg stage.

From the available evidences it is clear that container breeding species are the most favorites to be passively introduced and established in new regions. This pose a number of questions related on the possible regulation of the international trade of goods that may be exploited by these species, which appears to be the best strategy to develop in order to reduce the risk of new invasions.

It may also be underlined that surveillance programs based on ovitraps are of limited usefulness in term of detection of possible new species because of the difficulties in discriminating the species at the egg stage, and the amount of labor required in egg hatching and larval development to achieve older stages (larva or adult).

Established species

In the case of Dengue and Chikungunya viruses, both strictly connected to the binomial *Ae.albopictus*/human, the vector population density play a key role in the epidemiological equation (Fine, 1981; Reisen, 1989).

$$R_0 = \frac{m \ b \ S_m \ V \ S_v \ p \ i}{(- \log_e p)}$$

where:

m is the mean number of bites per human per day;
b corresponds to 1/GC, were GC is the duration of the gonotrophic cycle;

S_m is the species vector competence;

V is the period during which the infected host has a sufficient viremia to infect the mosquito vector;

S_v is the proportion of the human population sensitive to the infection;

p is the female mosquito daily survival rate;

i is the duration of the extrinsic incubation period of the virus in the vector.

Of course the number of bites/human/day depends on several factors, some of which relate to the human socio-economic condition determining the

level of exposure to mosquito bites, including the vector population density.

We may directly influence vector density through mosquito control campaigns.

In this context it is important to know if the vector density in a certain area may be able to sustain an epidemic in case the introduction of the virus through a viremic person. Thus an adequate system of quantitative monitoring has been implemented in the Emilia-Romagna region to provide real time data at a fine geographic scale through the use of ovitraps positioned in a statistical sound network using the Taylor model (Albieri *et al.*, 2010; Carrieri *et al.*, 2011b).

The number of ovitraps to produce values of Relative variation in the range $0.2 < D < 0.3$ has been considered sufficient (about 2,800 ovitraps). This method provides several advantages over other methods, including high sensitivity, ease of field management, and low management costs. Ovitrap data reliability, in terms of quantitative estimation of adult population densities, is controversial and questionable in the tropics while it seems sufficiently sensitive and precise in temperate regions (Tab. 3) (Carrieri *et al.*, 2011a, 2011c).

This system has also some disadvantages such as the strict selectivity for container breeding species, the unavailability of adults for virus screening, the difficulty in determining species at the egg stage (so in case a new *Aedes* species is introduced the system may not be able to discriminate the eggs).

Standard ovitraps currently in use allow a biweekly inspection requiring 10-11 checks per season, producing data that are processed with GIS geostatistical analysis to obtain maps, as the one reported in Figure 1, with information which are regularly publicized on a dedicated website (www.zanzaratigreonline.it).

In the case of WNV surveillance plan in Emilia-Romagna, the entomological and veterinary surveillance, when properly organized, have proven useful to early detect the virus circulation 3-4 weeks before the appearance of human cases (Fig. 2). When validated for a sufficient number of years the surveillance system may allow for the adoption of public health measures only in case they are really needed.

Table 3 - Pearson product moment correlations (R) between mosquito population indices and the mean number of eggs/ovitraps/week collected the week before, the week of and the week after the inspection (from Carrieri *et al.* 2011a)

Population Indices	Mean number of eggs/week/ovitrap		
	Previous week	Inspection week	Week after inspection
HI - House Index	0.0867	-0.1117	-0.3778
CI - Container Index	0.3194	0.0482	-0.4175
BI - Breteau Index	0.0623	-0.1465	-0.4313
PPI - Pupae/premise	-0.0289	-0.2553	-0.5118
PHI - Pupae/ha	0.1703	0.3396	0.8622*

*P < 0.01. HI: percent of houses with at least one positive container. CI: percent of infested containers. BI: Number of positive containers/100 houses. PPI: number of pupae per premise. PHI: number of pupae per hectare.

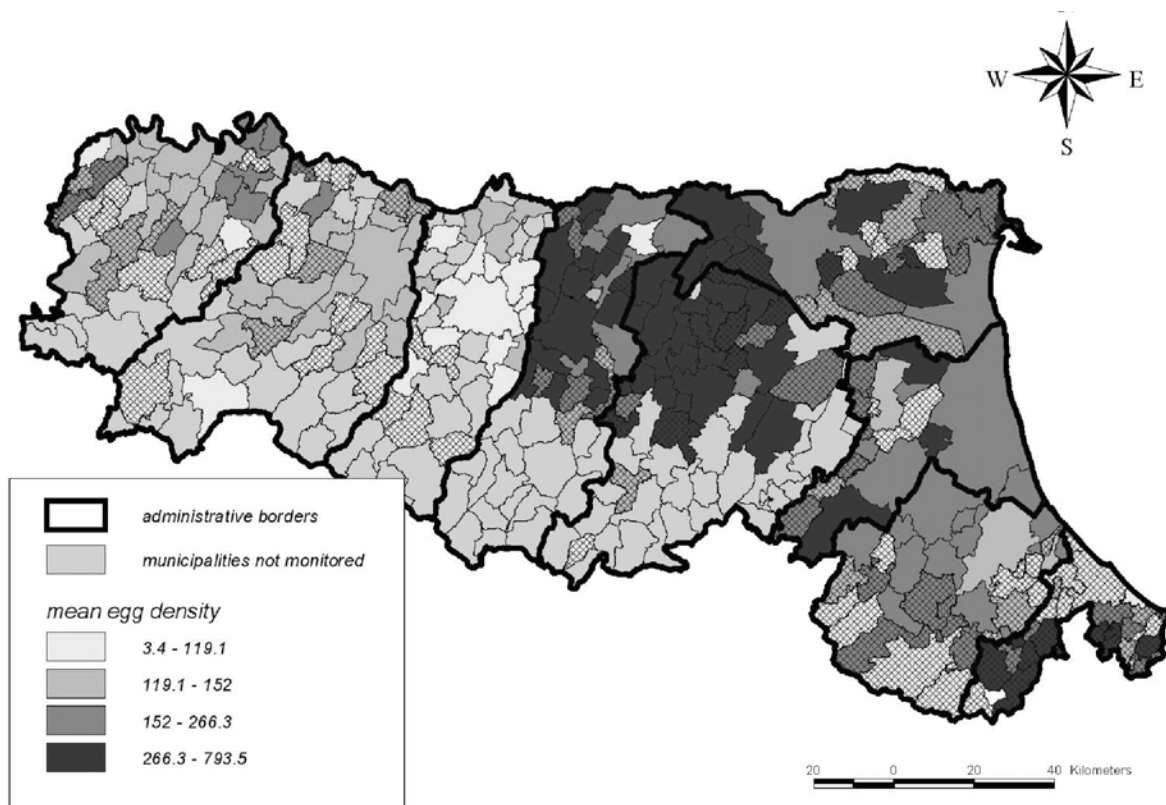


Figure 1 - Choropleth map of *Aedes albopictus* mean egg density (number of eggs/ovitraps/week) during the season 2011 in the Emilia-Romagna Region (Italy) calculated for 10 bi-weekly data. Legend values are subdivided into quartiles; wired polygons represent municipalities with sampling designs that were not statistically efficient for measuring true population densities for $RV \leq 0.3$.

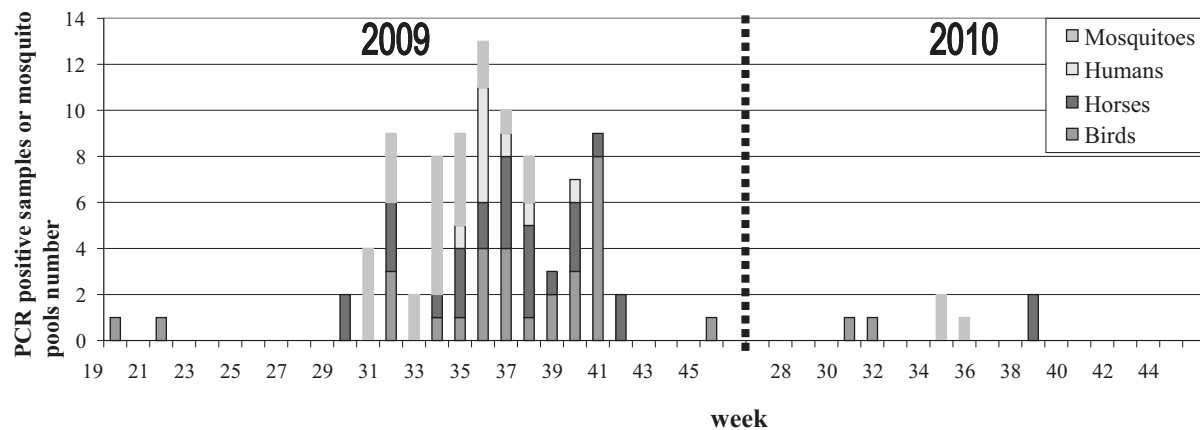


Figure 2 - Seasonal dynamic of WNV positivity obtained during the 2009 and 2010 seasons.

These measures include the information campaign regarding personal protection precautions to be adopted by citizen in risky areas, eventually vector control operations, policy on blood and organs donations.

The WNV surveillance plan in the Emilia-Romagna region detect another arbovirus which resulted highly active in the last three years (2009-2011): Usutu virus (USUV). This virus seems to co-circulate with the WNV, using *Cx. pipiens* as the main vector, and several birds species as hosts. Its

epidemiology needs to be investigated to clarify the role of non-bird hosts and of *Ae. albopictus* as possible secondary vector (Weissenböck *et al.*, 2003, Tamba *et al.*, 2011).

CONCLUSIONS

Vector monitoring and vector-borne disease surveillance programs when applied in a properly organized way, are showing to be of high usefulness for the: (i) early detection of invasive exotic mosquito species and relative infested area definition; (ii) risk

assessment of vector borne diseases such as Dengue and CHIK throughout vector density estimation at local scale; (iii) surveillance of arboviruses activity in large areas; (iv) support to epidemiological understanding of vector borne diseases.

The entomological surveillance may be conveniently integrated with medical and veterinary surveillance activities to produce a more comprehensive understanding of the situation and to better planning the use of resources.

When integrated with meteorology, environmental spatial techniques and informatics/statistics the entomological surveillance may be able to produce output of information that go to a fine scale, allowing explanation or hypothesis to understand observed phenomena.

Modeling may produce the best outcome in term of explanation and/or prediction when monitoring/surveillance are well planned on statistical bases and on baseline knowledge of biology of the involved species and ecology of the interested territory.

The capacity to early detect the presence of invasive species and define the size of the colonized area is fundamental to increase the chances of elimination of invaders at the beginning of the colonization process with much less efforts than it would need in case of wide infested areas. A number of eliminations of new populations of *Ae. albopictus* have been achieved in Italy, France and Serbia when the species was confined to a used tire company and surrounding area or border crossing.

It has been demonstrated on several occasions within different countries and environmental conditions that it is possible, and perhaps highly convenient in term of cost-benefit balance, to eliminate an invading mosquito species by promptly applying intensive suppression methods if the colonized area is still well delimited.

A country level specific evaluation of the most probable way/sites of entry of the species could be conducted to assist a focused surveillance plan aimed at the prompt detection and elimination of the species. Cost comparative analysis of this approach compared with the control cost (including the sanitary cost and risk) in case of wide colonization of the country may produce an indication of the financial support to be invested in the preventative measures.

The main concern the southern Europe countries are facing is named *Ae. aegypti*, tropical widely present species, which recently implanted a bridgehead in Madeira island and Abkhazia (Almeida *et al.*, 2007). Frequent and rapid connections from these areas to climate prone regions pose a clear risk of introduction and establishment in the continent. It may be of significance to recall that this species was quite commonly found in the Mediterranean ports until the beginning of the 19th century, and still is one of the major vector of Dengue worldwide. A coordi-

nate international effort aimed at the elimination of *Ae. aegypti* from Madeira and Abkhazia must be evaluated as a preventative measure to protect southern Europe.

The ability of European countries to obtain data on the presence and abundance of invasive species and to develop efficient control programs and tools for their evaluation needs to be rapidly and consistently improved in order to increase the chances for early detection and elimination of invaders at the beginning of the colonization process.

Where the invading species is established on a large area (large town or region), monitoring of population abundance is needed with standardized methods on a long term basis to perform a risk assessment of arbovirus transmission such as dengue and Chikungunya, obtain data about the evolution of the vector density and guiding the planning of information/control campaigns.

On the basis of the current available technology the possibilities to develop well focused and cost benefit surveillance programs are certainly increasing with important benefit for the prevention of vector borne diseases.

WNV surveillance based on mosquito collection coupled with the rapid laboratory screening for arbovirus presence has proven reliable in terms of sensitiveness and precociousness in the detection of virus circulation. This is particularly useful in the case of periodical incursion of the virus in a geographic area, with silent periods sometimes lasting many years. By adopting an active entomological surveillance it is possible to assist the public health authorities in the target adoption of preventative measures only in case of real need.

The efficiency of the surveillance program may be optimized and related costs reduced, by the introduction of GIS satellite supported technologies, by the progressive understanding of the role played by environmental determinants, and by the introduction of more efficient methods of sampling.

Depending from the collection methods utilized, the entomological surveillance may provide information on the possible activity of other arboviruses. In the case of CO₂ baited traps, considering their efficacy in collecting several haematophagous species, it is possible to maintain under surveillance not only mosquito borne viruses, but sand fly, biting midge and black fly borne pathogens as well.

ECDC is engaged in stimulating the awareness and the capacities of European countries to organize and conduct surveillance and control of mosquito vector-borne diseases. The European Spatial Agency (ESA) is also on the ground by promoting better exploitation of remote sensing satellite capacities in the field of vector surveillance and by supporting the VECMAP initiative inside the Integrated Applications Promotion (IAP) ESA ESTEC, an integrated spatial tool and service for modeling the distribution of mosquito vectors of disease.

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Rift valley fever: the risk of introduction into Italy

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Summary - Rift Valley Fever (RVF) is a serious disease transmitted by arthropods which strikes humans, and domestic and wild ruminants. The virus is widespread in Africa and has also recently been found in some areas of Yemen and Saudi Arabia. Epidemic breeding grounds of RVF are particularly frequent in Africa and the Middle East, probably due to climatic and environmental conditions, and the increase in the commercial exchange of live animals. The probability of the introduction of the virus into Europe and of its diffusion on a large scale is considered to be low. Nevertheless, it is not possible to completely exclude the possibility that breeding grounds of the disease can appear, in particular, in the southern areas of Europe. If this happened, it is probable that human cases of the disease could be reported in particular categories at risk, such as breeders, veterinarians and butchers. Surveillance systems and diagnostic instruments are widely available but the instruments necessary for controlling the disease are limited; controlling the population of the vectors is an activity which is difficult to carry out, and vaccination is possible quickly and on a large scale only in ruminants. Furthermore, the current vaccines which can be used on animals have limited efficacy (inactivated vaccines) or can cause serious collateral effects (live attenuated vaccines). The most efficacious strategy for protecting Europe (and also the rest of the world) from the diffusion of RVF is the implementation of efficacious surveillance systems and programs for controlling the disease in endemic areas.

Key words: Rift Valley Fever, Italy, risk

INTRODUCTION

Rift Valley Fever (RVF) is an acute viral disease which strikes both animals and humans; it is transmitted by arthropods belonging to more than thirty different species. The disease strikes numerous species of domestic and wild ruminants causing acute hepatitis with high mortality in young ovines and bovines, and, in adults, abortion in all stages of gestation. Rift Valley Fever is a serious zoonosis characterised by an influenza-like syndrome in humans which is sometimes complicated by haemorrhage, visual disturbances and nervous symptoms. The World Health Organization, FAO and OIE consider RVF to be an extremely serious disease due to its potential negative effects on the health of humans and on the productivity of animals.

Rift Valley Fever was clinically detected for the first time in 1913 in the Rift Valley in Kenya; in that country, the virus was isolated for the first time in 1931. The disease was confined to the sub-Sahara in Africa for a long time but, in 1977, it spread first to Sudan and then to Egypt, along the Nile Valley, arriving at the delta. In 2000, it was seen in the humid zones of the Arabian Peninsula as a consequence of the importation of live animals from the

Horn of Africa while, more recently, it was reported in Senegal and Mauritania.

Rift Valley Fever is an exotic disease for Italy and the other European countries, and is considered to be at potential risk of introduction and diffusion (EFSA, 2005).

The Rift Valley Fever Virus (RVFV) belongs to the *Bunyaviridae* family, *Phlebovirus* genus. It is a virus with an *envelope* characterised by a genome with segmented RNA, a characteristic which conditions the possibility of recombination taking place; a single serotype has been reported and strains differ on the basis of their virulence. The RVF virus is antigenetically correlated with other *Phleboviruses* and this can create problems of specificity in the serological diagnosis of the infection.

It is considered a rather resistant virus; in the blood and in serum, at a temperature of 4°C, it can resist for a number of months and, after heat treatment at 56°C for 120', it maintains its infective capacity. It is inactivated by lipid solvents, by low concentration formalin (0.25%) and by high concentrations of sodium and calcium hypochlorite.

Virus isolation can be performed in suckling or weaned mice by intracerebral or intraperitoneal

inoculation or in a variety of cell cultures including Vero, BHK21 or mosquito line cells. The RVFV can be identified in cell cultures by immunofluorescence, virus neutralisation, reverse transcriptase polymerase chain reaction (RT-PCR) and/or genome sequencing. Virus isolation is the gold standard for RVF diagnosis.

Epidemiology

Geographic spread

Rift Valley Fever is endemic to or has been reported in numerous eastern, southern and western African countries (Fig. 1). In 2000, it was also observed in the Arabian Peninsula as the result of the importation of live animals from the Horn of Africa and was reported in the humid zone of Yemen and Saudi Arabia (Tihama region) (Davies and Martin, 2006). During the period of religious festivals in Mecca, 10 to 15 million small ruminants are imported from the Horn of Africa to Saudi Arabia. In May 2007, RVF was diagnosed on the French island of Mayotte in a young boy who had been evacuated from Anjouan, one of the other islands of the Comoros archipelago. The RVFV was probably introduced there by the

trade of live ruminants imported from Kenya or Tanzania during the 2006-2007 epidemics.

Epidemics of RVF have a 3-5-year cycle according to the rainfall which conditions the replication of the vectors capable of transmission and, consequently, the possibility of diffusion of the disease.

The construction of large artificial swamps increases the extension of the ideal reproduction habitat of the vectors; in Egypt in the 1970s, the construction of the Ashwan Dam strongly influenced the appearance and successive endemization of the disease in this country. A similar situation was also verified in Western Africa following the construction of the Dama Dam on the Senegal River where the disease was seen in 1987 and has since appeared cyclically.

Transmission

More than 30 mosquito species were found to be infected by RVFV (Lefèvre *et al.*, 2003; McIntosh and Jupp, 1981), belonging to seven genera of which *Aedes* and *Culex* are considered to be the most important from the point of view of vector competence (other genera are *Anopheles*, *Coquillettidia*, *Eretmapodite*, *Mansonia* and *Ochlerotatus*). Temperature and relative humidity

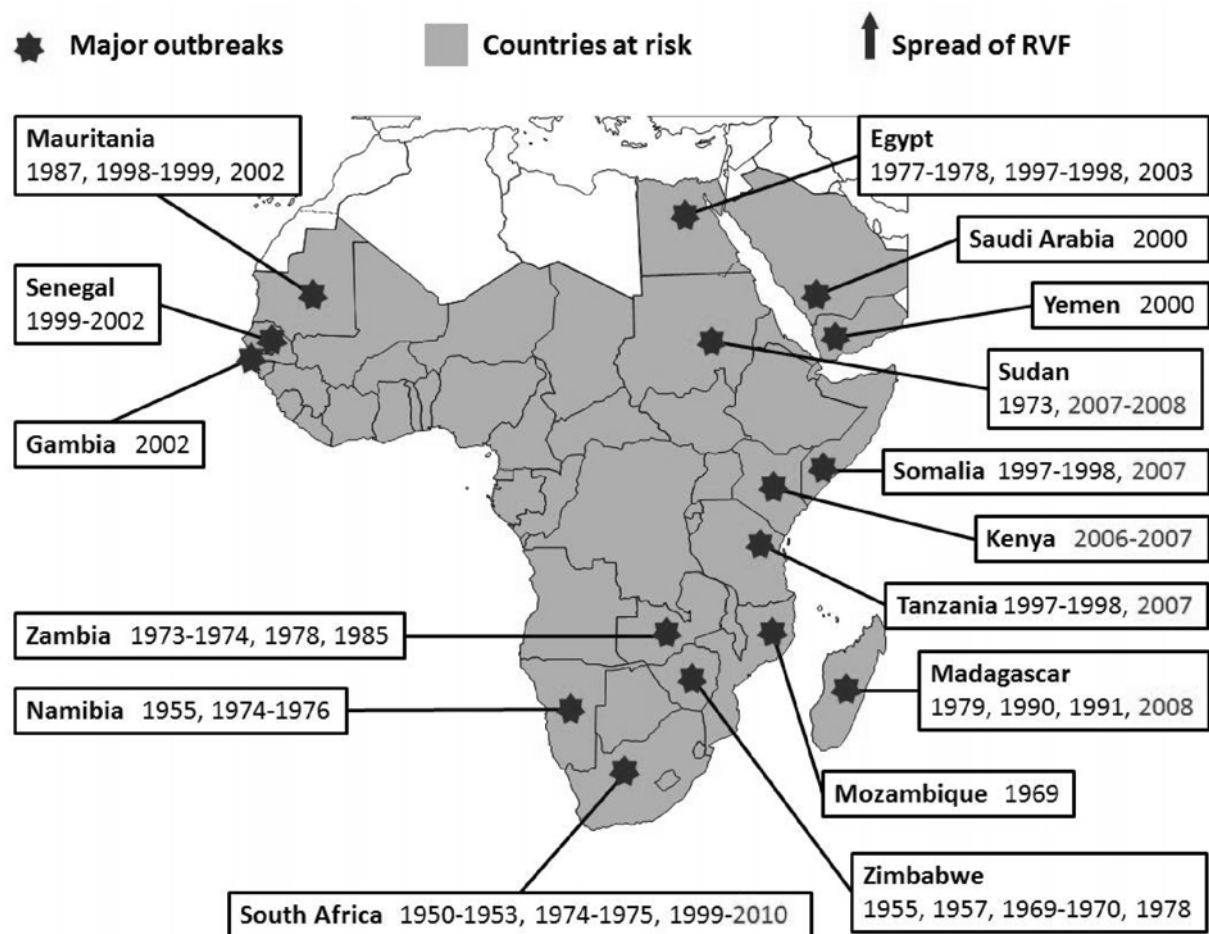


Figure 1 - Rift Valley Fever distribution, outbreaks and spread

are factors limiting the replication of these insects and, therefore, also condition the diffusion of RVF in a specific geographical area. The increase in precipitation, the mild/hot climate, the elevated relative humidity, the construction of large artificial swamps and the presence of irrigated areas represent important risk factors for the presence and diffusion of the disease.

Furthermore, vertical transmission of the virus from the adult female to the eggs has been reported in *Aedes mcintoshi*, which consequently become the reservoir of the virus in inter-epidemic periods when other clinical cases of RVF are not reported. Transovarial transmission also seems to be probable in many other species, including the *Ae. vexans* species complex which is also widespread in Europe. In the case of drought, the eggs can remain viable for 4-5 years and the successive development to the larval stage will come when optimal climatic conditions are realised, with elevated percentages of relative humidity and temperatures above 15-20°C. Larvae and then already infected adult insects capable of transmitting the infection can develop from these eggs. The other species of arthropods carry out an important role in the epidemic diffusion of the disease but do not play any role in long-term maintenance of the virus since, in the different species of *Aedes*, transovarial transmission has never been reported. Moreover, the epidemic diffusion of RVF will depend, other than on the presence and density of the vectors, also on the populations of receptive vertebrates.

The flying capacity of the mosquitoes of the *Aedes* e *Culex* genus are rather limited and goes from a few hundred metres to a few kilometers (Ba *et al.*, 2006; Bogojevic *et al.*, 2007). Nevertheless, these distances are sufficient to allow local diffusion of RVF.

In animals, the virus can also be transmitted mechanically by other arthropods, as, for example, tabanids, culicids and phlebotomes, which, however, play a marginal epidemiological role exclusively internal to active breeding grounds.

In the majority of cases, the transmission of the RVF virus to humans takes place directly, by means of contact with blood or infected organs, fetuses and fetal membranes at the moment of slaughtering.

Humans can also be infected via the respiratory tract, by means of aerosol of the virus present in the blood, during slaughtering by means of the jugular vein, as in the Islamic rite. Another possible mode of infection is through food by means of unpasteurised milk, the consumption of partially cooked meat or entrails or by being bitten by an infected mosquito. It follows that RVF is very frequently an occupational zoonosis which strikes veterinarians, laboratory technicians, husbandry workers and butchers. Direct human-to-human transmission has not been reported. Transplacental RVFV transmission may occur in vertebrates, including humans. It

results in abortion and elevated newborn mortality rates (Arishi *et al.*, 2006).

Clinical features

Animals

In epidemic breeding grounds, the percentage of diseased ovines and bovines can vary from 20-90% and the period of incubation, in the acute forms, is approximately 24-48 hours. Clinical manifestations vary depending on age and animal species. In calves and lambs under 6 weeks of age, a hyperacute form is often reported with very elevated mortality due to cardiocirculatory collapse and acute hepatitis. In ovines and adult bovines, an acute form with fever, sensory depression and high titre viraemia is habitually reported; successively, a purulent nasal mucous discharge, lachrymation, jaundice and nasal regurgitation of ruminal material appear. Three days after the appearance of the first symptoms, diarrhoea, generalised lymphadenopathy and haemoglobinuria are reported. Abortion occurs in 85-100% of cases in all periods of gestation; contextually, a decrease in milk production is reported. Mortality, in the breeding grounds of first onset, can fluctuate from 20-70%. Breeds indigenous to sub-Saharan Africa present an elevated natural resistance to the infection and the subacute forms of the disease are therefore frequent. In areas where the disease is endemic, the only clinically evident symptom in adult bovines is often abortion. Rift Valley Fever sometimes evolves in an asymptomatic form. The wild buffalo (*Syncerus caffer*) is receptive to the infection, presents transitory viraemia and can also abort; in dromedaries, the virus can cause viraemia, abortion or neonatal mortality. Viraemia and the possibility of abortion, events nevertheless difficult to demonstrate in nature, have also been reported in wild ruminants.

Humans

In most cases, human infections remain inapparent, or have mild, influenza-like symptoms (Chevalier *et al.*, 2010). Nevertheless, a serious form has been described, similar to Dengue, with fever from 38-40°C, headache, asthenia, perspiration, and muscular and articular pain. Moreover, general weakness, dizziness, hepatic tenderness, jaundice, nausea, vomiting, nosebleeds and photophobia are observed. The course of the disease is approximately two weeks. During the vast epidemic in Egypt in 1977, many cases of ocular complications with detachment of the retina were observed; in the epidemic in Somalia in 2000 which caused approximately 2000 deaths, cardiorespiratory and haemorrhagic complications were principally reported. Furthermore, a meningoencephalic form was described which appears at 4 weeks post-infection and is characterised by memory loss, hallucinations, confusion, dizziness, convulsions and lethargy; this form is rarely lethal but neurological deficits remain.

Human case-fatality rates have been under 1% in the past; however, an increase has been reported since 1970 (Gubler, 2002). In the RVF epidemic in Saudi Arabia in the year 2000, the fatality rate reached 14% (Balkhy and Memish, 2003).

Risk of introduction of RVF into Italy

The OIE has added RVF to the list of notifiable diseases. The reason for this choice lies in the fact that: a) RVF has a potential of international diffusion, b) it is a zoonosis and c) the consequences for the health of humans can be serious. For these reasons, it is opportune that RVF-free countries develop a risk analysis in order to identify the possible means of introduction of the disease, the possible consequences on the economy of the country and on public health, and efficacious measures for reducing the hazard (Tolari *et al.*, 2009). The principal aim of import risk analysis is to provide importing countries with an objective and defensible method of assessing the disease risks associated with the importation of animals, animal products, animal genetic material, foodstuffs, biological products and pathological material.

The risk analysis of introduction begins with identifying the possible means of introducing the virus into the European Union (EU) (Fig. 2) and with estimating the level of risk which is proportional, for example, to the eventual prevalence of the disease in the exporting country, to the presence of epidemic breeding grounds, to the level of health control at

departure and arrival, and to the volume of goods exchanged and their typology.

The importation of infected ruminants represents the principal hazard of introducing RVF into the EU.

Disease-free status regarding RVF is necessary to be able to export live animals and products of animal origin into the EU. This status depends on the capacity that the country has, based on observable evidence, of activating an efficient system of surveillance regarding the disease and on its willingness to report possible epidemics of RVF. The risk of introduction is, therefore, related to the level of the health guarantees of the exporting country. These guarantees include the presence and the intensity of the controls, the organisation of the veterinary services, the typology of the laboratory examinations carried out on the live animals and on the products of animal origin and their sensitivity. Furthermore, it is necessary to verify the epidemiological situation in the countries bordering the exporting country and the probability of legal and illegal trade of animals and products of animal origin between these countries. These constraints are the same as those foreseen for Foot and mouth disease and for other exotic diseases (Council Directive 72/462/EEC). It follows that the introduction into the EU of live ruminants coming from Africa and the Middle East and their products is forbidden. Nevertheless, it is probable that illegal importation and/or triangulation occur between the Middle East and central Europe, and between

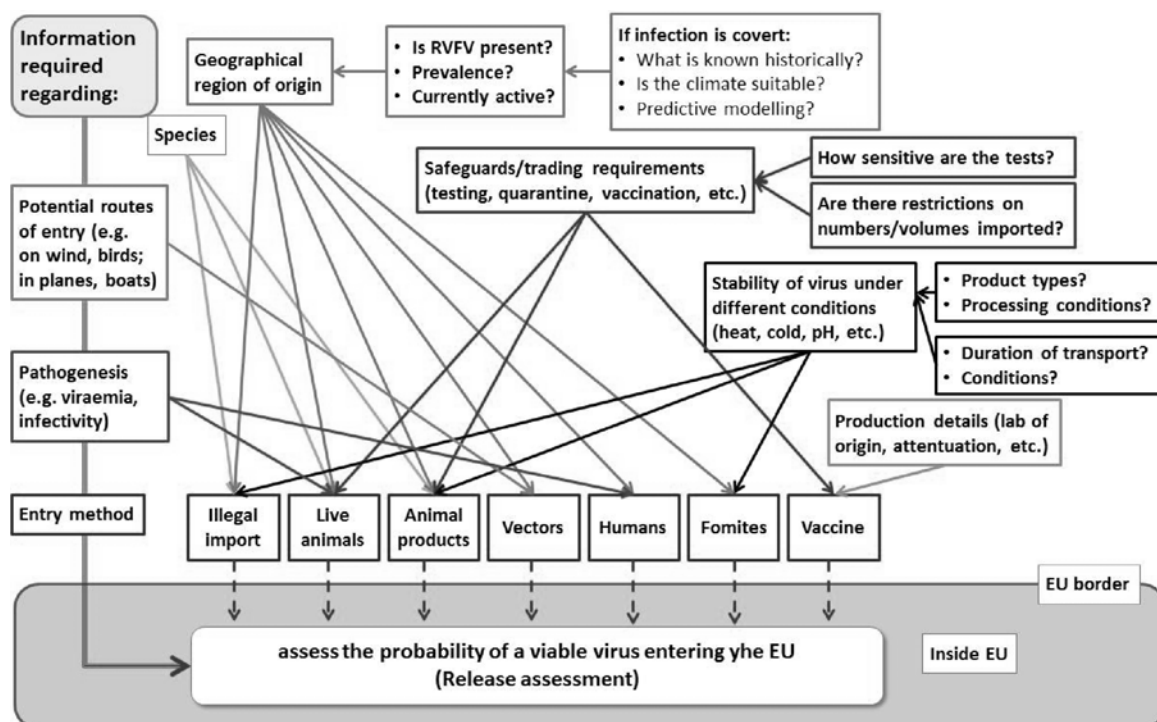


Figure 2 - The potential routes of entry of the RVF virus into the EU and the types of data required (by route) to assess the probability of entry (EFSA, 2005, modified)

Northern and Southern Africa. This represents one of the principal components of the risk of the introduction of numerous exotic diseases, also those of a zoonotic character (peste des petits ruminants, foot and mouth disease, Crimean-Congo haemorrhagic fever, etc.). Nevertheless, recent risk analyses have concluded, for example, that the risk of introduction of peste des petits ruminants virus from Maghreb to France is extremely low, from 0 to 2 on a scale that goes from 0 (impossible event) to 9 (certain event) (Miller *et al.*, 2009).

The risk of introduction from live animal trade could increase if, in the exporting country, rapid diffusion epidemic breeding grounds are present while this risk would be lower if the epidemiological situation is that which occurs in the interepidemic phase. The first situation can be evaluated on the basis of long-term meteorological forecasts relative to rainfall; in the second case, it could be opportune to carry out serological controls in sentinel animals. The risk could be reduced by carrying out controls in order to identify preclinical carrier animals, utilising high sensitivity serological and virological tests. The risk level is also conditioned by the geographic location and the typology of commercial trade internal to the exporting country; North African countries which border on the Mediterranean Basin probably have commercial relationships with other African countries in which the disease is endemic. Moreover, another hazard, probably marginal, is represented by the importation of zoo animals.

The risk of introduction by means of products of animal origin is correlated to the state of viraemia potentially present at the moment of slaughtering. European Union countries annually import a considerable amount of bovine and ovine meat from countries of Southern Africa recognized to be free from RVF but, also in this case, the risk is minimal in relation to the current normative constraints.

The home range of the vectors of RVF is relatively modest (maximum a few kilometres) and this limits the diffusion of the disease over long distances. Nevertheless, the transport of insects for long distances (even over 200 km) by means of wind has been demonstrated for vectors of other diseases; in 2000, the passive transport of *Culicoides* sp. caused the diffusion of the bluetongue virus from North Africa to Sardinia. The probability of this happening is related to the epidemiological situation of the infected country and to its distance from the borders of the EU. Currently, the disease is present in Mauritania, Saudi Arabia and Southern Egypt but the introduction of the RVFV by means of this modality would require an epidemic-type epidemiological phase and a series of particular meteorological and climatic conditions: constant wind at a velocity of more than 50 km/h which blows for long periods of time in the same direction. Theoretically, the passive transportation of infected arthropods inside an aeroplane could also represent a source of

introduction of the virus if none of the direct prophylactic measures are activated during the period of greatest activity of the mosquitoes in the infected countries.

People returning from countries where RVF is present after having been infected in areas in which the disease is endemic can also represent a potential source of introduction of the virus. Nevertheless, human cases observed in the countries in which the disease is endemic are rare and, therefore, the probability of introduction by this means is considered negligible.

Vaccines in which the process of inactivation or attenuation of the vaccine strain was not carried out in an efficacious way and drugs polluted with RVFV constitute another, theoretical, means of introduction. Empiric evidence leads us to consider this highly improbable mode of infection; Egypt has imported millions of doses of vaccine from South Africa without having ever reported a case of RVF ascribable to the administration of the vaccine. Introduction by means of fomites is a completely theoretical eventuality.

Risk of diffusion of RVF in Italy

The risk of diffusion of RVF in EU countries, including Italy (Fig. 3), is principally related to: 1) the presence and density of the populations of receptive animals, 2) the entity of movement of live animals belonging to receptive species and 3) the presence and density of competent vectors for transmission in the territory. The first two points are widely satisfied even if the density of the receptive species and the entity of their movement present significant differences in the EU countries.

Since this disease is transmitted almost exclusively by means of insect vectors, the third point is the most difficult to evaluate.

Many vectors potentially capable of transmitting RVF are present in the EU (*Aedes vexans vexans*, *Ochlerotatus caspius*, *Culex theileri*, *Culex pipiens*, *Culex perexiguus*). These vectors are generally active from May-October according to the climatic conditions, the altitude and the latitude. Differences in climate, seasonal variations of vector and host density, and genetic drift may result in differences in vector competence (the biological suitability of the vector to transmit the pathogen) and vectorial capacity (external factors such as the number and lifespan of the vectors, and the feeding preferences of the host) as compared to the situation in Africa. Moreover, the introduction and spread of new vector species represent additional risks. For example, *Ae. albopictus* can transmit RVFV (Moutailler *et al.*, 2007), and many epidemiological concerns arise from the current distribution of this species in Europe (ECDC, 2008).

Surveillance and prophylaxis

The diverse procedures for evaluating the risk of

introduction and the diffusion of RVF in the EU are all in agreement that the risk is low. Nevertheless, the recent appearance of the disease in East Africa, including Sudan, the Nile Valley, and the Indian Ocean, has demonstrated that the circulation of RVF is very active and is probably influenced by climatic, environmental and socio-economic changes. These changes, together with the demographic growth of human population and the consequent increase in the request for meat could cause a significant increase in controlled and uncontrolled movements of farm animals. Consequently, the countries in the Mediterranean area, Central Europe and the Middle East could undergo an increase in the risk of introduction of RVF. It is therefore

important to promote and realise risk analyses based on accurate estimates of the movements of ruminants between the endemic areas and RVF-free areas. Furthermore, the high risk ecosystems should be identified and catalogued, with regular updating of the data in order to take into consideration the climatic-environmental variations.

The EU should finance active surveillance activity in the countries in which RVF is present in order to identify the presence of events of an epidemics at an early stage and implement a network of sentinel farms in the countries of Northern Africa and Southern Europe so as to identify the eventual circulation of the virus at an early stage. The specific training of technical and veterinary personnel is also

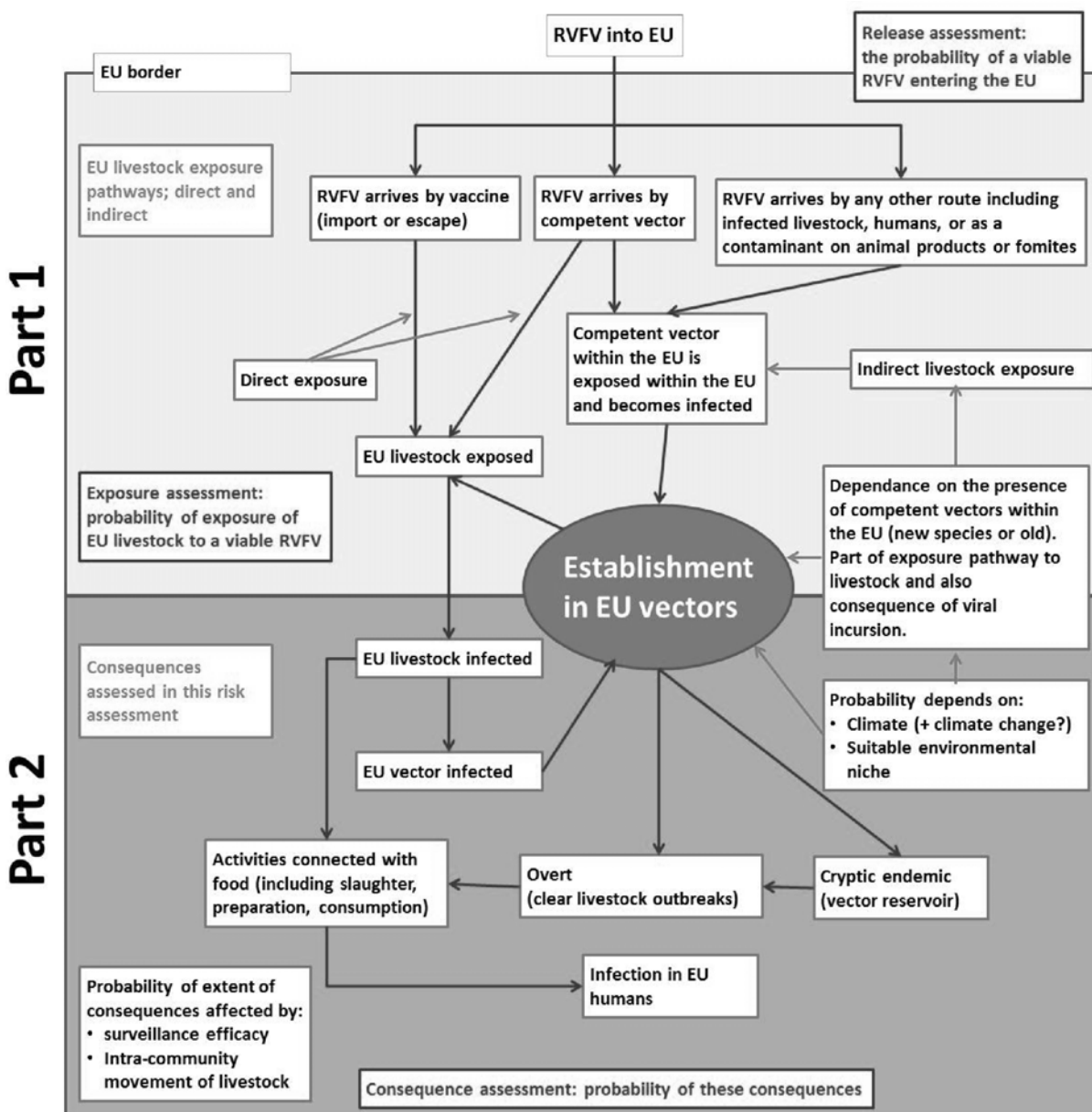


Figure 3 - Diagrammatic summary of potential exposure (Part 1) and consequence assessment pathways (Part 2) after the entry of the RVF virus into the EU (EFSA, 2005, modified)

of great importance. An emergency plan could be put into effect for the countries of Southern Europe, taking into consideration the epidemiological parameters described without neglecting the hypothesis of a vaccination plan which takes into account the quality and the availability of the vaccines on the international market.

The Smithburn vaccine is a live attenuated vaccine which is considered very economical and efficacious when used on sheep, goats and bovines. It is capable of inducing long-term immunity and prevents abortion caused by infection with the RVFV. Nevertheless, it can have collateral effects, in particular, when administered to pregnant animals. Despite these disadvantages, its use is recommended by the FAO (Geering *et al.*, 2003) and is currently the most used vaccine for controlling RVF in Africa. The inactivated vaccine is less immunogenic and more costly, and frequent boosters are necessary to induce an adequate level of protection; this limits the possibility of its use in countries in which farming is nomadic. However, this vaccine has been used both in Israel and in Egypt, and could also be used in Europe since it is, for the most part, innocuous and safe with respect to the live attenuated vaccine. Regarding vaccination in humans, in the past, an inactivated vaccine has been used, produced in the USA and administered exclusively to personnel at high risk working in endemic areas; currently, this vaccine is not available.

The most efficacious long-term strategy is, however, represented by controlling RVF in areas in which the disease is endemic. This would allow the attainment of a double objective: significant reduction in the risk of introducing the disease in places which are currently RVF free (including Europe), and improvement of the economic and health conditions in the countries in which the disease is present.

In order to reach these objectives, notable efforts are, however, necessary in order to better understand the ecology of the RVF virus, its biological vectors and the epidemiological cycles of the disease in African countries.

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Usutu virus in Emilia-Romagna region (Italy): implications for the public health.

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Summary - Usutu virus (USUV), an African Flavivirus, was detected for the first time in Europe in 2001. This virus had rarely been associated with disease in human but in Emilia-Romagna region USUV-related illnesses were reported in two immunocompromised patients in 2009 and USUV IgG-specific antibodies were recently identified in this region in four healthy blood donors with no history of flavivirus infection.

In 2009 and 2010 a widely presence of USUV was detected in Emilia-Romagna region through a multidisciplinary surveillance system. The circulation of the virus in the environment was demonstrated by the relevant number of PCR-USUV positive mosquito pools, 147 out of 4,900 tested, and PCR-USUV positive birds 23 out of 2,483 tested, while the virus was PCR undetected in the tested human samples from 30 subjects with clinical symptoms of meningoencephalitis. The relevant level of USUV detected in the environment, in mosquitoes and birds, without human detections, suggest a low capability of USUV to infect humans.

This capability, though at low level, raising the potential pathogenicity of USUV for humans, at least in immunocompromised individuals, and pointing out the necessity to know the potential circulation of this virus in the environment and to test its presence in the blood bags.

Key words: Usutu virus, flavivirus, mosquito, bird, surveillance.

INTRODUCTION

In 2009 the West Nile virus surveillance system (WNV) in the Emilia-Romagna region (Angelini *et al.*, 2010) allowed the unexpected detection of another flavivirus of the Japanese encephalitis antigenic complex (Calisher *et al.*, 1989), phylogenetically closely related (Kuno *et al.*, 1998; Bakonyi *et al.*, 2004) to WNV: Usutu virus (USUV) (Tamba *et al.*, 2011). Active circulation of this virus on the regional territory was also detected in the 2010 season in birds and mosquitoes (Calzolari *et al.*, 2010a; Calzolari *et al.*, 2010b), as well as in the 2011 (CEREV, 2011), throughout the surveillance system.

The first detection of USUV in Europe dates back to 2001 in Vienna (Weissenböck *et al.*, 2002), where the virus caused the death of hundreds of wild birds, and in followed years spread to neighbouring countries into Hungary (Bakonyi *et al.*, 2007), Italy (Manarolla *et al.*, 2010), Switzerland (Steinmetz,

2011) Czech Republic (Hubálek *et al.*, 2008a), Poland (Hubálek *et al.*, 2008b) and Germany (Jöst *et al.*, 2011). The USUV was also detected in other European countries, like Spain (Busquets *et al.*, 2008), by PCR in a pool of mosquito, and England (Buckley *et al.*, 2003; Buckley *et al.*, 2006), by serology in birds. The first serological detection of the virus in Emilia-Romagna date back to 2007, when virus was detected in sentinel chicken tested for WNV (Lelli *et al.*, 2008).

Even if USUV ecology is less known than the WNV one, the two viruses seem to show biological cycle similarities: the principal vectors of WNV and USUV are largely ornithophilic mosquitoes, mainly of the genus *Culex*, wild birds are principal reservoirs of WNV and they are suspected of being also of USUV, both viruses can be pathogenic for these animals (Hubálek and Halouzka, 1999; Weissenböck *et al.*, 2003; Gratz, 2006; Chvala *et al.*, 2007). Besides, migratory birds are considered

to play a key role in the diffusion of the two viruses in Europe from Africa (Weissenböck *et al.*, 2002; Malkinson and Banet, 2002; Hubálek, 2008), where they were discovered (Smithburn *et al.*, 1940, Woodall *et al.*, 1964),

Like a large part of arbovirus, the presence of USUV in the environment is strongly influenced by a complex interaction of ecological factors, like abundance of vectors and reservoirs and environmental conditions, such as temperature and rainfall. Despite the similarities described between the two viruses, some ecological differences also emerged between them during their simultaneous detections in Emilia-Romagna region in 2009 (Calzolari *et al.*, 2010b).

While the WNV risks for human health is well recognized, medical importance of USUV is not fully understood, but in summer 2009 USUV-related illness were reported in two immunocompromised patients in Emilia-Romagna region (Cavrini *et al.*, 2009, Pecorari *et al.*, 2009), furthermore, USUV was detected in serum of two organ donors tested in a retrospective WNV screening performed in Italy in 2009 (Capobianchi *et al.*, 2010), the virus was detected in cerebrospinal fluid from 3 patients with a suspected meningoencephalitis (Cavrini *et al.*, 2011). Furthermore USUV was isolated from two patients in Africa, one with fever and rash, one, 10-years-old, with fever and jaundice (Nikolay *et al.*, 2011) and detected in a patient with rash in Austria (Weissenböck *et al.*, 2007). USUV IgG-specific antibodies were recently identified in four healthy blood donors with no history of flavivirus infection (Gaibani *et al.*, 2011).

Materials and Methods

Field surveillance. Analysis were performed on mosquito, wild birds and human samples collected in the multidisciplinary surveillance plan performed on the Emilia-Romagna region portion of the Pianura Padana (Angelini *et al.*, 2010). Mosquito pools sized ≤ 200 specimens, grouped according to date, location and species, were obtained by carbon dioxide baited traps distributed on all the survey area. The pooled mosquitoes were stored in polypropylene cryotubes and frozen at -80° . For biomolecular analysis mosquito pools were grinded by vortexing samples with copper plated round balls and PBS, centrifuged and an aliquot was collected. Further details on this method are present in other publications (Calzolari *et al.* 2010a, Calzolari *et al.*, 2010b).

A monthly sample of about 40 wild birds caught or shot within specific wildlife population control programs was collected. Monitoring was mainly focused on the European Magpie (*Pica pica*), the Hooded Crow (*Corvus cornix*), and the Eurasian Jay (*Garrulus glandarius*), these corvids are subject to control plans for their wide diffusion and eating habits in surveyed area. Passive surveillance on wild

birds was carried out on animals found dead in the field or died in the wildlife rehabilitation centres. Birds were necropsied and for each specimen organ samples (brain, spleen, heart and kidney) were pooled, ground and submitted to biomolecular analysis.

The plasma, serum, and cerebrospinal fluid (CSF) samples of 30 subjects with clinical symptoms of meningoencephalitis were referred to the Regional Reference Centre for Microbiological Emergences at St. Orsola-Malpighi Hospital, Bologna, in 2010.

Virus survey. RNAs present in samples were extracted using Trizol[®] LS Reagent (Invitrogen, Carlsbad, CA); cDNA synthesis was achieved using random hexamer (Roche Diagnostics, Mannheim, DE) and SuperScript[®] II Reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions.

Presence of USUV in mosquito pools and birds was detected by a specific traditional PCR (Manarolla *et al.*, 2010) and a Flavivirus genus PCR (Scaramozzino *et al.*, 2001). Obtained fragment were sequenced by an automated fluorescence-based technique following the manufacturer's instructions (ABI-PRISM 3130 Genetic Analyzer, Applied Biosystems, Foster City, CA). Human samples were tested for WNV or USUV by RT-PCR assay based on specific TaqMan[®] probes (Tang *et al.*, 2006; Cavrini *et al.*, 2011).

The obtained sequences were employed to perform basic local alignment search tool (BLAST) in the GenBank library (GB) to confirm the specificity of positive reaction and to estimate the degree of identity of detected strains. The obtained sequences were aligned with available GB sequences by ClustalW of the freeware program MEGA 5 (Tamura *et al.*, 2011) and a phylogenetic tree was produced by the same program.

To estimate the proportion of infected mosquitoes in *Culex pipiens* and *Aedes albopictus* populations the maximal likelihood estimation index (MLE) was calculated on all the two species samples by the Poolscreen2 program (Biggerstaff, 2006); MLE index estimates the proportion of infected mosquitoes on 1000 specimens sampled.

Results

In the seasons 2009-2010, a total of 629,074 mosquitoes (grouped in 4,900 pool) were analyzed (Tab. 1); 147 pools were USUV-positive, of which 143 were composed by *Culex pipiens* and 4 by *Aedes albopictus* (*Cx. pipiens* was also the most abundant sampled species). The MLE resulted 0.26 (with a lower limit of 0.22 and an upper limit of 0.31) for *Cx. pipiens* and 1.30 for *Ae. albopictus* (with a lower limit of 0.43 and an upper limit of 3.10). Pools of other abundant species, like *Ae. caspius* and *Ae. vexans*, result always USUV-negative (Tab. 1).

In the two years of survey 2,483 birds were tested

for the presence of USUV and the virus was detected by PCR in 23 birds. The large part of tested birds were actively sampled throughout control plan and belong to Passeriformes Order, particularly three corvids species being most represented: European Magpies (*Pica pica*), Hooded Crows (*Corvus cornix*) and Eurasian Jays (*Garrulus glandarius*) (Tab. 2). Interestingly, a large number of positive birds (8 on a total of 23 positive bird) were blackbirds (*Turdus merula*), a species not actively sampled. Despite the greater number of birds sampled by the active surveillance (1,130 on 1,276 birds tested) in 2010, 11 out of the 12 USUV positive birds were from passive surveillance. In the two years 63 partial NS5 gene USUV sequences, obtained by the flavivirus-genus PCR, were obtained from mosquitoes and birds. These sequences showed a good identity, ranged from 100

to 98.3% on 199 positions which were compared. Moreover in 2010, 87 sequences were obtained by specific USUV PCR targeting *E* gene, 12 from birds, 75 from *Cx. pipiens* pools and one from *Ae. albopictus* pool. These sequences showed an identity ranging from 98.3 to 100 %, on 354 positions which were compared, with a number of base differences per sequence of 1.40 from averaging over all sequence pairs. The consensus sequences of both PCR products showed an high score in BLAST analysis with the two USUV strains isolated in Budapest (GB accession number: EF206350) and in Vienna (GB accession number: AY453411), with a 96.5% and 99% identity for partial NS5 gene and partial *E* gene, respectively. Moreover different homologue sequences of *E* gene detected in central Europe showed a high affinity for sequences obtained in this study (Fig. 1).

Table 1 - Total number of specimens, PCR pools and PCR-positive pools collected for every mosquito species in 2009 and 2010 surveys.

Species	N	2009		N	2010		N	Total		
		Pool	USUV +		Pool	USUV +		%	Pool	USUV +
<i>Aedes albopictus</i>	1,227	108	2	1,855	131	2	3,082	0.5	239	4
<i>Ae. caspius</i> *	29,283	314		18,135	367		47,418	7.5	681	
<i>Ae. detritus</i> *	5	2		46	2		51	<0.1	4	
<i>Ae. dorsalis</i> *	13	1					13	<0.1	1	
<i>Ae. geniculatus</i> *	8	3		532	5		540	0.1	8	
<i>Ae. vexans</i>	4,597	60		17,697	185		22,294	3.5	245	
<i>Aedes</i> spp.								<0.1		
<i>Anopheles maculipennis</i> s.l.	82	14		167	17		249	<0.1	31	
<i>An. plumbeus</i>	2	2		15	4		17	<0.1	6	
<i>Coquillettidia richiardii</i>				167	4		167	<0.1	4	
<i>Culiseta annulata</i>				1	1		1	<0.1	1	
<i>Culiseta</i> spp.				1	1		1	<0.1	1	
<i>Culex modestus</i>	246	26		1,107	27		1,353	<0.1	53	
<i>Cx. pipiens</i>	155,053	1,259	54	398,835	2367	89	553,888	88.0	3,626	143
<i>Culex</i> spp.								0.0		
	190,516	1789	56	438,558	3,111	91	629,074		4,900	147

* *Ochlerotatus* taxon was considered an *Aedes* subgenus (Savage and Strickman, 2004)

Table 2 - Birds tested in 2009 and 2010 with USUV-positive specimens.

Species	Common name	2009		2010		Total	
		N	USUV/+	N	USUV/+	N	USUV/+
Passeriformes							
<i>Pica pica</i>	European Magpies	607	4	601	1	1,208	5
<i>Garrulus glandarius</i>	Eurasian Jay	96	2	88	1	184	3
<i>Turdus merula</i>	Blackbird	30	3	11	5	41	8
<i>Sturnus vulgaris</i>	European Starling	98	1	56		154	1
Non Passeriformes							
<i>Streptopelia decaocto</i>	Collared Dove			11	1	11	1
<i>Asio otus</i>	Long-eared Owls			4	2	4	2
<i>Caprimulgus europaeus</i>	Nightjar			1	1	1	1
<i>Alectoris rufa</i>	Partridge			1	1	1	1
<i>Larus sp.</i>	Gull	8	1			8	1
Other tested species		368		503		871	
Total		1,207	11	1,276	12	2,483	23

In 2010, between July 31 and December 1, 30 patients, all living in the Emilia-Romagna region, with clinical indications of meningoencephalitis were evaluated by RT-PCR for the presence USUV genome in plasma/serum and/or cerebrospinal fluid (CSF). The male/female ratio was 16:14 and the median age was 48 (range 17 to 83 years). No patients tested positive for USUV in plasma/serum or CSF.

DISCUSSION

The high homology detected between sequences of both *NS5* and *E* gene in birds and mosquitoes, suggests that a single USUV strain circulated in the survey area. This hypothesis is reinforced by the high homology detected also with the human sequence isolated in 2009 from Bologna and present in GB (GB accession number: JF826447) (Fig. 1). Moreover these data highlights a high rate of identity between the 2009 and 2010 *NS5* sequences, strongly suggesting the USUV overwintering between 2009 and 2010 in Emilia-Romagna region. Unfortunately USUV sequences present in GB are scarce, among these both the *E* and the *NS5* USUV sequences show the highest nucleotide identity rates with strains originating from Vienna and Budapest, compared to the South African sequences, implying that the USUV strain detected in central Europe is the same circulating in Emilia-Romagna region. This result was further confirmed by the affinity between *E* sequence from this study and these from

Germany and Austria present in GB (Fig. 1).

The 88% of the 629,074 tested mosquitoes belongs to the species *Cx. pipiens* and 143 pools USUV-positive (out of 147) belonged to this species, indicating the *Cx. pipiens* mosquito as the principal vector of this virus. Four USUV-positive pools were composed by Tiger mosquito (*Ae. albopictus*), indicating that this species may play some role in the USUV circulation. The MLE results seem to confirm this hypothetical role, with an estimation of 1.30 positive *Ae. albopictus* specimen out of 1000 mosquitoes of this species tested (MLE 1.30), index highest than that obtained for *Cx. pipiens* (MLE 0.26).

If this hypothesis will be proved the USUV capability to spread in urbanized areas will have to be considered, taking into account the ability of the Tiger mosquito to colonize inhabited environments and its opportunistic blood-feeding habits (Valerio *et al.* 2010), with the consequent high risk for humans of being exposed to the virus, in respect to *Cx. pipiens*, a less anthropophilic mosquito. Further experimental studies are needed to confirm the vector competence and the role of the Tiger mosquito in the USUV cycle. From our evidences the other sampled mosquitoes species don't showed any involvement in the USUV cycle.

Obtained data pointed out the high Blackbirds sensitivity to USUV, as widely reported in Europe by the finding of a large dead subject number tested positive by direct tests (Weissenböck *et al.* 2002,

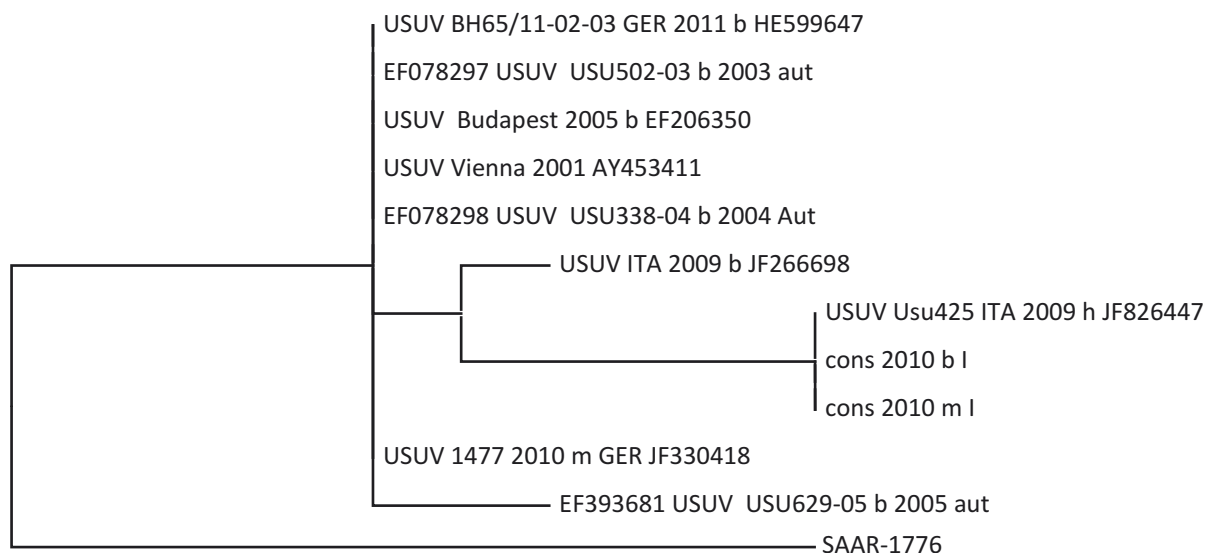


Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model, this model was chosen between 24 different nucleotide substitution models for the lowest BIC value (Bayesian Information Criterion). The tree with the highest log likelihood (-725.5742) is shown. Initial tree for the heuristic search were obtained automatically by BIONJ method with MCL distance matrix. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 429 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.* 2011). Abbreviation: b, from bird; m, from mosquito; h, from human; Ger, from Germany; Ita, from Italy; Aut: from Austria; cons, consensus sequence.

Weissenböck *et al.* 2003; Chvala *et al.* 2007; Bakonyi *et al.* 2007; Manarolla *et al.* 2010; Steinmetz *et al.* 2011; Savini *et al.* 2011). Also the high sensitivity to USUV for the owls, reported in the literature (Weissenböck *et al.* 2002; Steinmetz *et al.* 2011; Manarolla *et al.*, 2010), was confirmed by the presence of two positive Long-eared Owls.

The 2010 data highlights a pathogenic action of USUV for wild birds with 11 USUV-positive birds from passive surveillance; only one actively sampled bird tested USUV-positive, although most of the specimens came from this type of surveillance. This relevant USUV pathogenicity for wild birds could be due to its recent introduction and to the lack of adaptation of the virus to the European reservoirs, but a potential involvement of vertebrate reservoirs other than birds in the USUV cycle could also be hypothesized, requiring further experimental studies to elucidate this possibility.

The current data support reports in the literature on the capacity of USUV to become endemic in Europe, highlighting the need to monitoring its presence and diffusion. However, the relevant 2010 USUV circulation did not correspond with the detection of symptomatic USUV infections in humans in the same study period. The identification of the first 4 USUV seropositive healthy blood donors in 2010 in the Emilia-Romagna region (Gaibani *et al.*, 2011) is in contrast with the elevated WNV seropositivity found among blood donors in the same area in 2009 (Pierro *et al.*, 2011); this finding suggests that USUV is likely able to asymptotically infect humans. The relevant level of USUV detected in the environment, in mosquitoes and birds, without human detections, suggest a low capability of USUV to infect humans.

This capability, though at low level, raising the potential pathogenicity of USUV for humans, at least in immunocompromised individuals, and pointing out the necessity to know the potential circulation of this virus in the environment and to test its presence in the blood bags. Monitoring USUV circulation is difficult also due to the lack of specific and useful serological tests for both human and animal samples, to detect previous contact with the virus and to allow the differential diagnosis from other flaviviruses (Tamba *et al.*, 2011; Vazquez *et al.*, 2011).

The use of an integrated surveillance, targeting also mosquitoes and wild birds, may be able to identify the circulation of the USUV in the environment and to estimate the intensity of viral circulation, like achieved from the described system. The mosquito analysis can also provide indices connected to viral circulation estimating the number positive mosquitoes in the sampled population, like minimum infection rate or maximum likelihood estimation, which, properly sized to a specific period and in a particular area, could be used as an indicator of virus-connected risk and of when to start controlling blood bags, with a relevant resource savings.

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Genetic diversity of *Leishmania* from Sudan and possible correlation to clinical signs: preliminary results

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Summary - *Leishmania* species can cause diseases with a wide spectrum of clinical manifestations. Different types of diseases are caused by the same species group, whereas different species may cause the same type of disease. In this study we report the preliminary results of a multilocus molecular and phylogenetic analysis of *Leishmania* isolated from Sudanese patients. Samples were obtained from the eastern part of the country where the disease is endemic and from the Institute of Endemic Diseases, University of Khartoum. PCR and direct sequencing were used to analyse the polymorphisms in gp63 and minicircle kDNA genes of *Leishmania* strains from patients showing different clinical manifestations. The interspecific difference in the gp63 sequences ranged from 1.8% to 2.7%. A higher interspecific difference (0.8%-7.7%) was recorded for the kDNA sequences. The intraspecific nucleotide variation ranged from 0.0% to 0.8% for both genes. Phylogenetic analysis of the *Leishmania* species sequenced in this study and of those available in GenBank™ were concordant in clustering *Leishmania* in two major clades, corresponding to *L.donovani* complex and *L.major*. Patients with visceral leishmaniasis, mucocutaneous or Post Kala-azar Dermal Leishmaniasis were infected by members of *L.donovani* complex. Patients with cutaneous lesions were infected by *L.major* with the exception of two patients infected by *L.donovani*.

Key words: Leishmania, clinical signs, minicircle kDNA, gp63.

INTRODUCTION

Leishmaniasis is a vector-borne parasitic disease caused by intramacrophage protozoa of the genus *Leishmania*, transmitted generally by at least 30 species of sandflies (either *Phlebotomus* or *Lutzomyia* genera) and rarely by congenital and parenteral routes such as organ implantation, blood transfusion, needle sharing and laboratory accidents (Magill, 1995; Herwaldt, 2001). Depending on the species of *Leishmania* involved, human and a wide range of mammals can act as reservoir (Ashford, 1996). At least four major clinical forms of Leishmaniasis are recognised: cutaneous leishmaniasis (CL), either diffused or localized, mucocutaneous leishmaniasis (ML), affecting the nose and the upper respiratory tract, visceral Leishmaniasis (VL) and the Post Kala-azar Dermal Leishmaniasis (PKDL).

Leishmaniasis as a Neglected Tropical Disease

represents an increasingly significant medical problem. It is endemic in the tropical and subtropical regions of 88 countries, including 16 developed countries and 72 developing countries, 13 of them among the least developed (Desjeux, 2004; WHO, 2000). Population displacements and increasing cases of *Leishmania/HIV* co-infection brought new dramatic concerns to the disease (Alvar *et al.*, 2008). Leishmaniasis affected individuals are estimated in about 12-13 millions worldwide. Annually, 1-1.5 millions new cases of cutaneous leishmaniasis occur, over 90% of these cases are reported from seven countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. Regarding VL, there are 500,000 new cases occurring annually, 90% of these cases are reported from five countries: Bangladesh, Brazil, India, Nepal and Sudan (Desjeux, 1996; WHO, 2004). However, the real burden of leishmaniasis is greatly underestimated

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because substantial number of cases remain unrecorded and misdiagnosed (Guerin *et al.*, 2002, Murray *et al.*, 2005).

Leishmaniasis in Sudan

Sudan is known as one of the most endemic areas of leishmaniasis in the world. The disease represents a serious health problem and outbreaks occur periodically causing high number of fatalities (Zijlstra *et al.*, 1994; Osman *et al.*, 2000; Khalil *et al.*, 2008). Many different clinical forms of leishmaniasis co-exist. The most serious VL is caused by *Leishmania donovani* and transmitted by *Phlebotomus orientalis* (El Hassan & Zijlstra, 2001a; Elnaiem *et al.*, 1998). VL is endemic in the eastern part of the country (El Hassan *et al.*, 1995; Khalil *et al.*, 2002); however scattered cases have been reported from areas not known to be endemic in the Southern, Northern and Western parts (Osman *et al.*, 2000). Both anthroponotic and zoonotic transmission were thought to occur (Zijlstra *et al.*, 1995; Elnaiem *et al.*, 2001; Dereure *et al.*, 2003; Hassan *et al.*, 2004). CL is caused by *L. major* and transmitted by *P. papatasi*. It is endemic in the central and northern part (El-Safi *et al.*, 1991), PKDL occurs in high rate during or shortly after treatment. At least 50% of VL patients develop PKDL, this percentage is higher than in any other VL endemic area (WHO, 2010; Zijlstra *et al.*, 2003; 1995). PKDL patients are thought to act as human reservoir for the parasite (El-Hassan & Khalil, 2001). Sudanese Mucosal Leishmaniasis (SML) is a rare and particular form of ML. Unlike ML, SML starts usually as primary mucosal disease, without being preceded or accompanied by cutaneous lesions. SML occurs in areas endemic for VL (El Hassan & Zijlstra, 2001b). *Leishmania/HIV* co-infection is a growing concern particularly in the east (Alvar *et al.*, 2008).

Leishmaniasis Control

Since 2001, leishmaniasis control program has been merged with the Malaria and Schistosomiasis control program under the National Malaria, Schistosomiasis and Leishmaniasis Administration within the Division of the Endemic Disease Control created by the Federal Ministry of Health. The organization Médecins Sans Frontières Holland (MSF-H) has been involved in the VL control programme since its first response to an outbreak in Gedarif state in eastern Sudan in 1996. From 2001 to 2004, MSF-H supported five hospitals in Gedarif state and two hospitals in Sennar State to improve diagnosis and treatment and to introduce a surveillance system for *HIV/VL* co-infection. In 2010 Médecins Sans Frontières Suisse (MSF CH) has started to support Tabarak Allah hospital in Gedarif state through diagnosis, treatment and medical follow up (WHO, 2010).

Aim of the Study

Leishmania parasite species revealed a high degree of genetic diversity resulting in different types (Herwaldt, 1999; Cunningham, 2002; Dedet *et al.*, 1999). Nonetheless, different types of diseases are sometimes caused by the same *Leishmania* species group (Dedet *et al.*, 1999), and the same phenotype of the disease may be caused by different *Leishmania* species. In this study, in order to detect the correlation between different clinical manifestations of Sudanese patients and the genetic profile of *Leishmania*, two genes have been selected and subjected to biomolecular methods and subsequent sequencing. The selected genes were the *Leishmania* minicircle kDNA that is present in different copy numbers within and between *Leishmania* strains (Lambson and Barker, 2002; Rogers and Wirth, 1987), and the gp63 gene, also known as Major Surface Glycoprotease, that is expressed on the surface of promastigotes in all *Leishmania* species and has been identified as a parasite virulence factor due to its ability in protecting promastigotes from complement lyses inside the vertebrate host (Bouvier *et al.*, 1989; Etges *et al.*, 1986; Yao *et al.*, 2003; Brittingham *et al.*, 1995). The study is part of a wider research, aimed to unravel the role of animal species in disease transmission through studying the genetic structure of *Leishmania* from human and animal isolates.

MATERIALS AND METHODS

Clinical samples

Eighty-three clinical samples were collected from 44 patients. Sixty samples were collected from 21 patients at the outpatient clinic in Gedarif city. From each of these 21 patients, bone marrow (HBM), blood (HB) and lymph node (HLN) aspirates were taken. Eight samples were collected at MSF-CH leishmaniasis clinic inside the local Tabarak Allah hospital. Ten samples from different leishmaniasis patients were provided as extracted genomic DNA by the Institute of Endemic Disease, University of Khartoum, Sudan (IEND), and other five samples were collected previously at the IEND from patients with cutaneous lesions (Tab. 1). In some patients leishmaniasis was confirmed either serologically or by culture. Clinical samples were spotted on Whatman filter paper #3; each filter paper sample was stored in a separate polyethylene bag at room temperature for further analysis.

Parasite Culture

Aspirates were taken from bone marrow, lymph node or ulcer's edges of the leishmaniasis patients and inoculated into bottles containing biphasic media (NNN), incubated at 24°C (Ashford *et al.*, 1992). Cultures were examined microscopically for parasite growth, the successfully cultured isolates

Table 1 - Provenance, numbers and types of samples collected included in the study.

Sample Type	Number of samples	Leishmaniasis diagnosis	Clinical Manifestation	Geographic origin
HBM*	21	clinical	VL	Gedarif city
HB*	21	clinical	VL	Gedarif city
HLN*	18	clinical	VL	Gedarif city
HB	7	serology	VL	TabarakaAllah H
HLN	1	serology	VL	TabarakaAllah H
DNA	7	cultured parasite	VL	Khartoum-IEND
DNA	1	cultured parasite	PKDL	Khartoum- IEND
DNA	1	cultured parasite	ML	Khartoum- IEND
DNA	6	cultured parasite	CL	Khartoum- IEND

HBM: Human Bone Marrow, HB: Human Blood, HLN: Human Lymph Node.*: samples from same patients

were mass cultured in 200 ml of RPMI-1640 supplemented with 10% Foetal Calf Serum (FCS), and 1% of Penicillin/Streptomycin solution.

Biomolecular Assay

DNA Extraction

Extraction from culture

DNA of the cultured parasites was extracted using phenol/chloroform method as described previously by Maniatis *et al.*, (1986).

Extraction from biological material

Filter papers with spotted biological material (Lymph node, bone marrow or blood aspirates) were punched out with a paper puncher. To prevent DNA contamination from one sample to the next, clean sheet of paper sprayed with ethanol 90% was punched several times. In sterile eppendorf 1.5 ml tube, 350 μ l of PBS were added to 5-7 punched out discs, mixed by vortex and centrifuged 8.000 x g for 1 min, 200 μ l of the supernatant were taken for extraction. DNA extraction was performed using DNeasy Blood e tissue (Qiagen, Hilden, Germany), according to manufacturer's instructions.

Real Time PCR Assay

As an initial screening, a real time PCR was conducted to investigate the presence of *Leishmania* complexes (*L.viannia*, *L.mexicana*, *L.donovani/infantum*, and *L.major*) in all samples. The primers Lid-f and Lid-r which generate a 80bp fragment of GPI gene (Wortmann *et al.*, 2005) were used with the probe TaqMan MGB Lid-probe 5-ATCG-GCAGGTTCT-3 labeled with the fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5' end and with the fluorescent quencher dye TAMRA (tetra-methyl carboxyrhodamine). Real time PCR were performed in a final volume of 20 μ l containing 3 μ l of DNA, 10 μ l of FastStart TaqMan Probe Master (Rox)1 \times (Roche Mannheim, Germany), 0.4 μ M of both primers, 0.3 μ M of the probe. The thermal cycling profile consisted of an initial activation at 95°C for 10 min, followed by 45

cycles each consisting of denaturation at 95°C for 15sec and annealing/extension at 60°C for 30sec. Negative (sterile water) and positive (DNA extracted from African *L.donovani* ZMON-18, MHOM/SD/2S, kindly supplied by the ISS, Rome-Italy) controls were included in each run of real time PCR reaction. Real time PCR was carried out on a 7900HT fast Real-time PCR system (Applied biosystems).

To determine the genetic profile of *Leishmania* spp., DNA from resulted positive samples was subjected to amplification of two highly polymorphic genes (minicircle kinetoplast kDNA gene and major surface protease gp63 gene).

kDNA PCR

All the positive samples were tested by two PCRs, using the two couple of primers AJS3/R1 and F2/DBY (Lambson and Barker, 2002) which amplify respectively a 620 bp and 250 bp fragments of the kDNA gene (Fig. 1).

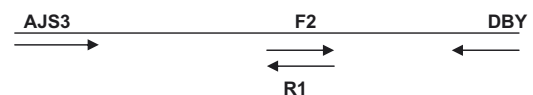


Figure 1 - The DNA segments of the selected minicircle kDNA class used for amplification

The two PCRs had the same amplification conditions. The reactions were performed in a final volume of 50 μ l containing 5 μ l of DNA, 5 μ l of PCR buffer 1 \times (Applied Biosystems, Foster City, CA), 2 mM of MgCl₂ (Applied Biosystems, Foster City, CA), 0.5 μ M of each primer, 0.2 mM of dNTPs (Applied Biosystems, Foster City, CA), 2 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). Amplifications were carried out in a GeneAmp®PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) with the following thermal cycling profile: denaturation for 10 min at 95°C, followed by 35 cycles each consisting of 30 sec at 94°C, 30 sec at 63°C, 30 sec at 72°C and a final extension step for 7 min at 72°C.

Negative and positive controls were included in each run of PCR as described above.

Gp63 PCR

All the positive samples were tested using the two couple of primers gp63-1/gp63-2 and gp63-3/gp63-4 (El Tai *et al.*, 2001), which amplify respectively a 442 bp and 374 bp fragments of the gp63 gene (Fig.2).

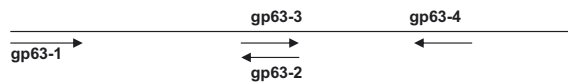


Figure 2 - The two DNA segments of the GP63 gene selected for amplification.

The same amplification conditions were used for the two PCRs. The reactions were performed in a final volume of 50 μ l containing 5 μ l of DNA, 5 μ l of PCR buffer 1X (Applied Biosystems, Foster City, CA), 2 mM of MgCl₂ (Applied Biosystems, Foster City, CA), 0.4 μ M of each primer, 0.2 mM of dNTPs (Applied Biosystems, Foster City, CA), 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). Amplifications were carried out in a GeneAmp®PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) with the following thermal cycling profile: denaturation for 10 min at 95°C, followed by 35 cycles each consisting of 30 sec at 94°C, 30 sec at 64°C, 1 min at 72°C and a final extension step for 7 min at 72°C. Negative and positive controls were included in each run of PCR as described above.

All the PCR products were analyzed on 7% acrylamide gel, visualized by silver staining and subsequently subjected to sequencing.

sequenced in a 16-capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). Sequence data were assembled and edited with SeqScape software v2.5 (Applied Biosystem, Foster City, CA, USA). Sequences obtained were aligned and compared with representative sequences of *Leishmania* available in GenBank.

Phylogenetic analysis of the sequences was conducted by using the neighbor-joining method with 1000 bootstrap replicates implemented in MEGA 4 software (Tamura *et al.*, 2007). Only sequences with more than 750 bp were used for the phylogenetic analyses.

RESULTS

Overall the diagnosis of leishmaniasis was confirmed by real time PCR in 36 patients out of 44 (81%).

The real time PCR confirmed 49 samples out of 83 (59%) as *Leishmania* positive. In particular 100% of the cultured samples resulted positive, 75% (6/8) of those diagnosed by serology and 47% (28/60) of samples from patients diagnosed on the basis of the clinical symptoms. The positivity in real time PCR was higher (57%) for HBM samples compared to HB (33%) and HLN (50%).

The PCRs targeting the kDNA and gp63 genes produced the expected amplicates in all the real time PCR positive samples, with the exception of few blood samples for both PCRs and few lymph node samples for kDNA-PCRs only. Ninety percent of the amplicons (74/82) from kDNA-PCRs and 92% (79/86) from gp63-PCRs were then successfully sequenced (Tab. 2).

Table 2 - Results of real time PCR and sequences obtained with the PCRs

Sample type	No of samples	Real Time PCR +ve (n° of sequences obtained)	kDNA PCRs products (n° of sequences obtained)	gp63 PCRs products
HBM*	21	12 (57%)	24 (23)	24 (22)
HB*	21	7 (33%)	5 (5)	4 (3)
HLN*	18	9 (50%)	13 (8)	18 (17)
HLN	1	1 (100%)	2 (2)	2 (2)
HB	7	5 (71%)	8 (6)	8 (6)
DNA	7	7 (100%)	14 (14)	14 (13)
DNA	1	1 (100%)	2 (1)	2 (2)
DNA	1	1 (100%)	2 (2)	2 (2)
DNA	6	5 (100%)	12 (12)	12 (12)

HBM: Human Bone marrow, HB: Human Blood, HLN: Human Lymph Node. *: samples from same patients.

Note: PCR products refer to the amplicates of the two gene segments.

Sequencing and Phylogenetic Analysis

PCR products were sequenced using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystem, Foster City, CA, USA). The products of the sequencing reactions were purified using PERFORMA DTR Ultra 96-Well kit (Edge BioSystems, Gaithersburg, MD, USA) and

The results of the biomolecular analyses are shown in table 2. Sequence analysis of both genes (kDNA and gp63) showed that all the samples associated with VL, PKDL and ML clinical forms and one sample associated with CL symptoms belonged to the *L.donovani* complex. Other 3 CL samples attributed to *L.major* by gp63 gene gave doubtful results

with the kDNA gene. Overall the homology of the sequence samples of this study with the reference sequences deposited in GenBank ranged from 93% to 96%.

Phylogenetic trees inferred by both kDNA and gp63 genes (Figs. 3-4) confirmed that Sudanese samples cluster into two distinct groups, corresponding to *L. donovani* complex and *L. major* species. All cutaneous samples were not included

neous samples were classified as *L. major*, with the exception of one sample which clustered with *L. donovani*. All the other sequence samples (from VL, ML and PKDL patients) clustered within the *L. donovani* complex and were closer to *L. donovani* than to *L. infantum*.

The intraspecific nucleotide variation in the gp63 between our sequences and the positive control

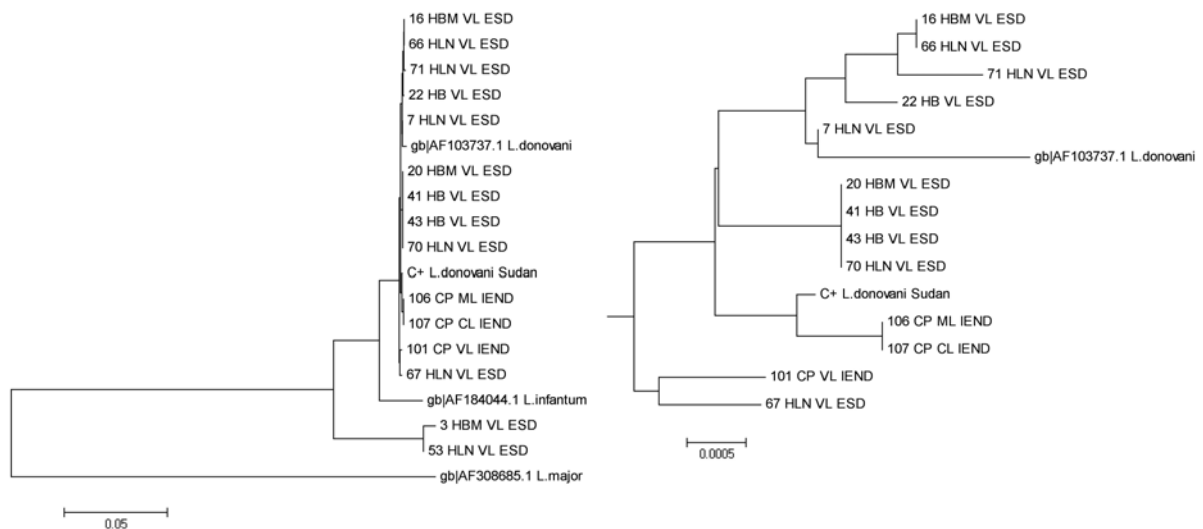


Figure 3 - Phylogenetic tree and sub tree based on 15 kDNA sequence data, the positive control (C+ *L. donovani* Sudan) and *L. donovani*, *L. infantum*, *L. major* sequences available in GenBankTM. The trees were constructed using Neighbour-Joining (NJ) method.

Note: cutaneous samples were not included

HBM= human bone marrow; HLN=human lymph node; HB= human blood; VL=visceral leishmaniasis; ML=mucocutaneous leishmaniasis; PKDL=post kala-azar dermal leishmaniasis; CP=cultured parasite; ESD=eastern Sudan; IEND=samples from Karthoum hospital

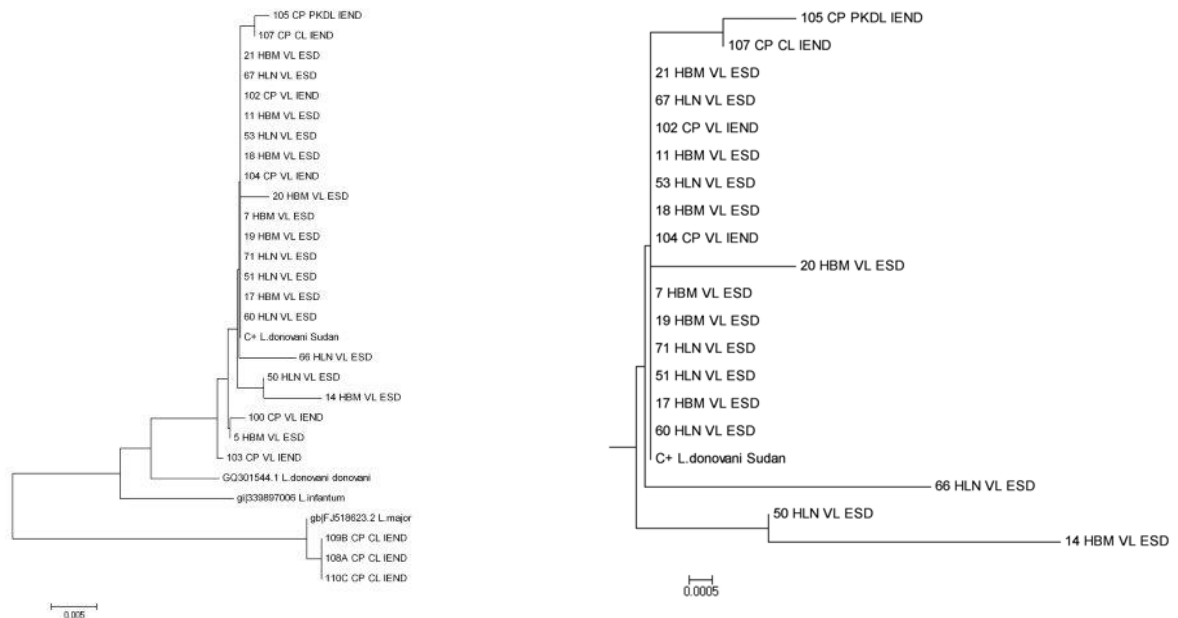


Figure 4 - Phylogenetic tree and sub tree based on 26 gp63 sequence data, the positive control (C+ *L. donovani* Sudan) and *L. donovani*, *L. infantum* and *L. major* sequences available in GenBankTM. The trees were constructed using Neighbour-Joining (NJ) method.

HBM= human bone marrow; HLN=human lymph node; HB= human blood; VL=visceral leishmaniasis; ML=mucocutaneous leishmaniasis; PKDL=post kala-azar dermal leishmaniasis; CP=cultured parasite; ESD=eastern Sudan; IEND=samples from Karthoum hospital

ranged from 0.0% to 0.6%, while the interspecific nucleotidic variation between the positive control and *L.donovani donovani* and *L.infantum* sequences deposited in GenBank™ were respectively 1.8% and 2.7% (Tab. 3). Intraspecific variation in the kDNA sequences ranged from 0.2% to 0.8%, while the interspecific variation between the positive control and *L.donovani* and *L.infantum* sequences deposited in GenBank™ were respectively 0.8% and 7.7% (Tab. 4). Regarding *L.major* the interspecific nucleotidic variation in the gp63 gene were 7.4% and 8.1% for *L.infantum* and *L.donovani* respectively (Tab. 5).

The phylogenetic tree inferred by minicircle kDNA

samples (Tab. 4).

The gp63 gene sequences comparison including the 53HLN sample (Tab. 3) did not reveal noteworthy variation.

DISCUSSION

The modified real time PCR used in this study revealed to be a useful tool for the screening of a large number of samples. 81% of the patients were confirmed positives by real time PCR. As expected, all samples from cultured parasites were positive by real time PCR while only 50% of the other samples from symptomatic patients were found positive.

The sensitivity and specificity of the method need to

Table 3 - Intra and inter-specific nucleotide differences among *L.donovani* isolates of this study (gp63 gene sequences) isolates (kDNA gene sequences).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. C+ <i>L.donovani</i> Sudan																									
2. 44 HB VL ESD	0.000																								
3. 51 HLN VL ESD	0.000	0.000																							
4. 53 HLN VL ESD	0.000	0.000	0.000																						
5. 60 HLN VL ESD	0.000	0.000	0.000	0.000																					
6. 71 HLN VL ESD	0.000	0.000	0.000	0.000	0.000																				
7. 67 HLN VL ESD	0.000	0.000	0.000	0.000	0.000	0.000																			
8. 7HBM VL ESD	0.000	0.000	0.000	0.000	0.000	0.000	0.000																		
9. 11HBM VL ESD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000																	
10. 17HBM VL ESD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000																
11. 18HBM VL ESD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000															
12. 19HBM VL ESD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000														
13. 21HBM VL ESD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000													
14. 104 CP VL IEND	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000												
15. 102 CP VL IEND	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000											
16. 107 CP CL IEND	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002										
17. 5HBM VL ESD	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003									
18. 100 CP VL IEND	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.005	0.002								
19. 50 HLN VL ESD	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.005	0.005	0.006							
20. 105 CP PKDL IEND	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.002	0.005	0.006	0.006						
21. 20HBM VL ESD	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.005	0.005	0.006	0.006	0.006					
22. 103 CP VL IEND	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.005	0.002	0.003	0.006	0.006	0.006				
23. 14HBM VL ESD	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.006	0.006	0.008	0.002	0.008	0.008	0.008			
24. 66 HLN VL ESD	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.008	0.008	0.010	0.010	0.010	0.010	0.010	0.011		
25. GQ301544.1 <i>L.donovani donovani</i>	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.020	0.016	0.018	0.018	0.021	0.021	0.015	0.020	0.025	
26. gi339897006 <i>L.infantum</i>	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.029	0.029	0.031	0.027	0.031	0.031	0.029	0.029	0.031	0.020

Table 4: Intra and inter-specific range of nucleotidic differences of *L. donovani* isolates (kDNA gene sequences).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. gbIAF103737.1 <i>L.donovani</i>															
2. 7 HLN VL ESD	0.004														
3. 22 HB VL ESD	0.006	0.002													
4. C+ <i>L.donovani</i> Sudan	0.008	0.004	0.006												
5. 16 HBM VL ESD	0.008	0.004	0.006	0.008											
6. 66 HLN VL ESD	0.008	0.004	0.006	0.008	0.000										
7. 71 HLN VL ESD	0.008	0.004	0.002	0.008	0.004	0.004									
8. 41 HB VL ESD	0.010	0.006	0.008	0.006	0.006	0.006	0.010								
9. 43 HB VL ESD	0.010	0.006	0.008	0.006	0.006	0.006	0.010	0.000							
10. 20 HBM VL ESD	0.010	0.006	0.008	0.006	0.006	0.006	0.010	0.000	0.000						
11. 70 HLN VL ESD	0.010	0.006	0.008	0.006	0.006	0.006	0.010	0.000	0.000	0.000					
12. 67 HLN VL ESD	0.010	0.006	0.008	0.006	0.009	0.009	0.009	0.008	0.008	0.008	0.008				
13. 106 CP ML IEND	0.010	0.006	0.004	0.002	0.009	0.009	0.006	0.008	0.008	0.008	0.008	0.008			
14. 107 CP CL IEND	0.011	0.008	0.006	0.004	0.011	0.011	0.008	0.010	0.010	0.010	0.010	0.009	0.002		
15. 101 CP VL IEND	0.011	0.008	0.010	0.008	0.011	0.011	0.011	0.010	0.010	0.010	0.010	0.006	0.010	0.011	
16. gbIAF184044.1 <i>L.infantum</i>	0.082	0.077	0.079	0.077	0.073	0.073	0.077	0.073	0.073	0.073	0.073	0.075	0.079	0.081	0.077

gene sequences (Fig. 3), showed that two samples of the same patient taken from bone marrow and lymph node (3HBM and 53HLN) clearly clustered differently from other sequences. the two sequences were aligned with the *L.donovani* and *L.infantum* from GenBank™ (Fig. 5). Alignment showed a nucleotidic difference of 18.1% and 19.9%, respectively. This nucleotidic variation is much higher than the interspecific variation showed by the other Sudanese

Table 5 - Intra and inter-specific range of nucleotidic differences of *L. major* isolates (gp63 gene sequences).

	1	2	3	4	5	6
1. gbIFJ518623.2 <i>L.major</i>						
2. 110C CL IEND	0.003					
3. 108A CP CL IEND	0.004	0.001				
4. 109B CP CL IEND	0.004	0.001	0.000			
5. gi339897006 <i>L.infantum</i>	0.074	0.075	0.075	0.076		
6. GQ301544.1 <i>L.donovani donovani</i>	0.076	0.079	0.078	0.078	0.019	
7. C+ <i>L.donovani</i> Sudan	0.081	0.084	0.082	0.082	0.044	0.026

#gb AF103737.1_L.donovani	ATG	GGC	TCC	CCT	GGG	CTG	GAT	TGG	GCT	COC	CTG	GGC	TGG	ATT	GGG	CTG	GAA	COC	TGG	GST	TGG	-AG	GCT	TGG	TTT	TGG	
#3HEM_EASTERN_SD	..T	...	CT.	GG.GTC	...	---AC	...	
#53HLN_EASTERN_SD	..T	...	CT.	GG.GTC	...	---AC	...	
#gb AF184044.1_L.infantum-T	..TTAATCGAC	...
#gb AF103737.1_L.donovani	GTG	GGC	TAG	CAT	TTT	TGG	TGT	TTT	AAT	GGT	TCG	GG-	TGC	CTT	TGA	TGT	GGC	TGG	TGG	TTA	TGC	GGC	TTT	AGT	GGG	TTG	
#3HEM_EASTERN_SD	..CT	GT.	...	G.T	..AGT	..G	..-TA	---GG	..-	
#53HLN_EASTERN_SD	..CT	GT.	...	G.T	..AGGT	..G	..-TA	---GG	..-	..-	
#gb AF184044.1_L.infantumG-TG	..TG	..T	
#gb AF103737.1_L.donovani	TAG	CCT	GGC	GGA	GGC	TCG	TGC	CTT	GGT	GGG	GST	GCC	CTG	GTG	GGG	GTG	CTT	CGG	G-T	TA-	CTC	TAC	GGG	AGT	CTT	TGA	
#3HEM_EASTERN_SD	...	---	...	T.G	..T	..TA	C.AC	..ATTG	T.T	..GC	T..	C..	GC.C	..GG	...	GTG	...	T.GT	
#53HLN_EASTERN_SD	...	---	...	T.G	..T	..TA	C.AC	..ATTG	T.T	..GC	T..	C..	GC.C	..GG	...	GTG	...	T.GT	
#gb AF184044.1_L.infantum	G..TTAT	..AGTG	..-TG	
#gb AF103737.1_L.donovani	TGA	TGA	TGG	GGG	TGG	G-T	TGG	CTT	TGG	TGT	TGG	GGT	T-G	ATC	GAT	GGT	GGT	CTT	GGG	TTG	COC	TTA	GGT	GG-	TGA	TTC	
#3HEM_EASTERN_SD	CTTGA	..T	..GST	TT.	..AG	..G	C..	
#53HLN_EASTERN_SD	CTTGA	..T	..GST	TT.	..AG	..G	C..	
#gb AF184044.1_L.infantumCT	G..	..T-	..AAA-A	GC.A-G	C..	
#gb AF103737.1_L.donovani	TCT	TTA	ATA	TTA	ATA	TTA	AAG	GTA	AGT	GCT	T-G	GGC	GTA	TGA	TAT	AGC	CAT	AGC	GCT	TTA	GAA	TAG	TTC	GGC	TCC	GAA	
#3HEM_EASTERN_SDCG	G..T	..T	A.G	..TG</							

Figure 5 - Nucleotide alignment of the 3HBM and 53HLN kDNA sequences with *L.donovani* and *L.infantum* reference sequences obtained from GenBank™. The two samples obtained from the same patient.

be determined in the next future. However, it was evident that the method was more sensitive in bone marrow samples (57%) compared to lymph node (50%) and blood samples (33%) from the same patients, confirming the bone marrow as the best sample for *Leishmania* diagnosis.

Even if PCR amplification of each target gene from individual DNA samples resulted in amplicons of the expected size, the Basic Local Alignment Search Tool (Blast) analyses of the sequences did not clearly classify the samples into a particular *Leishmania* species. This could be due either to the genetic difference in the amplicons or to the scarcity of sequences of the same target genes in GenBank™ unequivocally classified at the species level. However phylogenetic analysis helped to clarify the scenario when samples with more than 750 bp were included.

The phylogenetic analysis of both genes indicated the existence of two monophyletic groups and they were concordant in clustering VL, ML and PKDL samples with *L. donovani* positive control and *L. donovani* sequences available in GenBank, and in clustering 4 out of 5 CL samples with *L. major*. Two patients with CL clinical signs resulted infected by *L. donovani*. This species has been reported to cause cutaneous leishmaniasis in Sudan (Elamin *et al.*, 2005; 2007) as well as Leishmanioma (ulcerating swellings at the site of inoculation of the parasite by the phlebotomine vector (El-Hassan and Zijlstra, 2001a).

The only two samples from patients with ML and PKDL clustered in the *Leishmania* complex as well.

PKDL clinical form seems to be caused by *L. donovani sensu stricto* in Sudan and no particular zymodeme has been found to be associated with it (El-Hassan and Zijlstra, 2001b).

Two samples from the same patient with VL symptoms showed a remarkable nucleotidic difference compared to the other sequences of the *L. donovani* complex (Fig. 5) suggesting the possible presence of a new different *L. donovani* genotype. The nucleotidic difference was revealed only by one of the two target genes, this highlights the need of a multilocus approach to detect polymorphism in the genetic structure of Leishmania.

The results presented in this study are still preliminary and further genetic studies are needed in order to examine whether the genetic population differences of *Leishmania* spp. in Sudan are related to their capacity of causing different clinical signs. Particularly, more samples from PKDL, ML and CL patients should be obtained and analysed.

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Active monitoring of ticks and tick-borne zoonotic pathogens (TBP) as part of a 'one-health' surveillance strategy: a case study from the Colli Euganei Regional Park, north-eastern Italy.

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Summary - A survey on ticks and tick-borne zoonotic pathogens was carried out in 2009 and 2010 in Colli Euganei Regional Park (Veneto Region, Italy). Fiftyfive sites were sampled once or monthly to assess the presence and seasonality of ticks. In 67 out of 191 dragging 359 ticks were collected: *Ixodes ricinus* was the most abundant species with 341 specimens (54 adults, 95 nymphs, 192 larvae), followed by *Rhipicephalus sanguineus* (11 larvae), *Dermacentor marginatus* (6 adults) and *I. acuminatus* (1 adult). The overall average density for *I. ricinus* was 2.05 ticks/100m². PCR was applied to identify the zoonotic agents. *B. burgdorferi* s.l. was found in 16/61 adults (26.2%; 95%CI 15.2% - 37.2%), in 2/32 pools of nymphs (5.0%; 95%CI 1.6% - 11.3%) and in 5/35 pools of larvae (1.0%; 95%CI 0.1% - 3.6%). *Rickettsia* spp. was found in all the tick species excepted for *I. acuminatus*. The species of *Rickettsia* identified in *I. ricinus* were *R. monacensis* (26/118 - 22.0%) and *R. helvetica* (4/118 - 3.4%). *R. monacensis* was identified in one pool of *Rh. sanguineus* larvae. *R. slovac*a was identified in one adult of *D. marginatus*. All the examined ticks were negative for TBE virus, *A. phagocitophilum*, *Bartonella* spp. and piroplasms.

Keywords: Tick-borne zoonoses, natural park, epidemiology, Italy

INTRODUCTION

According to WHO, zoonoses represent about the 75% of the emerging human diseases. Among these, vector-borne diseases (VBD) are relevant and emerging for many reasons: climate change, use of natural environment, increase of recreational human activities, expansion of wild animal populations. This is an emergent public health concern also in Italy (Romi, 2010).

In the last two decades, many short-term and medium-term ticks and tick-borne zoonotic pathogens (TBP) monitoring projects has been implemented in different part of the world and particularly in areas where an increased incidence of human clinical cases was reported (Cinco *et al.*, 2006; D'Agaro *et al.*, 2009; Kollaritsch *et al.*, 2011; Rizzoli *et al.*, 2011). The main objectives of these projects were to investigate the ecology of the different tick species, to understand the epidemiology of the associated infections and possibly to identify risk factors and to design accurate risk prediction models and maps (Rizzoli *et al.*, 2007;

Carpi *et al.*, 2008; Rizzoli *et al.*, 2009). This activity, focused on animal and vector populations, was intended to support and integrate surveillance and control programs mainly based on improvement of diagnosis, prevention and treatment of the diseases in humans.

Small or large-scale field surveys of the animal reservoirs and of the vector populations have been implemented in Italy. This is the case of Lyme borreliosis (LB) and of Tick Borne Encephalitis (TBE) in northeastern Italy (Rizzoli *et al.*, 2007; D'Agaro *et al.*, 2009; Nazzi *et al.*, 2010), tick-borne lymphadenopathy (TIBOLA) in Tuscany (Selmi *et al.*, 2009) and of Mediterranean spotted fever (MSF) in southern Italy (Torina and Caracappa, 2007).

North-eastern Italy is one of the geographical areas of higher occurrence of reported human cases of LB (Nazzi *et al.*, 2010; Pistone *et al.*, 2010; Calderaro *et al.*, 2011) and TBE (Cinco *et al.*, 2004; D'Agaro *et al.*, 2009), mainly in northern alpine forested areas. However, information on ticks distribution and on associated TBP is lacking for the Colli Euganei

Regional Park, an isolated hilly area of volcanic origin in the central part of the Veneto Region, supporting intense human activities such as agriculture, rural tourism and recreation and potentially at risk, because of its climatic and environmental characteristics and because of the high level of human-wildlife interactions.

In this context during 2009 and 2010 a project funded by University of Padova was carried out in the Colli Euganei Regional Park, in order to investigate the presence, distribution and ecology of ticks as well as to assess the prevalence of the main TBP (i.e. *Anaplasma phagocytophilum*, *Borrelia burgdorferi* s.l., *Rickettsia* spp., *Bartonella* spp., *Babesia* spp., *Theileria* spp., and TBE virus). The overall objective of the project was to provide a baseline survey of TBD in the area and to implement a pilot project for the detailed analyses of the environmental factors affecting tick abundance and TBP prevalence. The final expected output is the design of risk prediction models and risk maps, to be extended at regional level.

MATERIAL AND METHODS

Tick collection

The survey was conducted from April to November 2009 and from March to November 2010 in the area of the Colli Euganei Regional Park (Fig. 1).

Ticks were collected by dragging using a 1m² white flannel cloth along transect of 100 meters; the cloth was examined with interval of five meter to avoid detachment of ticks.

During the two-years study 55 sites distributed all over the area have been surveyed accounting for a total of 191 catches. Since there was no information on the presence of ticks in this area, during the first year 51 sites were sampled once to assess the presence and the density of ticks in the Park. Four other sites have been sampled monthly (28 draggings) to assess ticks seasonality. During the second year we surveyed monthly 16 sites (112 draggings) out of the 55 previously surveyed, selected according to different geographical exposures and ticks abundance.

Each site was georeferenced using GPS (Juno SB®, Trimble, USA) and at each sampling relative humidity (RH) and temperature (T°) were reported using a portable thermo-hygrometer.

Tick specimens were registered according to site and date of collection, identified (Manilla and Iori, 1992) and stored at -80°C.

GIS database

It has been used as much open-source software and free development tools as possible. Implementation has been carried out mainly in the PostgreSQL object-relational database, in tight conjunction with

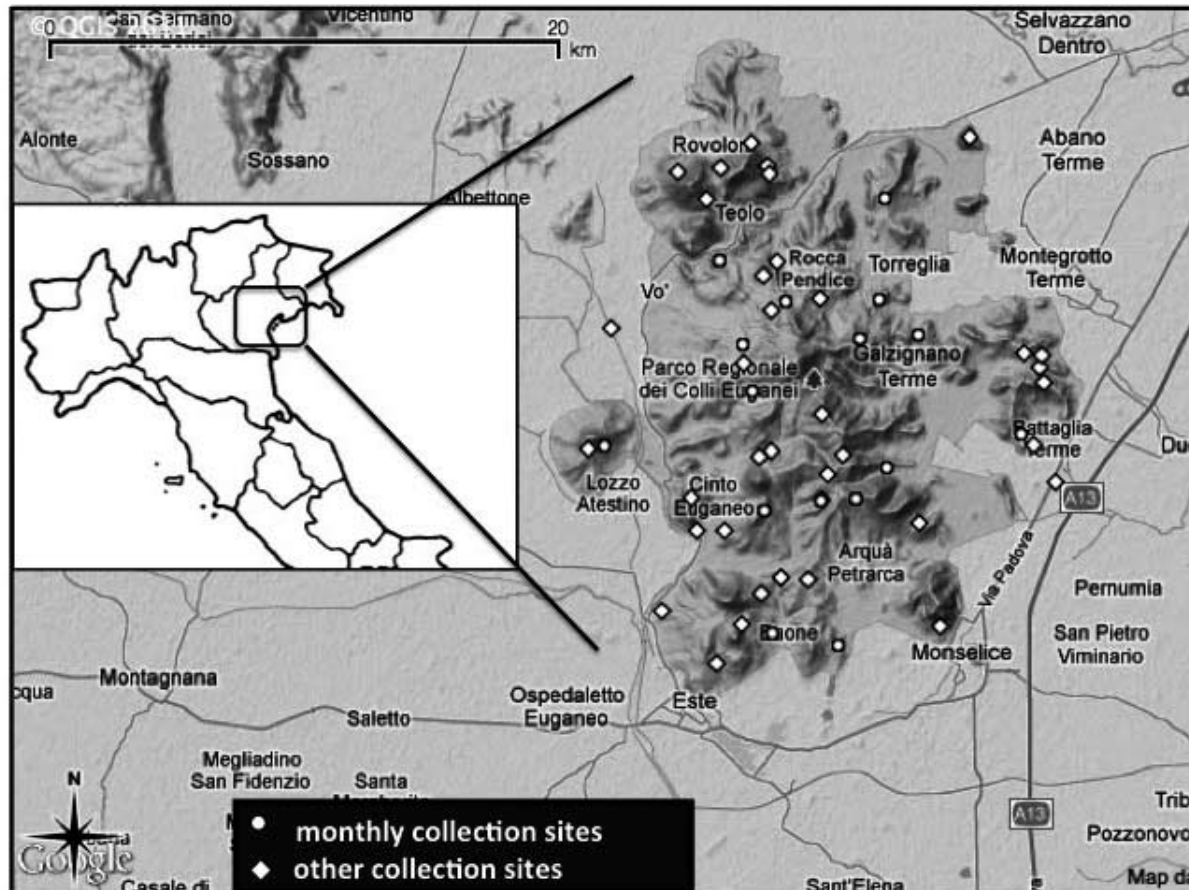


Figure 1 - Map of Colli Euganei Regional Park

its extension PostGIS allowing the PostgreSQL server to be used as a back-end spatial database for geographic information systems. A server-client approach has been chosen in order to achieve higher flexibility and the possibility to integrate also spatial data into the same unique environment. Existing and freely available regional cartography and maps have been used. Height points and contour lines have been extracted to generate a digital terrain model (DTM) and the derived products such as aspect and slope maps. Slope and aspect maps had a grid resolution of 10 m.

Since GPS-acquired site coordinates and the existing cartography have different reference systems (WGS84 and Roma40-Gauss Boaga, respectively), particular attention has been paid to all problems related to coordinate transformations.

OpenOffice has been adopted as user front-end to access the database. More specifically, Base can connect to PostgreSQL and offers tools to inspect data, define queries, create forms and, finally, it is integrated with the spreadsheet Calc, also included in the office suite. For geospatial analysis, PostgreSQL/PostGIS can be interfaced with Quantum GIS (the data inspection and visualisation tool of choice in this project) and with GRASS GIS, a feature-rich GIS environment, as well as with R language, for more specific statistical analyses.

DNA/RNA extraction

DNA and RNA were extracted from Ixodide ticks using All Prep DNA/RNA mini Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's instructions; for the other ticks species collected we extracted DNA using DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany). Extraction was carried out on single adult ticks and on pooled sample of nymphs (1 to 10) and larvae (1 to 20).

Biomolecular analysis

Anaplasma phagocytophilum/*Borrelia burgdorferi* s.l

To detect *Anaplasma phagocytophilum* and *Borrelia burgdorferi* s.l. a multiplex realtime PCR was conducted using primers and probes described by Courtney *et al.*, (2004) on the Roche LightCycler 480. The assay was run in a volume of 10 μ l consisting of 5 μ l of Kapa Probe Fast qPCR reagent (Kapa Biosystems Ltd, Cape Town South Africa), primers ApMsp2F (ATGGAAGGTAGTGTGGTTATG-GTATT) and ApMsp2R (TTGGTCTTGAAGCG CTCGTA) at a concentration of 1,25 μ M, primers Bb23SF (CGAGTCTTAAAGGGCGATTTAGT) and Bb23SR (GCTTCAGCCTGGCCATAAATAG) at a concentration of 0,87 μ M, probes ApMsp2 (FAM-TGGTGCCAGGGTTGAGCTTGAGATTG-IBFQ) and Bb23S (Cy5-AGATGTGGTAGACCC-GAAGCCGAGTG-IBRQ) at a concentration of 1 μ M and 2 μ l of target DNA. Two plasmids

harbouring the gene target sequences served as positive controls. Briefly the PCR condition were 1 cycle of denaturation at 95°C for 90 sec followed by 45 cycles consisting of denaturation at 95°C for 2sec and annealing/extension at 60°C for 20sec. Fluorescence data collection was performed at the end of each annealing/extension step.

Rickettsia spp

Rickettsia spp. detection was performed with a conventional PCR targeted at *gltA* gene described by Regnery *et al.*, (1991). A 50 μ l assay run contained 1x PCR Buffer (Genespin, Milan, Italy), 1.5 mM MgCl₂, 0,2 mM of each dNTP, 1 mM primer Rp877 (GGGGGCCTGCTCACGGCGG), 1mM primer Rp1258n (ATTGCAAAAAGTACAGTGAACA), 1U Xtra Taq Polymerase (Genespin, Milan, Italy) and 5 μ l of DNA. PCR was carried out on a 2720 Thermal Cycler (Applied Biosystems) with the following cycling profile: 5min denaturation at 95°C; 50 cycles of 30sec at 95°C, 45sec at 45°C and 45sec at 65°C; a final 7min extension step at 65°C. DNA extracted from a positive tick was used as positive control. The PCR products were separated on 2% agarose gel along with the molecular weight marker pUC Mix marker 8 (Fermentas GmbH, St Leon-Rot, Germany). Results were visualised using a Gel Doc Imaging System (Bio-Rad Laboratories, Hercules, USA) and its Quantity One Analysis Software.

Both strands of purified amplicons obtained from positive ticks were sequenced using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and the consensus sequences were subjected to Blast analysis to identify *Rickettsia* species. In case of uncertain species identification, a second PCR targeted either on *ompA* (Roux *et al.*, 1996) or on *ompB* (Choi *et al.*, 2005) gene was performed.

Bartonella spp

Intergenic region between 16S and 23S rRNA encoding genes (ITS) of *Bartonella* spp. was amplified with the primers described by Jensen *et al.*, (2000). A 20 μ l assay run contained 1x PCR Buffer (Genespin, Milan, Italy), 1,5mM MgCl₂, 0,2mM of each dNTP, 0,2 μ M primer F (CTCTTTCTTCA-GATGATGATCC), 0,2 μ M primer R (AACCAACT GAGCTACAAGCCCT), 1U Xtra Taq Polymerase (Genespin, Milan, Italy) and 2 μ l of DNA. PCR was carried out on a 2720 Thermal Cycler (Applied Biosystems) with the following cycling profile: 5min denaturation at 95°C; 50 cycles of 20sec at 95°C, 30sec at 60°C and 30sec at 72°C; a final 7min extension step at 72°C. DNA extracted from ATCC either *Bartonella henselae* or *Bartonella claridgeiae* strain was used as positive control. The PCR products were separated on 3% agarose gel along with the molecular weight marker 5 (Fermentas GmbH, St Leon-Rot, Germany). Results

were visualised using a Gel Doc Imaging System (Bio-Rad Laboratories, Hercules, USA) and its Quantity One Analysis Software.

Babesia spp. and *Theileria* spp.

To identify *Babesia* spp. and *Theileria* spp. the following primers were used: Crypto-F (AACCTG-GTTGATCCTGCCAGT; Herwaldt *et al.*, 2003) and RLB-R (CTAAGAATTTCACCTCTGACAGT; Centeno-Lima *et al.*, 2003), amplifying the 800 bp of the 18S rRNA. PCR was done in 30 μ l of reaction mixture containing 1x buffer, 2 mM MgCl₂, 0.2

marginatus (6 adults) and *I. acuminatus* (1 adult). The overall average density recorded for *I. ricinus* (N ticks/100m²) was 2.05 (adults: 0.33; nymphs: 0.57; larvae: 1.16). The temperature was significantly lower in sites with presence of adult whereas RH was not significantly associated with presence of ticks. In 2010, the seasonal trend of adults showed a peak in spring and autumn, nymphs showed a peak during June and larvae in summer, from July to September (Fig. 2).

The results of biomolecular analyses (Tab. 1) showed a high prevalence of *B. burgdorferi* s.l.,

Table 1 - Results of the biomolecular analyses.

PATHOGEN	<i>Ixodes ricinus</i>			<i>Ixodes acuminatus</i>	<i>Dermacentor marginatus</i>	<i>Rhipicephalus sanguineus</i>
	Adult (individual)	Nymphs (pool)	Larvae (pool)	Adult (individual)	Adult (individual)	Larvae (pool)
<i>Rickettsia</i> spp.	11/54	10/35	9/29	0/1	1/6	1/3
<i>Borrelia burgdorferi</i> s.l.	15/54	5/35	2/29	0/1	1/6	0/3
<i>Anaplasma phagocitophilum</i>	0/54	0/21	0/12	0/1	NI*	NI*
<i>Babesia</i> spp.	0/54	0/21	0/12	0/1	0/3	0/2
<i>Bartonella</i> spp.	0/54	0/21	0/12	0/1	0/3	0/2
<i>TBE virus</i>	0/54	0/21	0/12	0/1	NI*	NI*

*Not Investigated

mM dNTPs, 0.5 μ M primer and 1 unit of Taq polymerase (Platinum Taq Invitrogen). The reaction cycle is as follows: 2min denaturation at 94°C; 17 cycles of 30sec at 94°C and 30sec at 64°C with a decrease of 0.25°C each cycle, 25 cycles of 30sec at 94°C, 30sec at 60°C and 30sec at 72°C; a final 7min extension step at 72°C. The amplification was performed in Tpersonal termocycler (Biomtra, Gottingen Germany).

TBE virus

A realtime RT-PCR was used for TBE detection using the primers and protocol suggested by Schwaiger and Cassinotti, (2003).

Statistical analysis

T-test was used to assess differences in the presence of ticks according to RH and T°; Kruskal-Wallis test and Mann-Whitney test were used to analyze tick density according to month of sampling.

The estimated individual tick prevalence (p) was calculated according to method 3 of Cowling *et al.*, (1999) using the following formula: $p = 1 - (1 - x/m)^{1/k}$ (where x = positive pools; m = examined pools; k = mean number of ticks for each pool).

RESULTS

Totally, 67 out of 191 catches gave positive results and 359 ticks were collected: *Ixodes ricinus* was the most abundant species with 341 specimens (54 adults, 95 nymphs, 192 larvae) followed by *Rhipicephalus sanguineus* (11 larvae), *Dermacentor*

with 16/61 (26.2%; 95%CI 15.2% - 37.2%) positive adult, 2/32 positive pools of nymphs (5.0%; 95%CI 1.6% - 11.3%) and 5/35 positive pools of larvae (1.0%; 95%CI 0.1% - 3.6%). *Rickettsia* spp. was identified in all the tick species excepted for *I. acuminatus*. The two species of *Rickettsia* identified in *I. ricinus* were *R. monacensis* (26/118 - 22.0%) and *R. helvetica* (4/118 - 3.4%). Moreover *R. monacensis* was identified in one pool of *Rh. sanguineus* larvae. Finally we report the identification of *R. slovaca* from one adult of *D. marginatus*.

Instead, all *I. ricinus* ticks resulted negative to TBE virus and there were no positive samples for *A. phagocitophilum*, *Bartonella* spp. and piroplasms.

DISCUSSION

The present study demonstrated the presence of Ticks and TBP in the Colli Euganei Regional Park.

I. ricinus was the most abundant species as in others alpine and sub-alpine areas (Rizzoli *et al.*, 2011); other ticks species found were *D. marginatus*, probably as consequence of the recent introduction of wild boar in this area, and *R. sanguineus*, mainly associated with domestic dog, as consequence of the presence of residential as well as visiting dogs.

Tick density recorded in this area showed lower values and slightly different seasonality compared to others surveys in North-eastern Italy (Picolin *et al.*, 2006; Nazzi *et al.*, 2010; Tagliapietra *et al.*, 2011).

Dynamics and ecology of the specific tick populations should be better investigated according to some risk factors influencing their presence: host

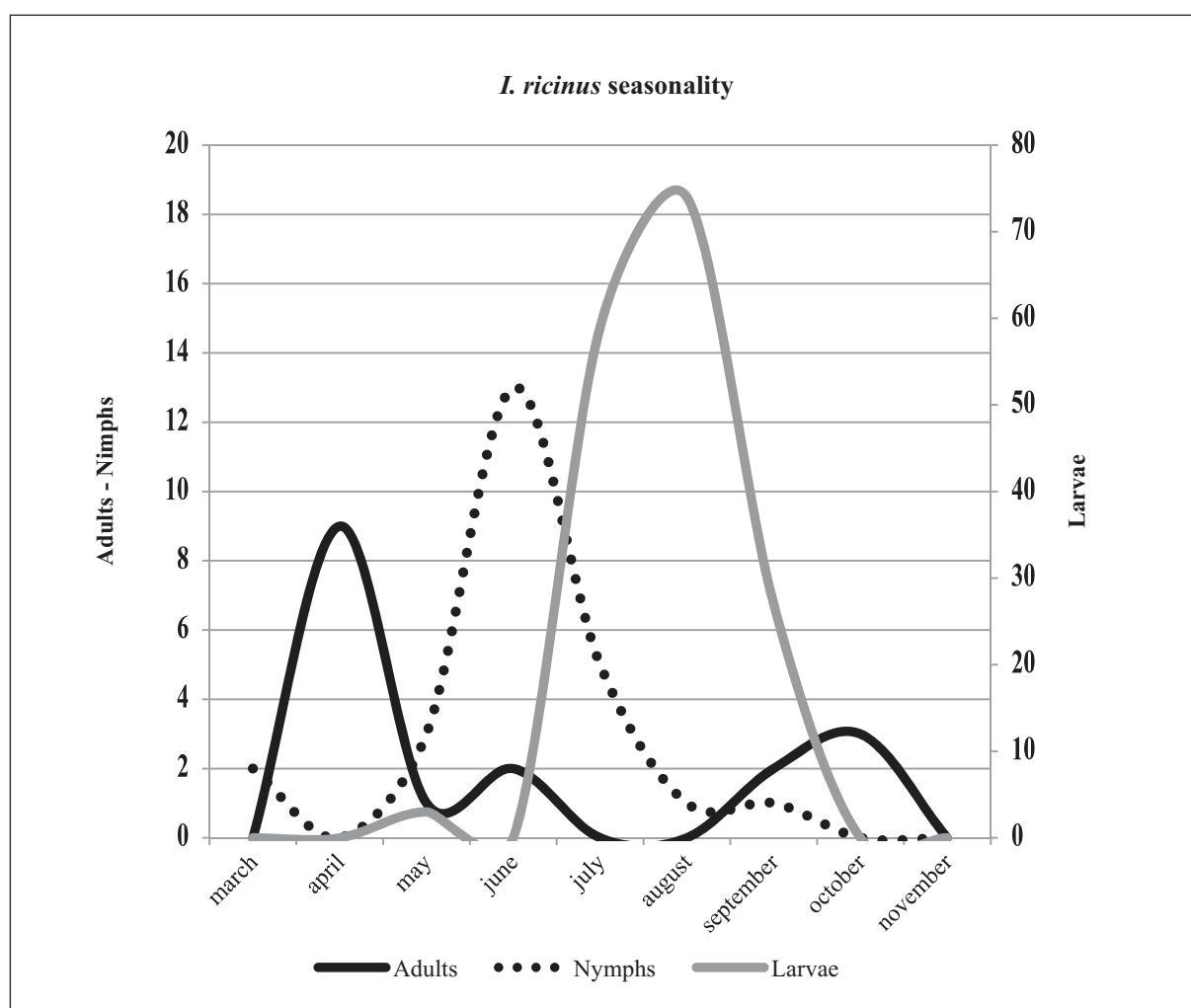


Figure 2 - Total number of *I. ricinus* per stage collected in 2010 from the 16 monthly collection sites.

animal populations, vegetation index, land use, local climate effect and environmental characteristics. Regarding the investigation on zoonotic agents, the most relevant aspect of our investigation are:

- 1) The high prevalence of *B. burgdorferi* s.l confirms that this infection is widespread in Italy as reported by other authors (Calderaro *et al.*, 2011; Di Renzi *et al.*, 2010; Nazzi *et al.*, 2010; Pistone *et al.*, 2010).
- 2) The presence of *Rickettsia* spp. in *I. ricinus* confirms the emergence of new rickettsial agents in Italy besides *R. conorii*. *R. helvetica* is included in the rickettsia spotted fever group and has been reported in other studies both in ticks and humans (Beninati *et al.*, 2002; Fournier *et al.*, 2004; Cinco *et al.*, 2006; Boretti *et al.*, 2009; Silaghi *et al.*, 2011); also *R. monacensis* was reported in other surveys in *I. ricinus* (Floris *et al.*, 2008) and seems to be associated to human clinical cases (Jado *et al.*, 2007).
- 3) The identification of *R. slovaca* in a questing *D. marginatus* tick claims for a better understanding of the real magnitude of this infection which

implies monitoring the wild boar population, recently introduced in the Park, and their associated tick population. In fact, a focus of Tick-borne Lymphadenopathy (Tibola) was recently described in a similar hilly area of Tuscany, Central Italy, where wild boar population has been suspected as the reservoir (Selmi *et al.*, 2008 and 2009).

- 4) The negative results for TBE virus suggests its absence in the area. This represents a positive finding given its potentially high pathogenicity to humans and its presence in the neighboring alpine forested areas. Piroplasms and *Bartonella* spp. also appear to be absent in the area. These infections are rarely reported in north-eastern Italy and their zoonotic potential is still debated. Nevertheless, it is important to include these pathogens among the surveyed ones, given the emerging zoonotic potential of some strains, such as the *Babesia* EU1 strain, which was identified as causative of human babesiosis in Europe (Hertwald *et al.*, 2003) and recently reported in ticks (Cassini *et al.*, 2010) and wild ruminants

(Duh *et al.*, 2005; Tampieri *et al.*, 2008) in the surrounding areas.

The outcomes of this study may suggest that human health service should pay particular attention to clinical cases referred to local hospitals with symptoms referable to TBD and particularly to LB and to TIBOLA. Besides, since the Colli Euganei Regional Park is densely populated by people for recreational and working activities, TBP should be continuously surveyed in the Park and information and education programs may be implemented by concerned authorities.

The active monitoring of the tick borne pathogens, also in absence of human clinical cases, may represent an important system to alert and prepare the public health services, in order to properly address diagnostics and care of patients. It is therefore highly recommended the integration between veterinary and public health services in the perspective of “one health - one medicine” concept.

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Factors influencing the risks of emerging animal diseases in the Mediterranean Regions: a review.

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Summary - In the last decades the detection of new infectious agents and the increasing spread of infectious diseases into new geographical areas led to the formulation of a new category of pathogens, responsible for the so called emerging and re-emerging diseases. Most of these emerging infectious diseases (EIDs) are zoonoses, being capable of infecting both animals and humans and sometimes to cause significant economic and public health consequences. Nowadays the European and Mediterranean countries are facing more frequently the occurrence of EIDs, which are challenging and forcing veterinary and public health Institutions to find new and more holistic control approaches. The aim of this paper is to discuss the main factors driving today the emergence of EIDs in Europe and in the Mediterranean Basin. Some vector-borne diseases (bluetongue, West Nile disease, Rift Valley fever, Crimean-Congo Haemorrhagic fever) have been considered as examples and the different aspects influencing their emergence or re-emergence are taken into account.

Key words: One World-One Health, emerging infectious diseases, bluetongue, West Nile virus, Rift Valley fever, Crimean-Congo Haemorrhagic fever.

INTRODUCTION

Although the catchphrase "One World - One Health" (OWOH) was firstly used in 2004 (WILD CONSERVATION SOCIETY, 2004), the "One Medicine" concept is not really new, but it has recently gained international attention (Zinsstag *et al.*, 2011). This approach leads to recognize that health of humans and other animals is one unique entity and man and non-human animals are sharing the same world and had a mutual influence with the environment where they lived in.

The massive changes of the last decades, especially the increased urbanization have led to large movements of populations and the opening up of much needed new areas for food production. These phenomena, coupled with the increased trade of meat, milk and other products of animal origin, and the expansion of air and land connections between countries, contributed to extend the impacts of some infections far beyond their geographical areas of origin, playing a substantial role in the worldwide globalization of diseases (Seimenis, 2008).

In the last decades the detection of new infectious agents and the increasing spread of infectious diseases into new geographical areas led to the formulation of a new category of pathogens, responsible for the so called emerging and re-emerging diseases. Most of these emerging infectious diseases

(EIDs) are zoonoses, being capable of infecting both animals and humans (Pfeffer and Dobler, 2010) and sometimes to cause significant economic and public health consequences. Their emergence is thought to be driven largely by socio-economic, environmental and ecological factors (Jones *et al.*, 2008). The interaction between living beings, including men, animals and pathogens, which share the same environment, should be seen as a unique system.

In the interaction between men, animals and pathogens different components may play a role:

1. the effects of globalization of trade of animals and animal products and the need for a multinational approach,
2. the role of wildlife and, more in general, of environmental factors in the spread and maintenance of infections,
3. the fundamental importance of the integration of veterinary and medical medicines into a "one medicine" strategy and, more in general, the necessity to pursue a multidisciplinary approach,
4. the need for a comprehensive view over the whole production chain, following a "farm to fork" approach.

Nowadays the European and Mediterranean countries are facing more frequently the occurrence of EIDs, which are challenging and forcing veterinary and public health Institutions to find new and more

holistic control approaches.

The aim of this paper is to discuss the main factors driving today the emergence of EIDs in Europe and in the Mediterranean Basin. Some vector-borne diseases will be considered as examples and the different aspects influencing their emergence or re-emergence will be taken into account.

Bluetongue: the role of climatic variables and animal trade.

Bluetongue virus (BTV) belongs to the genus *Orbivirus*, family *Reoviridae*. It causes an infectious, non-contagious disease of ruminants. Twenty four serologically and immunologically distinct serotypes of the virus are known. The virus replicates in all ruminant species, but severe disease is mostly restricted to sheep, although the recent epidemic of serotype 8 (BTV-8) in northern Europe was characterised by the presence of severe clinical signs in cattle.

BTV is transmitted by the bites of some species of *Culicoides* midges. Transmission is seasonal, and most cases occur between late summer and early autumn when adult vectors are abundant. Adult *Culicoides* are not strong fliers but they can be passively dispersed by the wind, up to several hundred kilometres, especially over the sea (Braverman and Chechik, 1996).

Until the previous century, for decades the disease was restricted to areas where the competent vector species live. Until the end of the last century many BTV serotypes have been circulating on the fringes of Europe (in sub-Saharan Africa, Turkey and the Middle East), but among European countries, only Greece sporadically experienced the introduction of the infection in some territories.

In the summer of 2000, however, serotype 2 (BTV-2) occurred in Italy (Sardinia), where it caused the loss of more than half million of sheep in two years, due to the severity of the disease. Before the epidemic of BTV, Sardinia had an intensive cattle trade with the rest of Italy, particularly with the northern regions. Due to the BTV infection, trade of ruminants came to a complete standstill until 2002, when the mass vaccination of all sheep, goats and cattle reared in Sardinia permitted the partial restore of animal trades to the free zones of the rest of Italy (Caporale and Giovannini, 2010). It was the first time that live-attenuated vaccines available at that time were successfully used in cattle with the aim of permitting the safe trade of vaccinated animals (Caporale and Giovannini, 2010).

Although the introduction of BTV into Sardinia by the movement of animals or animal products has been ruled out, the investigations carried out and the epidemiological characteristics of the epidemic induced to consider the passive windborne transportation of infected vectors from BTV-2-infected regions of North Africa as the most likely route of BTV introduction (Calistri *et al.*, 2004). The

summer of 2000 in Italy, in fact, was characterised by several dust storms, originating in North Africa and moving across to southern Italy and the Italian islands (Calistri *et al.*, 2004).

In August 2006, BTV-8 was notified in the Netherlands, close to the borders with France, Belgium and Germany. This was the onset of a rapidly spreading BTV-epidemic in north-western Europe that affected cattle and sheep holdings in almost all Western Europe, reaching latitudes as high as in Norway (Caporale and Giovannini, 2010). It is uncertain how BTV-8 reached north-western Europe, but the absence of BTV-8 circulation in the Mediterranean Basin suggests that it was not a linear extension of earlier European outbreaks but was introduced by a distinct route and mechanism. The 'wait and see' approach adopted at the beginning of BTV-8 epidemic by the affected countries, the lack of a vaccination policy until 2008 and the permission of animal movements within the infected areas contributed to the rapid spread of the infection all over the European territory (Caporale and Giovannini, 2010). These animal movements and the absence of vaccination might well explain the rapidity and the extent of BTV-8 spread, which resembled what happened in Sardinia in the absence of vaccination in the first four weeks of the 2000 epidemic. Furthermore, it must be noted that in northern Europe the spread in one year affected a geographic area that was larger than that affected over eight years in the Mediterranean Countries, where vaccination, surveillance and animal movement controls had been applied (Caporale and Giovannini, 2010).

What happened in Europe learns that climate-driven effects may facilitate the introduction and probably the persistence of diseases like bluetongue, but the animal trade, whenever not subjected to rigorous control measures, play a substantial role in the spread of the infection over large geographical areas.

West Nile disease and the role of wild animals.

West Nile virus (WNV) is a mosquito-transmitted *Flavivirus* belonging to the Japanese encephalitis antigenic complex in the family *Flaviviridae*. The ecological aspects of WNV infection, involving mosquitoes, birds and humans, were first described in the 1950s in Egypt (Taylor *et al.*, 1956). The infection in humans is usually asymptomatic, but 10–20% of infected people develop an acute, influenza-like, self-limiting febrile illness. Neurological disease in both humans and horses was reported for the first time in the late 1950s in Israel. WNV invaded North America in 1999 (Lanciotti *et al.*, 1999). From the original point of invasion (New York) the WNV dispersed within a few years over the whole U.S.A. and southern parts of Canada and also spread into Central America and parts of South America.

In the last 30 years several cases of West Nile infection in horses and humans were reported in Europe and in the Mediterranean Basin. Phylogenetic relatedness of the WNV segregates the isolates in five lineages even if only two of them currently are affecting the European territories (Bondre *et al.*, 2007). The lineage 1 includes WNV strains from Europe, North America e North Africa while lineage 2 was thought to comprise low pathogenic group of viruses endemic to sub-Saharan Africa. Recently, strains belonging to lineage 2 have been correlated to clinical disease in South African horses (Venter *et al.*, 2009) and also reported in Hungary and Austria affecting birds and horses (Bakonyi *et al.*, 2006). Furthermore lineage 2 has been proven to be highly pathogenic in humans (Papa *et al.*, 2011). A third lineage has been proposed to include the Rabensburg virus, an European strain isolated in Czech Republic, a fourth independent lineage which comprises an isolate from Caucasus and lineage 5, for an Indian isolate (Bakonyi *et al.*, 2006; Bondre *et al.*, 2007).

WNV is maintained in an enzootic cycle, transmitted primarily between avian hosts and mosquito vectors. The role of migratory birds in relation to the introduction of virus into Europe and the Mediterranean Basin is clearly assessed by numerous studies. According to this hypothesis the migratory birds, which may be infected in their African wintering places, are carrying the virus northward during spring migrations to European sites. This hypothesis would explain why outbreaks often occur in or near wetlands and urban areas, where migratory bird populations, vectors and amplifying hosts are often contemporaneously present. It would also explain the timing of outbreaks, with the introduction of WNV in April and May during the northward spring birds migration, the following amplification of the virus through the infection of local bird populations and the occurrence of viral infection in humans and equines from July to September (Calistri *et al.*, 2010a).

An epidemic of WNV encephalitis occurred in Italy during the late summer 1998, among horses in proximity of Padule di Fucecchio marshes, a wetland area in Tuscany, where, however, no significant wild bird deaths or rise in human neurological cases were detected (Cantile *et al.*, 2000). Following the WNV epidemic in Tuscany, the Italian government established a national WNV surveillance programme, which was in place in 15 Italian wetlands since 2001 (Calistri *et al.*, 2010a). After the epidemic in Tuscany, no further WNV clinical outbreaks were observed in Italy either in horses or in humans until August 2008, although sporadic evidences of WNV introduction and local circulation were detected between 2003 and 2007 by the national surveillance programme in some of the 15 risk areas (Calistri *et al.*, 2010a). The first clinical

signs of disease were observed on the 20th of August 2008 in a racehorse living in a stable in Ferrara Province, closer to Po river and less than 50 km far from the Comacchio wetlands, one of the risk areas selected in the framework of the WNV national surveillance programme. The suspicion of West Nile encephalitis was confirmed by virus neutralization test, RT-PCR and virus isolation (Calistri *et al.*, 2010b). As on 31 December 2008, the infection affected eight Provinces in three Regions (Emilia Romagna, Veneto, Lombardy), where a total of 794 cases of WNV infection in 251 equine stables were detected on the basis of the clinical signs and as a result of a serological screening in horses living in the area (Calistri *et al.*, 2010b). Since 2008, WNV is constantly circulating in Italy, progressively involving new areas, never infected before (Calistri *et al.*, 2010c). In particular two large epidemics occurred in Sicily and in Sardinia. During 2010, 46 outbreaks of WNV infection in horses were notified in the westernmost zones of Sicily (Trapani province), in an area where no previous evidences of WNV circulation were observed. In 2011, a severe WNV epidemic occurred in western Sardinia (Oristano province), causing 31 outbreaks in horse stables,

The significant number of virological positive birds found in Italy in 2008 and 2009 among magpies (*Pica pica*), carrion crows (*Corvus corone*) and rock pigeons (*Columba livia*) suggests a possible role of resident bird species in the maintenance of local virus transmission (Monaco *et al.*, 2011a). At the moment the lack of data on the length and the load of viraemia in these species does not permit to make any solid inference on the possible contribution of these birds in WNV endemisation and in the maintenance of the infection during the inter-epizootic periods (Calistri *et al.*, 2010a). While the role of resident birds in WNV endemisation is not clear, more information is available in relation to the vectors. Vertical transmission of WNV in mosquitoes (i.e. passage of virus from infected female to her offspring) was demonstrated in the laboratory (Baqar *et al.*, 1993) and in the field (Miller *et al.*, 2000). In addition, the detection of WNV in pools of overwintering hibernating *Culex pipiens* mosquitoes in New York City in 2000 clearly indicates the capacity of the virus to overwinter and to survive in hibernating infected adult mosquitoes. The survival of adult mosquitoes during winter and even a certain level of adult activity were sometimes observed in the Mediterranean countries and in other temperate areas (Romi *et al.*, 2006).

The mechanisms governing the bridging of virus infection to human and equines populations are not fully understood. The role of ubiquitous mosquito species, feeding both on humans and birds, is considered to play an important role and particularly the *Culex pipiens* species complex.

Usutu: an emerging zoonotic disease

Usutu virus (USUV) belongs to the Japanese encephalitis serogroup within the mosquito-transmitted viruses of the genus *Flavivirus* of the family *Flaviviridae*. USUV represents the typical example of an emerging pathogen. In Europe the virus was firstly detected in Austria in 2001, where it caused a widespread mortality among the population of Eurasian blackbirds (*Turdus merula*) and some other bird species. USUV was probably introduced into Austria via viremic birds returning from their winter migration from Africa to Europe. Another potential way of introduction was considered the transport of virus-infected mosquitoes from Africa to Austria via airplane, given the location of the emergence of infection in Austria, near the international airport of Vienna (Meister *et al.*, 2008). In the following years the presence of USUV was associated to mortality of birds in 2005 in Hungary (Bakonyi *et al.*, 2007) and the following year in Switzerland (Steinmetz *et al.*, 2007). In Italy a silent virus circulation was detected since 2007 (Lelli *et al.*, 2008; Savini *et al.*, 2011).

In 2009, USUV was shown to exhibit human pathogenicity when it was for the first time proven to cause neuroinvasive infection in two patients with immune deficiency (orthotopic liver transplantation, B cell lymphoma) in Italy (Pecorari *et al.*, 2009; Cavrini *et al.*, 2009).

USUV is thought to be maintained in nature by a mosquito-bird transmission cycle. In Italy, the virus was detected in a pool of *C. pipiens* and was isolated from organs of dead blackbirds suggesting that blackbirds and *C. pipiens* might play an important role in the Italian endemic cycle (Savini *et al.*, 2011). The fact that many horses often had neutralising titers to both WNV and USUV seems to imply that both viruses might share the same vector/s (Savini *et al.*, 2011). These observations coupled with the proven capacity of the virus to cause human illness in some instances, highlight the importance of implementing a specific surveillance system for these pathogens.

The influence of environmental perturbation in the ecology of Crimean Congo Haemorrhagic Fever

Crimean-Congo haemorrhagic fever (CCHF) is a viral infection transmitted by the bite of ticks or through the blood or body fluids of CCHF patients. It was firstly reported in the Crimean Peninsula in 1940 (Purnak *et al.*, 2007). Causative agent is a virus belonging to the genus *Nairovirus* of the *Bunyaviridae* family. *Hyalomma* genus ticks, and particularly *Hyalomma marginatum marginatum*, play an important role in the spread of the infection as a reservoir (Zavitsanou *et al.*, 2009). Ticks of the genus *Hyalomma*, in fact, serve as vectors and reservoir of the CCHF virus and the geographic distribution of the disease overlaps with the global distribu-

tion of *Hyalomma* ticks (Karti *et al.*, 2004).

Humans become infected through tick bites or contact with infected blood or tissues. Healthcare professionals may be infected as a consequence of the direct exposure to infected blood and body fluids of patients.

Although it has been shown that the majority of birds is resistant to infection the potential role of migratory birds in the disease dissemination could not be ignored. Migratory birds could carry infected ticks and could be implicated in the CCHF virus long distance spread. In addition, recently the CCHF virus has been detected in a specimen of *Hyalomma lusitanium* collected from a deer in southern Spain (Anonymus, 2011). This episode is the first evidence of CCHF virus presence in Western Europe and it opens new scenarios on possible infection spread across southern European countries. To date the number of worldwide CCHF reported cases is increasing. In relation to the Mediterranean countries, Turkey is experiencing since 2002 a large epidemic. Until 2010 more than 5300 human cases have been notified in Turkey with around 5% of case-fatality rate. The spread of virus during the 2002 in Turkey was firstly attributed to the bird migrations from the Balkans (Zavitsanou *et al.*, 2009).

Although climatic variables may deeply influence the occurrence of tick-borne diseases, other factors, such as the land use and demographic changes, are considered to have played a significant role in the spread of CCHF in Turkey. Several ecological and socio-economic conditions have been considered to explain this epidemic: the increase in bush type vegetation due to the abandon of arable lands, the increase of wildlife population (i.e. hares) and the decrease of sheep and goat population as well as the poor conditions of people living in the involved areas.

Hares are considered the main host for immature ticks, whereas cattle are the preferred host for adult ticks. No clinical signs can be observed in animals, but the surveillance in the veterinary field is important for defining the extent of the infected geographical areas. In addition, the application of tick control measures in cattle farming is an important preventive action to reduce the exposure of human beings to the bite of infected ticks.

Furthermore, in case of not infected countries or zones, infected animals and animals carrying infected ticks may represent a significant risk for the introduction of the infection. For instance, the outbreak of CCHF in Pakistan in 2000 coincided with the movement of sacrificial animals from rural to urban areas for the festival of Eid-ul-Azha (Purnak *et al.*, 2007).

Rift Valley fever: a risk for the Mediterranean region

Rift Valley fever (RVF) is a mosquito-transmitted

zoonotic disease, which affects humans and ruminants. RVF is considered one of the most dangerous emerging diseases for Europe and the Mediterranean Countries, given its ability to adapt to several different ecological conditions and vector species (Anyamba *et al.*, 2009).

The disease was first described in 1930 - 1931 in Kenya. RVF virus (RVFV) belongs to the genus *Phlebovirus* of the family *Bunyaviridae*. It is transmitted in an enzootic cycle among wildlife by mosquitoes of at least six genera and probably over 30 different species (Anyamba *et al.*, 2009).

The impact of climate variables on RVFV infections is clearly relevant. In most semiarid areas, precipitation and green vegetation abundance are major determinants for arthropods like mosquitoes. There is a close relationship between green vegetation development and breeding and upsurge patterns of some insect pests and vectors of disease such as mosquitoes and locusts. The successful development and survival of mosquitoes that maintain, transmit, and amplify the RVFV in Africa is closely linked with rainfall events, with very large populations of mosquitoes emerging from flooded habitats after above-normal and persistent rainfall (Tucker, 1979). In eastern Africa the risk of Rift Valley fever increases when heavy rainfall fills local land depressions called "dambos," where the vector mosquitoes breed. This flooding results in the emergence of a single generation of *Aedes* mosquitoes. Whether these infections generate into epizootics depends upon the water pools remaining for four to six weeks or more, thus enabling the secondary vector mosquitoes to breed rapidly and generate the huge mosquito populations seen during RVF epizootic periods (Davies and Martin, 2006).

Human activities and water development projects have been clearly linked with the intensification of certain disease cycles. The occurrence of Rift Valley fever outbreaks in 1977-78 in Egypt were linked to the construction of Aswan dam and the consequent ecological perturbations the Nile course (Johnson *et al.*, 1978; Meegan, 1981). The Egyptian epidemic was characterized, for the first time, by the heavy public health consequences. More than 200,000 people became sick, 598 deaths occurred and livestock losses resulted in meat shortages (Gerdes, 2004; WHO, 1978). The way of RVFV introduction into Egypt was never proved, although the introduction with infected animal from Northern Sudan, following the Nile valley, was suspected (Abd El-Rahimet *et al.*, 1999).

Similarly in Mauritania and Senegal a RVF epidemic was associated with the creation of the Diama dam along the Senegal River in 1987-88 (Thonnon *et al.*, 1999). Many thousands of people became sick and about one fifth of those died (Gerdes, 2004; WHO, 1987; WHO, 1988).

During 1997-1998, flooding in northeast Africa, associated with El Niño, supported one of the most

important epidemic of RVF ever recorded. About 89,000 people were affected in Kenya and Somalia and 250 deaths were recorded (Gerdes, 2004). Eight year after, in 2006-2007, a second large epidemic occurred in Somalia, Kenya and Tanzania with a total of 1,062 human cases and 315 fatalities, and severe losses to livestock production (WHO, 2007).

The importance of animal trade in RVF spread was confirmed in 2000, when for the first time the infection escaped from Africa and reached the Arabian peninsula. In 2000 the virus was introduced across the Red Sea into Saudi Arabia and Yemen, with the movement of infected animals. The epidemic caused severe consequences among livestock and humans (CDC, 2000). In total 1,540 human cases of illness, with 207 deaths, were registered (WHO, 2000a; WHO 2000b).

More recently a severe RVF epidemic was observed in 2010 in the Republic of South Africa. characterised by the occurrence of 237 laboratory confirmed human cases and 26 deaths (Paweska *et al.*, 2011). Major complications of the disease observed in the patients were haemorrhagic fever, encephalitis, hepatitis and retinitis (Paweska *et al.*, 2011). In addition, the South Africa veterinary authorities notified 489 RVF outbreaks during 2010 to the OIE, with more than 14 000 cases and 8 000 animals dead. The epidemic started on January 2010 in the eastern Free State Province and progressively spread westbound to Western Cape and Northern Cape Provinces, towards the borders with Namibia. Close to these borders, Namibia experienced the RVFV transmission in 2010, in areas historically involved in previous outbreaks. Between May and June 2010, several suspected cases of RVF were reported on sheep farms in southern Namibia. Each clinical case was investigated by the staff of the Namibian Veterinary Services who received support from the Central Veterinary Laboratory (CVL) in Windhoek and the Institute 'G. Caporale' in Teramo, Italy. Samples of blood, heart, lung, spleen, liver, kidney and uterus were collected from sick animals and tested for RVF virus. The presence of the virus was confirmed in seven outbreaks. The epidemiological investigations as well as the sequence of the strains isolated suggest a possible common source of infection for the Namibian and South African RVF epidemics (Monaco *et al.*, 2011b).

The capacity of being disseminated through the movement of viraemic animals or passive transport of infected mosquitoes and the zoonotic characteristics make RVF one of the most dangerous emerging diseases at risk of introduction in the Mediterranean basin and in Europe.

DISCUSSION

Actions against diseases at the animal-human-ecosystems interface, before they become epidemic or pandemic, must be based on prevention. But

preventive medicine could not be effective and efficient unless the existence of a solid and indissoluble human-animal-environment interrelationship is not fully recognized. It is necessary to start from the evidence that man and non-human animals shared the same world and had a mutual influence with the environment they lived in. This integrative approach is a key tool to ensure sustainable public health in an era of climate change, resource depletion, land degradation, urbanization, hunger and development challenges.

The prevention of the crossing of ecosystem boundaries by diseases requires the existence of effective surveillance and early warning systems focusing on all relevant ecosystems involved or potentially involved and an appropriate multi-disciplinary and inter-sectorial cooperation. In planning surveillance systems at the animal-human-ecosystems interface, and in defining the actions to prevent the crossing of ecosystem boundaries, no single method is valid for all diseases and situations. The methods to be used are indeed rather intrinsic to the specific environment and disease we are facing, and should be defined consequent to an accurate retrospective and holistic analysis of the epidemiology of the disease under consideration, the environmental situation, the production systems involved and the historical information available.

The existence of effective surveillance and early warning systems are the pre-requisite for the prompt detection of infection introduction or (re)occurrence in given region. Reliable information about the distribution of these diseases in the countries helps to concentrate resources in the areas of highest potential risk of introduction and spread.

The global level of actual challenges, however, requires for a global responses, far beyond the national boundaries and interests. The control of infectious diseases in the global village requires:

- global coordination and concerted actions between all countries and Institutions involved,
- global infrastructure assuring the early warning a rapid responses on a global scale,
- the development of a culture based on transparency and trust between countries and Institutions, sharing common goals in the prevention and control of EIDs.

International Organizations, like the World Organization for Animal Health (OIE), the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), are working together for the development of common preventive actions and surveillance systems for EIDs. The networks of OIE Reference Laboratories and of OIE, FAO and WHO Collaborating Centre are carrying out twinning programs and projects supporting the activities of Member States. The three international organizations have initiated together a number of collaborative projects to help strengthen laboratory capacity and networking.

Because of the myriad interconnected factors that promote the emergence of diseases, many of the actions needed for the control and prevention of emerging diseases and zoonoses are multidisciplinary and far wider than simple veterinary responsibility.

A high level of collaboration between different professionals and competencies (veterinarians, public health officers, entomologists, ornithologists, climatologists) is required for properly achieving the surveillance objectives. To date this level of coordination and collaboration is difficult to achieve in many of the involved countries. At the veterinary level, must be promoted:

- a more factual cross-disciplinary approach;
- the recognition of the importance of wildlife as reservoirs of infection;
- the improvement of vaccines and diagnostic tests;
- the training in the field of veterinary epidemiology;
- the communication with the public on the application of individual preventive measures.

Training of veterinarian is another key issue in the preparedness for EIDs. The knowledge on foreign animal diseases, the strengthening of the capacity of disease recognition and application of proper control measures, according to detailed contingency plans, are the cornerstone for a prompt early warning and rapid response system. E-learning methods may represent a valuable alternative to face-to-face training session, also for developing countries. Post-graduate training, however, should be coupled by effective learning University courses, during which veterinary students can be properly trained on the epidemiological aspects of EIDs and on the surveillance methods for those prevention and control.

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The SAPUVETNET Projects: experiences of intersectoral collaboration and research/training in veterinary public health across Latin America and Europe

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Summary - SAPUVETNET is the acronym of “Red de Salud Publica Veterinaria/Network of Veterinary Public Health”, a series of projects co-financed under the EU ALFA program, aimed to support an International network on Veterinary Public Health (VPH) constituted by Faculties of Veterinary Medicine from Latin-America (LA) and Europe (EU) (<http://www.sapuvetnet.org>). Since its start in 2002, SAPUVETNET has been continuously growing and expanding, and now it also includes several International collaborating institutions/organizations. The SAPUVETNET projects have envisaged a series of objectives/activities aimed to promote and enhance VPH research/training and intersectoral collaboration across LA and EU. Project partners use a mail-list and distance learning platforms (e.g. Moodle, Colibri) to organize common teaching activities. Major results so far achieved are: harmonisation/development of a common VPH curriculum; creation of common modules/courses on selected VPH topics; use of innovative teaching methods, based on problem solving approach/case studies; publication of videos (DVDs) and self-learning program (CD-ROM) on meat inspection/hygiene (in 3 languages); development of an on-line VPH teaching Manual (beta version in Spanish); organization of e-conferences on upcoming VPH issues; publication of a new International VPH Journal “Una Salud/One Health/Uma Saude” (in 3 languages); exchanges of teachers/researchers (e.g. bilateral visits LA-EU-LA) and coordinating meetings; participation in and/or organization of VPH seminars/congresses/conferences at National and International level; publication of scientific and popular articles on VPH issues related to project activities. SAPUVETNET didactic tools have been and/or are being tested/used by partner faculties/universities and other teaching institutions. Didactic material can be freely circulated and distributed, and can be used for distance learning, be modified/adapted to the local context of any country/geographical area, even outside LA and EU.

Key words: Inter-professional collaboration, *One Health* approach, Veterinary Public Health education, Latin America and Europe.

I progetti SAPUVETNET: esperienze di collaborazione intersettoriale e ricerca/formazione in Salute Pubblica Veterinaria tra America latina ed Europa.

Riassunto - SAPUVETNET è l'abbreviazione di "Red de Salud Publica Veterinaria/Rete di Salute Pubblica Veterinaria", una serie di progetti cofinanziati nell'ambito del programma UE ALFA, finalizzati a supportare una rete internazionale di Salute Pubblica Veterinaria (SPV) formata da Facoltà di Medicina Veterinaria dell'America Latina (AL) e dell'Europa (UE) (<http://www.sapuvetnet.org>). Fin dal suo inizio nel 2002, SAPUVETNET è continuamente cresciuto e si è ampliato, ed attualmente include anche numerose organizzazioni ed istituzioni partner internazionali. I progetti SAPUVETNET avevano previsto una serie di obiettivi ed azioni mirate a promuovere e migliorare la ricerca-formazione in SPV e la collaborazione intersettoriale tra AL ed UE. I partner del progetto utilizzano una *mail-list* e piattaforme per formazione a distanza (es. Moodle, Colibri) per organizzare attività formative comuni. I principali risultati finora raggiunti sono stati: armonizzazione/ sviluppo di un *curriculum* comune in SPV; creazione di moduli didattici/corsi comuni su specifici argomenti di SPV; uso di metodi didattici innovativi basati sull' approccio "soluzione di problema"/"studio di caso"; realizzazione di video didattici (DVD) e di un programma di auto-apprendimento (in CD-ROM) su ispezione ed igiene delle carni (in 3 lingue); sviluppo di un manuale didattico on-line su SPV (versione Beta in spagnolo); organizzazione di conferenze elettroniche su temi di attualità in SPV; pubblicazione di una nuova rivista internazionale di Salute Pubblica "Una Salud/Una Salute/Uma Saude" (in 3 lingue); intercambio di docenti/ricercatori (es. visite bilaterali AL-UE-AL) e riunioni di coordinamento; partecipazione in e/o organizzazione di seminari/congressi/conferenze su SPV a livello nazionale ed internazionale; pubblicazione di articoli scientifici e divulgativi su tematiche di SPV legate ad attività dei progetti. I materiali didattici SAPUVETNET sono stati testati/usati e/o sono in corso di validazione dalle Facoltà/Università partner dei progetti e da altre istituzioni. Tali prodotti possono essere usati anche per educazione a distanza, possono essere liberamente circolati e distribuiti, e modificati/adattati ad altri contesti locali di qualsiasi paese ed area geografica, anche al di fuori di AL ed UE.

Parole chiave: Collaborazione inter-professionale, Approccio *Una Salute*, Formazione in Salute Pubblica Veterinaria, America Latina ed Europa

INTRODUCTION AND BACKGROUND

According to the World Health Organization, the most recent and valid definition of Veterinary Public Health (VPH) is "the sum of all contributions to the physical, mental and social well-being of humans through an understanding and application of veterinary science" (WHO, 2002). According to this definition, Ortega *et al.* (2004; 2007) stated that veterinarians play an important role in different topics related to VPH, such as: i. improvement of management strategies on animal movement to prevent diseases emergence and their spread around the world; ii. animal welfare promotion as a way to improve food production methods and respond to consumer demands; iii. development of preventive strategies related to specific production systems, e.g. antimicrobial resistance and environmental impact; iv. sustainable management of the environment and the prevention of negative effects on human and animal populations; v. protection of both animal and public health through new health policies at national and international level; vi. International cooperation in the management of animals in areas affected by disasters; vii. humanitarian assistance to countries affected by wars where animal populations are a survival resource for humans.

The field of Veterinary Public Health has undergone drastic developments during the last decades (OPS-OMS, 2001; 2003): intensification of animal production systems, appearance of emerging and re-emerging zoonoses, as well as the risks of residues in the environment and in food of animal origin are issues of great concern, not only to decision-makers, but also to consumers. The globalisation of trade (incl. live animals, products of animal origin), increase in tourism and human movements in

general pose a constant risk for diseases to be spread around the World. At countrywide level, there is a strong need to have common plans for disease control -for both human and animal diseases- not only for those ones (diseases/zoonoses) already well known in the respective geographical contexts; all countries need to be prepared for the introduction of exotic diseases that might have an impact on human and animal health. Diseases such as Bovine Spongiform Encephalopathy (BSE), the recent outbreaks of Avian Influenza and other emerging zoonoses, the dioxin scandals and others have had a tremendous impact on animal production and on the consumer's concern. Veterinarians play an important role as they are involved in all issues concerning animals and their products in relation to human health. In addition to the involvement in food-safety, disease surveillance and control, veterinarians -and other health professionals alike- are more often confronted with new challenges which require closer intersectoral collaboration. New diagnostic tools as well as changing legislation demand for the veterinarian of the 21st century to meet the challenges of the changes in the profession, especially with regard to safeguarding public health. In fact the role of vets "curing animals" has been progressively shifting towards a more integrated approach in which the veterinarian has to assure the quality and safety of products of animal origin throughout the whole production process (From Farm to Fork), and to integrate within the so-called "One Health approach". This is a relatively new concept for establishing a more holistic approach to preventing epidemic/epizootic disease and for maintaining ecosystem integrity for the benefit of humans, domesticated animals and the biodiversity

(<http://www.oneworldonehealth.org/>) through the co-equal collaboration between health and environmentally related disciplines (vets, medical doctors, nurses, biologists, agriculturalists, etc.) (<http://www.onehealthinitiative.com/>).

Project(s) Aims and Objectives

The purpose of the SAPUVETNET projects was at first to establish a network in the field of education in Veterinary Public Health (VPH) involving Veterinary Faculties of Europe and Latin-America. Besides the development/harmonisation of the VPH curricula, the network also envisaged creating common training modules and to introduce innovative educational methods. The network also intended to evaluate and validate the newly introduced teaching methods/didactic tools for their suitability under the respective prevailing conditions, and subsequently disseminate the products to other Veterinary Faculties outside the SAPUVET network. After the first phases, the project network deemed it interesting to contribute more toward training of professionals adequately prepared to evaluate situations, and find solutions for public health-related problems within the “One World, One Health”. Besides trying to implement the concept of “One World, One Health”, the SAPUVETNET partners have been also actively working to contribute towards the Millennium Development Goals (MDG) (<http://www.un.org/millenniumgoals/>). In particular, the project partners strongly believe that searching for local solutions to global public health problems can play an important role to reach some of the MDGs. Education and research in Veterinary Public Health can play an important role in this respect. In the last phase of SAPUVETNET projects, the network partners are trying to bring LA and EU even more close by proposing common VPH courses and common rules for curricula accreditation among the participating veterinary faculties/schools.

The main aims and objectives of the SAPUVETNET projects, with special reference to training modules/teaching tools, have been reviewed by several project partners -amongst them- Ortega *et al.* (2003; 2004; 2005), De Meneghi *et al.* (2007; 2011), and Vilhena *et al.* (2011).

Partner Universities, collaborating Institutions /Organizations and Universities: the SAPUVET network was first initiated by Veterinary Faculties from 3 European countries (Italy, Spain, The Netherlands) and 5 from Latin-American countries (Argentina, Costa Rica, Cuba, Nicaragua, Peru), plus a collaborating Institution, the WHO/OIE/FAO Collaborating Centre for Training & Research in VPH. Eventually, during the development of the projects SAPUVETNET II and III, the number of European and Latin American partners more than doubled, totalling now 5 European and 11 Latin-

American official partner Universities, plus 12 collaborating Institutions/Organizations and Universities. Overall, the activities of the SAPUVETNET projects have been/are being developed through the collaboration and contribution of the following Universities and collaborating Institutions/ Organizations:

- Universiteit Utrecht (UU), Utrecht, The Netherlands*
- Università degli Studi di Torino (UNITO), Grugliasco-Turin, Italy*
- Universidad de Zaragoza (UNIZAR), Zaragoza, Spain*
- Universidad Agraria de La Habana (UNAH), S.Josè de las Lajas-La Habana, Cuba*
- Universidad Nacional de Costa Rica (UNA), Heredia, Costa Rica*
- Universidad Nacional Autónoma de Nicaragua (UNAN), León, Nicaragua*
- Universidad Peruana Cayetano Heredia (UPCH), Lima, Peru*
- Universidad Mayor de San Simon (UMSS), Cochabamba, Bolivia*
- Universidad del Salvador, Pilar-Buenos Aires, Argentina*
- Universidade de Évora (UEVORA), Evora, Portugal*
- Universidad de La Salle (UNISALLE), Bogotá, Colombia*
- Universidad de Buenos Aires (UBA), Buenos Aires, Argentina*
- Universidad Austral de Chile (UACH), Valdivia, Chile*
- Royal Veterinary College (RVC), London, United Kingdom*
- Universidad de la Republica (UR), Montevideo, Uruguay*
- Universidade do Estado de Santa Catarina (UDESC), Lages, Brasil
- Universidade de São Paulo (USP), São Paulo, Brasil*
- Universidad Autonoma de Baja California (UABC), Mexicali, Mexico*
- Universidad Nacional Autonoma de Mexico (UNAM), Mexico City, Mexico°
- Universidade Federal Rural do Rio de Janeiro (UFRRJ), Rio de Janeiro, Brasil°
- The Ohio State University, Veterinary Public Health Program, Columbus, USA°#
- Universidade Nova de Lisboa, Faculdade de Ciências Médicas, Lisbon, Portugal°
- Universidade Técnica de Lisboa, Instituto Superior de Ciências Sociais e Políticas, Lisbon, Portugal°
- WHO/OIE/FAO Collaborating Centre for Training & Research in VPH, Rome, Italy°
- Food and Agriculture Organization (FAO), VPH Unit, Rome, Italy°
- European College of Veterinary Public Health (ECVPH), Wien, Austria°

- Red de Salud Publica Veterinária (REDSPVET), Bogotá, Colombia°
- Conselho Federal de Medicina Veterinária do Brasil (CFMV), Brasília, Brasil°
- Consejo Panamericano de Educación en las Ciencias Veterinárias (COPEVET)°
- Sociedade Interamericana de Salud Pública Veterinaria (SISPVET)°.

Overall activities carried out, results and projects' products: during the nearly 10 years of project activity, many actions were carried out, and despite some draw-backs, slight delays and minor logistic difficulties, the expected results have been achieved, and almost all products have been finalised or will be finalised by the end of SAPUVETNET III (May, 2012).

The main results achieved during the SAPUVETNET projects, with special reference to training modules/teaching tools, have been described by several project partners –amongst them- Ortega *et al.* (2003; 2004), De Meneghi *et al.* (2007; 2011) and Vilhena *et al.* (2011). The experiences gained, and difficulties encountered in teaching VPH topics within International groups of students/teachers-including in SAPUVET I- have been reviewed by De Rosa and de Balogh (2005).

Hereunder, some of the major activities performed are described and the most interesting results obtained, and products developed, are presented:

Harmonisation/development of a common VPH curriculum: an harmonised curriculum in VPH is justified because of the following reasons: i) existence of sanitary problems of global impact; ii) integration/unification of diagnostic methods and preventive actions between countries; iii) shared social and cultural determinants; iv) trade globalization of animals products; v) improvement of quality of education at global scale, also facilitating students exchanges and performing common researches. The main themes proposed by SAPUVETNET to be included in a common VPH curriculum are: i.) history and global context of public health; ii.) Food safety, and animal production systems; iii) International trade; iv) Preventive medicine; v.) conservation medicine and Environmental management; vi.) Animal welfare; vii.) role of veterinary medicine in disasters; viii.) zoonoses epidemiology, surveillance and control. There are four themes which are considered transversal and common in all VPH courses given at partner Universities : i. one health approach, ii. professional ethics, iii. health education, iv. social and cultural determinants. According to the proposed common VPH curriculum, the minimum professional skills to be acquired

by undergraduate students, at the end of the VPH course, should be: i. to know how to perform ante and post-mortem visits on the farm or at slaughter plants, and assess animals welfare; ii. to be familiar with (veterinary) public health and the respective national, and international legal regulations; iii. to understand and apply concepts in prevention, risk assessment and epidemiological surveillance; iv. to have acceptable level of knowledge on the principles of law applicable to national and international level; v. to understand the importance of the situation analysis of risk-based processes.

Common VPH modules, case studies and e-learning course(s): novel teaching methodologies, mainly based on problem solving approach, were used for common training program on VPH. The SAPUVETNET teaching products can be used for distance learning ; e-learning programs/platforms such as Web-CT, Moodle, Colibri have been and/or are being used to make available selected teaching modules. A module consists of a group of case studies on emergency situations related to Veterinary Public Health (VPH), and the role that veterinarians have to play in this field. Case studies are usually based on fictitious epidemiological situations to simulate real situation which veterinarians can encounter in their day-to-day professional activity. Case studies so far developed during SAPUVETNET II have been divided in three mayor VPH areas: epidemic emergencies (including zoonotic diseases by direct transmission route, or by food chain); non epidemic diseases (including the impact of natural or induced disasters on animal and human populations); public health problems linked to animal management (as antimicrobial resistance transmission); issues related to animal welfare (e.g. botulism, cisticercosis, Rift valley Fever in epidemic emergencies, aggressiveness in dogs, chemical disasters in non-epidemic situation, effects of animal hoarding, multi-resistant Salmonella or swine streptococci, zoonoses related to tourism or migration, etc.). Every “case study ” includes a teacher’s guide, a student’s guide, and the case study in MS Powerpoint® format. Some theoretical/reference documents have been also included to help with the case study solution. These documents are presentations elaborated by the network members as well as other articles related to specific topics of VPH from other organizations, that can be provided as background material for solving the presented cases. Additional case studies have been developed during SAPUVETNET III and are being made available on the project webpage through a new user area specifically designed for students and teachers, so that all users (both students and teachers) can access to the

*official project partners who participated, at least, in one phase of the SAPUVETNET projects;

°collaborating partners (Universities, Institutions, Organizations) during the SAPUVETNET projects;

note: although The Ohio State University is not a Latin American University, it has active teaching/research VPH programmes in Latin American countries, including several SAPUVETNET partner Universities.

relevant information (students can visualize and/or download the information dedicated to them, and teachers will have access to their relevant information after registration).

In addition to the existing VPH modules and case studies, a new e-learning course, entitled “*Boas Práticas na Produção Primária*”, has been recently proposed and it is being discussed amongst the SAPUVETNET partners. This course will focus on good practices and HACCP (Hazard Analysis and Critical Control Points) in animal production, with special reference to beef cattle, pig and poultry production; this distance-learning course will be first offered to students of the SAPUVETNET partner universities; after having positively tested its format, this e-learning course -and eventually others on different VPH topics- could also be made available to students of other faculties/universities outside the network.

Didactic videos on meat inspection: a series of videos (DVDs) on meat inspection and good practices in animal slaughter were produced during SAPUVETNET II and SAPUVETNET III; so far, 3 videos on cattle, poultry and rabbit slaughter have been published by the Audiovisual Centre, Faculty of Veterinary Medicine, UNITO. The DVDs are available in 3 languages (Spanish, English and Portuguese), with audio comments and subtitles in the language selected, plus annexes with list of abbreviations/glossary, and the relevant legislation on European food hygiene /meat inspection.

Self-learning program on meat inspection/hygiene: an interactive training program on good abattoir practices (inspection of cattle carcass) and meat hygiene was jointly developed by the Audiovisual Centres of UNITO and UNAH. The programme is provided on CD-ROM, and it is available in 4 languages. It allows students to follow the whole learning process (in 3 sessions) through an interactive interface which includes: short video clips and audio comments, on-screen options of specific “hot spots/mouse sensitive areas” linked to the session of multiple choice questions to be clicked-and-selected. The program allows to count the time elapsed and the number of correct/wrong answers and attempts to answer.

Teaching Manual on VPH: the development of the Teaching Manual of Veterinary Public Health started during SAPUVETNET II. A beta version of the manual (in Spanish language) will be soon available online on the project webpage; the Portuguese and English translation will be also included. Besides being available online, the Manual will be also distributed on CD-ROM in order to allow students in areas with poor or absent Internet connexion to use this didactic tool. The manual is based on a hypertext approach, with various links to

additional off-line learning material, and it is composed by 12 chapters, namely: History of VPH; Risk assessment; Veterinary Preventive medicine; (Theoretical) Tools for intervention in public health; Food chain safety; Zoonoses (neglected, emerging and re-emerging); Antimicrobial resistance and VPH; Climate change and its impact on (re-)emerging diseases; Biodiversity; VPH activities in Disasters; Animal welfare; Environmental impact of the livestock industry. Each chapter of the VPH manual include an introductory section, orientation questions, technical contents, evaluation questions, bibliography, additional links, and off-line learning material.

E-conferences on upcoming VPH issues: during SAPUVETNET II, an electronic conference entitled “*Local practices of animal production and health with special reference to the use of veterinary drugs and development of resistance to antimicrobials: implications for Veterinary Public Health*” was held from 28th February to 2nd March 2007. Contributions from participants were presented and discussed in a forum which was moderated by a moderating group. Over 40 professionals (vets, animal productionists, medical doctors, chemists/pharmacists), and students from different countries and continents (North-Central-South America and the Caribbean, Africa and Europe) took part in the conference, sharing their experiences, voicing their points of view, making comments and presenting talks. During SAPUVETNET III three more e-conferences were organized and held: the first was called “*Preparing the World Rabies Day*”, and was held in June 2009. SAPUVETNET III project intended to contribute to the international debate –promoted by Global Alliance for Rabies Control- on the worldwide fighting against this important neglected zoonosis, which is still a serious threat in many countries of Africa, Latin America and Asia. More than 132 participants from several European and Latin American countries, as well as from USA, attended and contributed in the e-conference. The second one was entitled “*The New Veterinary Public Health; strategies and interaction of the Animal Health and Human Medicine*”, and was held in mid-December 2009. This challenging e-conference was aimed to discuss on common research and training needs/opportunities in Public Health across medical and veterinary professions; some burning questions, e.g. how to integrate teaching common Public Health topics in both Human and Veterinary Medicine courses, were posed to participants. The third e-conference was on the “*Prudent use of antimicrobials in animals: solution or utopia?*”, in September 2010. The initiative counted with 86 participants from 14 different countries. One additional e-conference on “*Population displacement and zoonoses -Surveillance networks: how to get cross cooperation between veterinary public health*

and humans public health?" is being organized, and will be held in March, 2012. Its goal will be to discuss the migratory flows (both human and animal) and their repercussions, consequences and impact on surveillance and control of zoonoses. The importance of good communication between the different health professions will be discussed, in order to improve risk management practices regarding zoonoses and food safety. The collaboration and participation of professionals/experts from other disciplines, e.g. medical doctors, biologists, epidemiologists, social sciences experts, etc is considered fundamental for the success of the event, and for strengthening intersectoral collaboration.

The SAPUVETNET e-Newsletter(s): the objective of the SAPUVET e-Newsletter is to circulate and share the products generated by the network and by its participants, by presenting selected VPH topics and news related to the developments and achievements of the project itself. By this mean, the network aims to promote the interest and collaboration from other groups/institutions dealing with PH. Four Newsletter issues (2 per year) were published during the SAPUVET II project (2003-2005), and five new issues have been already published throughout the duration of the SAPUVETNET III project (2008-2011), and one more is expected shortly.

The SAPUVET Revista de Salud Publica Veterinaria (in Spanish): during the SAPUVETNET II project, a Journal on VPH topics was published; 2 issues were planned (1 issue per year) and were published and distributed as hard copies, as well as as .pdf (to be downloaded from the project webpage http://www.sapuvetnet.org/antigo/Eng_PUB.htm). Unfortunately, it was not possible to continue with the publication of the journal, until the start of the third phase of the project, SAPUVETNET III, whence a new version of the SAPUVET Journal was started.

The International VPH Journal "Una Salud/One Health/Uma Saúde": the Journal is an official publication of the SAPUVETNET III project. It publishes original research paper, short articles and short communications. Professionals (veterinarians and medical doctors), researchers, and students -even if not members of the project- may publish their review articles and/or research papers. The articles published by the Journal are intended to disseminate the results of research studies in various VPH fields, such as the epidemiology of transmissible diseases of major importance in Public Health, food safety, animal welfare, anti-microbial resistance, interdisciplinary working projects on "One Health", risk analysis and other themes, aimed to strength the role of Public Health. The Journal is published every six months (in July and December); contributions can

be submitted in the following languages: English, Spanish and Portuguese. The *Una Salud/One Health/Uma Saúde* journal is available as hard copy version, as well as .pdf file to be downloaded from the project website (http://www.sapuvetnet.org/EN_frameset.html).

Project co-ordinating meetings: during SAPUVET I, the first phase of the SAPUVETNET projects, four coordinating meetings were organized; the first one was held in the Netherlands (Utrecht, March 2002), and served as kick-off meeting to allow all participants to get to know each other personally, and to present their respective faculties/universities and curricula. Further meetings were held in Cuba (La Havana, February 2003), then in Italy (Turin, September 2003), and finally in Costa Rica (Heredia, February 2004). Likewise during SAPUVETNET II, there have been four coordinating meetings, two in Europe (Zaragoza, Spain, June 2005 and in Evora, Portugal, February 2007), and two in Latin America (Lima, Peru, December 2005, and in Buenos Aires, May 2006). These face-to-face meetings were very fruitful in consolidating the network and advancing on the further development of the project's activities. Also during SAPUVETNET III, coordinating meetings were also organized, the majority of them in Latin America: in Colombia (Bogotá, February 2009), in Brazil (Brasilia, October 2009), in Peru (Lima, September, 2010), in Chile (Valdivia, March, 2011); only the final project meeting was held in Europe (Rome, Italy, September 2011). During the coordinating meetings, the results achieved during the various phases of the project(s) were reviewed and jointly discussed by old and new partners. Once again these face-to-face meetings were considered very fruitful in consolidating the network and further strengthening the personal and professional relationships of participants. The coordinating meetings were most appreciated by participants as they had the opportunity to visit besides the faculty, the teaching and research facilities, the University campus of the hosting Institutions, to also gain a more in-depth view of the country visited. In fact local installations, processing plants, farms, field research stations, etc where the hosting institutions/faculties carry out their practical teaching activities were also visited. During the last project meeting at FAO, Rome, future activities to be carried out after the official end of the SAPUVETNET projects (by May 2012) were discussed and planned.

Exchanges of teachers/researchers: a total of four bilateral exchanges/visits (EU-LA, LA-EU, LA-LA) took place during SAPUVET I, i.e. from Peru to The Netherlands and Italy; from Cuba to Costa Rica; from Argentina to The Netherlands; from Italy to Cuba). During SAPUVETNET II, the bilateral visits LA-EU were from Cuba to Portugal, and from

Nicaragua to Spain; the bilateral visits LA-LA were from Bolivia to Peru and Costa Rica; and from Peru to Argentina. During SAPUVETNET III, the bilateral exchanges LA-EU were from Argentina (UBA) to Portugal (UEVORA), while the bilateral visits LA-LA were from Chile (UACH), Peru (UPCH) y Uruguay (UR) to Cuba (UNAH). The exchanges were streamlined with ongoing activities at the respective faculties, in order to make best use of the bilateral visits. The visiting partners were able to actively participate in teaching events and meet with students and other lecturers, and researchers.

Participation/contribution in National and International congresses/conferences on VPH: teachers and researchers of the SAPUVETNET partner institutions participated in several congresses and conferences to present contributions (oral communication and/or poster presentation) on the activities of the SAPUVETNET projects, amongst which:

- Annual Scientific Conference of the European College of Veterinary Public Health (ECVPH), 25th-26th November, 2005, Glasgow, UK
- 1st Pan-American Congress on Zoonoses, 10th-12th May, 2006, La Plata, Argentina
- Annual Scientific Conference ECVPH, 7th-8th December, 2006, Lyon, France
- VI International Congress on Veterinary Sciences, 10th-13th April 2007, La Habana, Cuba
- Annual Scientific Conference ECVPH, 4 –5 December 2008, Thessaloniki, Greece
- 6th European Conference on Travel Medicine, 28th-30th April 2008, Rome, Italy
- III Congresso Nacional de Saúde Pública Veterinária, I Encontro Internacional de Saúde Pública Veterinária, Bonito, MS (Brasil) 25-28 October, 2009
- 2nd Iberian Conference on Epidemiology, 4th-5th February 2010, Barcelona, Spain
- IV Conference Cycles on Veterinary Public Health, 12th June 2010, Porto, Portugal
- III Congreso Internacional Sobre Cambio Climático y Desarrollo Sustentable, 8-11 August 2011, La Plata, Argentina
- 1st International Congress Impact of Pathogens on the human-animal's interface (ICOPHA), 15th-17th September 2011, Addis Ababa, Ethiopia.
- Annual Scientific Conference ECVPH, 5-7 October 2011, Brno, Czech Republic
- Congress of Veterinary Sciences 2011, 13th-15th October, 2011, Santarém, Portugal

For more information and details on other conferences/congresses, please visit the project webpage and read the SAPUVETNET Newletters (http://www.sapuvetnet.org/EN_frameset.html).

Organization of specific seminars/conference on VPH issues: SAPUVETNET partners were also active in organizing/promoting various confer-

ences/seminars/workshops on up-coming VPH issues, amongst which:

- Seminar on Food safety, 4th November 2005, Polo da Mitra, Evora, Portugal
- International Symposium on “Zoonoses and Human migrations”, 16th-18th February 2007, Evora, Portugal
- III Congresso Nacional de Saúde Pública Veterinária, I Encontro Internacional de Saúde Pública Veterinária, Bonito, MS (Brasil) 25-28 October, 2009
- World Rabies day, 28th September 2010, Universidad de La Salle, Bogota, Colombia

For more information and details on conferences organized and/or promoted by SAPUVETNET, please visit the project web page and read the SAPUVETNET Newletters (http://www.sapuvetnet.org/EN_frameset.html).

The SAPUVETNET student groups: in some partner Faculties/Universities, small groups of students and newly graduated vets with specific interest on VPH founded the *SAPUVET student groups*; all are working to disseminate knowledge in the field of VPH for the benefit of their own members and the local communities. So far, the following groups were started: the first one founded, *SAPUVET-Peru* in Lima; *Rede de Saúde Pública*, in Santa Catarina-Brazil; *Vector*, VPH group, Évora-Portugal, *SAPUVET-Argentina* in Buenos Aires, and also a group in Valdivia-Chile.

It is expected that the SAPUVETNET students groups will act as local catalysers of initiatives on up-coming VPH issues and will help to give continuity to project activities. Some of these students groups are already connected through a dedicated mail-list (sapuvetnet-estudantes@uevora.pt).

The SAPUVETNET project webpage: the SAPUVETNET project webpage was born during SAPUVET I, and has been expanding throughout SAPUVETNET II and III. The webpage is not only a way to communicate the project's evolution and activities, but it is also a source of teaching materials produced during the projects and made available for students and teachers, also outside the partner Universities. The webpage includes the most recent news/facts and feeds from interesting public health websites. The SAPUVETNET journal is also available for free download from the webpage. Teaching materials for download include articles and presentations from the projects partners, images related to several themes (risk analysis, food safety, animal welfare, etc.), videos (streaming), SAPUVETNET conferences abstracts, and several case studies that were produced in SAPUVET I, II and III, with a guide to the teacher and student. Information about all three projects can be seen. On the project website, reference is also made to some of the scientific and popular articles on VPH issues, and related

to project activities, published by project SAPUVETNET partners. The building up of the project webpage is an on-going process, so more sections are being added, and the contents (e.g. teaching material, etc.) are continuously updated.

CONCLUSIONS

The SAPUVETNET project, since its start in 2002, has shown a significant evolution and consolidation, as demonstrated by the integration of many education/research institutions, as well as International organizations, all sharing the theoretical framework and philosophy of SAPUVETNET. This process underlines a full understanding of the project goals and a shared need within the professional and scientific communities (both public health and veterinary public health) to work jointly within the concept of "One Health". SAPUVETNET partners have the consciousness that their contribution it is just a small piece of the great puzzle that is the public health; all SAPUVETNET products are a contribution to this common goal. Didactic tools produced by the SAPUVETNET projects have been and/or are being tested and used by partner faculties and other teaching Institutions/Organization for under and post-graduate courses. Teaching material can be freely circulated and distributed according to Creative Common policy (<http://creativecommons.org/>). Didactic tools can be also used for distance learning and can be modified/adapted to the local context of any country/geographical area, even outside Latin-America and Europe. A new version of the DVDs and CD-ROM on good abattoir practices and meat inspection is being prepared with the collaboration of FAO, VPH Unit, to include a French version (and eventually some major African languages) for distribution and circulation in most of the African continent. This will allow to make these teaching products by SAPUVETNET distributed and available also outside Latin America and Europe, giving an added value to the project. Teaching material produced by SAPUVETNET are available at the project webpage (<http://www.sapuvetnet.org>; under continuous updating) or can be obtained from the project coordinator(s) and/or the contact persons at the partner Faculties/Universities. All partners and collaborators of the SAPUVETNET project highly welcome any contribution from the scientific community, students and stakeholders towards a *Better Health* for all.

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Risk factors correlated to orf in Guantánamo Province

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Summary - Orf is a highly contagious viral disease that causes important economic losses in sheep and goat farms, and constitutes zoonosis. The employment of vaccines for the control of this illness is limited and it has been associated to outbreaks because of the insufficient attenuation of the vaccine strains. Even if it is known that Orf is wide spread in sheep and goats farms in Cuba, there is still a lack of epidemiological data on this disease. Considering the limitations of effective specific preventive measures against orf this work was directed to identify the risks factors correlated to the disease, by the means of a case-control study.

The risk factors for the animal population were assessed in order to identify additional means to control the disease. A total of ten flocks randomly selected were investigated. For gathering information a survey was carried out to identify the presumed risk factors. The survey allowed us to identify the risk factors significantly associated to the disease. The strength of the identified associations and etiological ratio at individual and population level, evidenced a real possibility to significantly reduce the orf incidence through an improvement of handling procedures.

Key words: orf, sheep, goats, case-control

INTRODUCTION

Orf is one of the most widespread viral diseases, worldwide affecting small ruminants and other species including wild animals (Robinson and Kerr, 2001; Hosamani *et al.*, 2009). The causative agent, orf virus (OV), is the type species of the Parapox virus genus within the family *Poxviridae*. OV is epitheliotropic, infecting damaged skin and replicating in regenerating epidermal keratinocytes causing one of the most distressing skin disease that affects both lambs and ewes and being characterised by scabby erythematous lesions normally around the nares and mouths of lambs. The virus does not appear to spread systemically from the site of initial infection, instead it is shed with scab material to seed the environmental pool. OV resists harsh environmental conditions, surviving in the environment for long periods with the possibility of being transmitted to susceptible hosts.

OV infections are ubiquitous in sheep and goat producing country worldwide however their impact both in financial terms and society is greatly underestimated. According to the 2001 United States Department of Agriculture Animal and Plant Health

Inspection Service's National Animal Health Monitoring System (USDA APHIS NAHMS) sheep survey, 40 percent of U.S. operations reported orf infecting their flocks in the previous three years (source CDC). The disease has a considerable economic impact on the agricultural sector (Gokce *et al.* 2005), particularly in the third world where it is regarded as one of the top twenty most important viral diseases of sheep and goats in terms of impact on the poor (Perry *et al.*, 2002). Within the developed world, in addition to its economic impact, it is regarded as one of the most important disease factors affecting the welfare of farmed sheep and goats (Haig and McInnes, 2002). From the farmer's perspective, financial losses can accrue not only from the death of animals, but also from various factors such as reduced live weight gain, extra feed costs, extra labor, veterinarian involvement and delay in finishing for market (Hosamani *et al.*, 2009). Orf virus is transmissible to man (Geerinck *et al.*, 2001) with disease considered an occupational hazard to farmers, shepherds, veterinarians, animal's handlers, meat and wool processors and is also associated with religious slaughter of animals (Guibal *et*

al., 1996; Ghislain *et al.*, 2001; Uzel *et al.*, 2005; Cea *et al.*, 2007; Malik *et al.*, 2009). Children may be also exposed to infected animals in occupational settings or during recreation (Lederman *et al.*, 2007). In the UK, OV infections are the most common viral zoonoses reported to the public health laboratory (Gilray *et al.*, 1998) and thousands of cases are reported annually in Australia and New

Zealand (Crumbie, 1998). In several countries, orf is highly communicable and the veterinary legislation concerning the disease base the control measures on vaccination and quarantine. Instead, in most of the countries the control of the disease appears to be at the level of managing an outbreak once it has started. There are no specific treatments for OV disease in animals and humans and none of the available vaccines endanger a complete long lasting immunity therefore vaccinated animals can be re-infected (Buddle *et al.*, 1984). Measures for prevention and control, such as disinfection, are necessary to interrupt the dissemination of OV and consequently to reduce the incidence of the disease in animals and humans (Gallina and Scagliarini, 2010). Although factors that increase the probability of being infected with OV have been pointed out, to our knowledge there are no studies estimating the magnitude of such risks. The retrospective analyses are considered useful tools to decisions making regarding future proposals to control endemic diseases. The objective of our study was to identify the risks factors correlated to orf in Cuba, with this aim a case-control study was performed.

$$n = \frac{\left(Z(a) \cdot \sqrt{\left(1 + \frac{1}{c}\right) \cdot p_m \cdot (1 - p_m)} + Z(b) \cdot \sqrt{p_1 \cdot (1 - p_1) + \frac{p_0 \cdot (1 - p_0)}{c}} \right)^2}{(p_1 - p_0)^2}$$

being

$$p_1 = \frac{p_0 \cdot RR}{1 + p_0 \cdot (RR - 1)} \quad p_m = \frac{p_1 + c \cdot p_0}{1 + c}$$

where

- Z(a) = value of the t of Student for the level of confidence specified.
- Z(b) = value of the t of Student for the power specified.
- p₀ = prospective proportion of exhibition among the healthy ones (control).
- p₁ = prospective proportion of exhibition among the sick (cases).
- P_m = prospective proportion of exhibition in the population (cases and control).
- 1-p_m = prospective proportion of not exposed in the population.
- C = Prospective relationship between sick and healthy.
- OR = Odds Ratio estimated of enough importance.

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MATERIAL AND METHODS

A case-control study was carried out for indepen-

The flocks investigated were selected through the simple probability method from the total affected and considering the number of cases and controls to be investigated, which invariably come from the same population base. Controls should not have any of the clinical symptoms of the disease. A designed and validated survey was carried out in each flock to gather information about the factors related either with the handling or the introduction of orf. Among these factors, the feeding, the number of drinking troughs, the combined grazing of sheep, goats and cattle, the density of animals in resting box, the grazing, animal density the proximity to other affected flocks, the presentation of the disease in previous periods and the quality of the grass have been taken into consideration. Concerning the factors directly related to the animals the followings were taken into consideration: species, sex, category and physiologic state. This last variable have been stratified as not pregnant, pregnant, early lactating and nursing. In order to identify the risk association Ji-square test (X²) was applied considering Yates correction and a significance of $p < 0.05$. The strength of the association was measured through the Odds Ratio (OR) and its confidence interval (IC) to 95%. The effect of the association on the animal population (cases and controls) was considered by

means of the etiological fraction and attributable population fraction. For the stratified factors, the association with the disease was identified by Cloth-Haenszel test based on the Ji-square and its significance ($p < 0.05$) as association interpretation.

The evaluation of the association of the physiological state factors and their strata (not pregnant, pregnant, early lactating and nursing) with the disease was carried out by Mantel-Haenszel and Breslow-Day test. The calculation of the associations was performed by using Win Episcope 2.0 (Thrusfield *et al.*, 2001) and EpiData 3.1 (Denmark, EpiData Association) softwares.

RESULTS AND DISCUSSION

Five out of eight factors, related with the handling of sheep and goats, were significantly associated with orf presentation (Tab. 1), although with diverse magnitudes. The total number of samples investigated was of 149 cases and 1669 controls.

The consumption of extremely fibrous grasses was identified as the most relevant risk factor in fact animals under this condition had an OR of 10.69 (5.986- 19.117 CI at 95%). The exposed cases to this factor were 90%, whereas 13% were attributable at a population level. OV virus penetrates the skin throughout abrasions, mainly located on the skin of the lips and nose (Lear *et al.*, 1996) so the lesions caused by fibrous grasses to these sites enhances OV

penetration. Furthermore this risk factor is also correlated with poor nutrition and animal welfare.

The probability of being infected with orf, in flocks with insufficient drinking ponds, was found to have an OR of 2.1523 (CI 95% 1.535-3.016). The highly contagious nature of the disease (Bofill *et al.*, 2007), which predominantly diffuses by direct contact among infected and healthy animals, explains the occurrence of higher number of cases in such condition. During the outbreaks, transmission is also propitiated through contact with contaminated objects (Jenkinson *et al.*, 1992), including fences, troughs, drinking troughs, beds, among others. While some of these objects are of occasional contact, the drinking troughs are widely used and, as a consequence, enhance the contact rate between sick and susceptible animals promote transmission. The etiological fraction for this factor indicated that 53% of the cases occurred after the exposition to this factor and 26% for the population (cases and controls). According to this observation, the enhancement of the drinking troughs could be of help to reduce disease transmission.

To reduce the risk of OV transmission the segregation of the sick animals from the healthy ones is also important. The closeness to affected flocks (1 Km) was identified as another risk factor which significantly explained orf presentation. For this factor the probability to get sick was three times higher (OR

Table 1 - Attributable factors to the handling of the flock.

Correlated variables	Cases/Control	OR	IC 95% Low/Hig (Woelf)	X2 Corrected of Yates	p	Ethiological fraction	Populational attributable fraction
Feeding (Grass vs. Grass + another food)	91/98 (1011/658)	1,021	0,724/ 1,439	0,001	0,974	0,020	0,012
Number of drinking . troughs (≤ 2 vs. > 2)	73/76 (515/1154)	2,1523	1,535/ 3,016	19,7413	0,0000	0,535	0,262
Combined grazing of ovines, caprines and cattle (present vs. absence).	43/106 (312/1357)	1,764	1,212/ 2,567	8,359	0,003	0,433	0,125
Density of animals in corrals of rest (+1 breeding (sheep and goats)/m2 vs. 1/m2).	106/43 (1052/617)	1,445	1,000/ 2,089	3,547	0,059	0,308	0,219
It loads animal in grazing (3 breeds/ha vs. 2/ha).	78/71 (954/715)	0,823	0,588/ 1,151	1,101	0,293	0,176	0,068
Proximity to other affected flocks (1Km vs. >1Km).	101/48 (651/1018)	3,290	2,301/ 4,703	45,534	0,0000	0,696	0,471
Presentation of the disease in previous periods (present vs. absence).	88/61 (1373/296)	0,311	0,219/ 0,441	45,213	0,0000	0,688	0,451
Quality of the grass (Dried up pasture vs. others).	23/126 (28/1641)	10.698	5.986/ 19.117	89.989	0.0000	0.906	0.139

3.290) for sheep and goats in flocks grazing in proximity with an affected one. The etiological fraction estimated that 69% of the exposed cases get sick for the presence of this factor and 47% for the population. The external isolation of animal farms is crucial to prevent the OV introduction into flocks. Means of transportation and personnel handling the animals can contribute further to diffuse the virus among animals of different production units (Torfason and Guonadottir, 2002; Ovejero, 2006). These kinds of particular factors could not be assessed in the study but have been considered as a further way of transmission of the virus from affected to healthy flocks. Although orf induce low mortality (Bofill *et al.*, 2007); the strength of the proximity to affected flocks as a risk factor, suggests the importance of increasing measures leading to a higher isolation. In this sense, it is worth considering the probable impact of more serious sheep and goat diseases that could be introduced with a similar way of transmission.

Others risk factors associated with the presentation of the disease were the common rearing sites for sheep, goats and cattle the previous disease presentation in the flocks. The flocks which combined grazing of vary species presented a higher probability of getting sick (OR=1,764) than those with simple grazing. Although there are not references of maintenance of OV virus in cattle, such association could be an effect of deficiencies in the biosecurity or inadequate perception regarding the importance of prevention measures by farmers that use to raise simultaneously several species. The previous presentation of the disease constituted a protection factor (OR=0,311). In this case the etiological ratio was interpreted as a fraction protected as a consequence of the exposition to the factor and corresponded to 68% of the exposed individuals that did not get sick, while at population level it match up 45%. Even though it is known that there is a controversial contribution of the immunity to prevent subsequent OV infections (McInnes *et al.*, 1998; Degraeve *et al.*, 1999; Friebe *et al.*, 2004), the protective effect in flocks previously exposed could

be influenced by the population immunity (Tortora, 1994; Guo *et al.*, 2003; Torfason and Guonadottir, 2002). Although the animals can be reinfected, the subsequent lesions are smaller and use to resolve faster than the primary ones (Jenkinson *et al.*, 1992; McInnes *et al.*, 1998). On other hand, the farmers experience of being able to recognize the disease and opportunely apply control measures such as segregation is also more probable in flocks previously affected.

No association with the disease was found considering the feeding type, the density of animals in resting box and the animal density in grazing. Among the factors attributable to animals (Tab. 2) only the sex and the productive category were associated to disease presentation. In the case of sex the cohort of females was much bigger than males, and it could influence the differences. Nevertheless these kinds of flocks use to raise in such ratio females and males. According to the category, the offspring were at a higher risk (OR=2.726) that the other category this is a further confirmation that orf has higher incidence in young animals (Bofill *et al.*, 2007). This is particularly evident on premises with high disease prevalence in which mainly young animals suffer the disease due to the contribution of the immunity to partially protect adult animals (Haig and McInnes, 2002; Guo *et al.*, 2003). However, recurrence of orf occurs even in presence of immune response (Haig and Mercer, 2002). Nonetheless the faster healing in lesions after reinfection (Jenkinson *et al.*, 1992; McInnes *et al.*, 1998) may lead to some adult animals resulting under-diagnosed.

Regarding the physiologic state of the females (Tab. 3), there were not pregnant sick, while nursing females presented a risk three times higher than others (OR = 3.170, IC 95% 1,562-6,435). This could be explained for bidirectional transmission of orf virus from the lambs to the udder and from the udder to the mouth of progeny (Torfason and Guonadottir, 2002). This result coincided with the progeny as more affected category. The test of OR homogeneity for the strata, shows that it doesn't exist ($p < 0.02$) therefore confusion could be

Table 2 - Attributable factors to the susceptible animal

Correlated variables	Cases/Control	OR	IC 95% Low/Hig (Woelf)	X2 Corrected of Yates	p	Ethiological fraction	Populational attributable fraction
Species (Sheep vs. Goats)	106/43 (1061/608)	1,412	0,977/ 2,041	3,088	0,078	0,292	0,207
Sex (Females vs. Males)	118/31 (1500/169)	0,428	0,280/ 0,656	14,862	0,0001	0,571	0,370
Category (Breeding vs. Others category)	65/84 (369/1300)	2.726	1.933/ 3.844	33.667	0.0000	0.633	0.276

Table 3 - Attributable factors to the stratified susceptible animal.

Correlated variables	Cases/ Control	OR Raw	IC 95% Low/Hig (Woollf)	OR Combined (M-H)	IC 95% Low/Hig (Woollf)	OR Pondered (M-H)	IC 95% Low/Hig. (Woollf)
Physiologic state	44/305 (123/962)	1,128	0,781/ 1,629	1,566	1,011/ 2,426	1,556	0,960/ 2,522
Empty females (Empty females vs. Other categories)	5/39 (74/360)	0,623	0,237/ 1,635	TEST OF HOMOGENEITY Ji-square gl Value p Combined (M-H) 7,9383 2 0,018 Pondered 7,9376 2 0,018			
Early lactating (Early lactating (goats and sheep) vs. Other categories)	6/38 (55/379)	1,088	0,439/ 2,692	TEST OF ASSOCIATION OF MANTEL-HAENSZEL Ji-square gl Value p 3,7465 1 0,052			
Nursing (Nursing vs. Other category)	33/11 (211/223)	3,170	1,562/ 6,435	TEST DE BRESLOW-DAY FOR HOMOGENEITY OF OR AMONG STRATA WITH MH-OR: Q(BD)= 8,292 Gl: 2 p: 0,021 NC: 97,859%			

excluded and the result is attributable to interaction (Ortega *et al.*, 1995). Such asseveration was demonstrated by the test of Breslow - Day with $p < 0.021$ as the evidence of interaction between the factor and disease presentation.

The biological interaction supposes dependence between two or more factors. If an acceptable biological mechanism exists the detection of frequencies higher than the prospective value is attributed to interaction (Thrusfield, 1990; Ortega *et al.*, 1995). In this sense it was expected that the offspring and breeders could represent the higher proportion of sick animals in the affected flocks. These categories have direct relationship that propitiate described transmission of orf (Jenkinson *et al.*, 1992).

The association measures establish the magnitude with which the exhibition associates to the presentation of the illness. Under certain circumstances these measures allow to carry out causative inferences, especially when they can be evaluated by means of a statistical function. In the case of OR a value higher than the unit indicates positive association for the risk factor, while lower values to the unit suggest protective effect.

In animal production the good handling is critical in order to reach performances nearest to the genetic potential of the species. When good handling is lacking this not only directly affects the return of value invested, but in some cases can increase the probability of disease presentation it consequent additional losses. Animal diseases not only affect animals, but can also affect the health and quality of life of people dependent on these animals for food or for their livelihood. Whilst much attention is paid to new and emerging diseases, endemic diseases are often overlooked. Orf infections, fall into the category of neglected zoonoses and there is a gap in our understanding of the epidemiology of orf for which information are frequently anecdotal and qualitative. Sheep and goat farms represent an important economic source for many rural communities in

Cuba for this reason any improvement in the control measures towards endemic diseases will enhance the quality of animal products and consequently and economic stability. Our study allowed us to identify the major risk factors associated to orf presentation in Cuba, we believe that our data might lead to alternative and improved animal husbandry and management practises that will contribute to a decrease of the disease incidence in animals and consequently to a reduction of the risk of transmission to humans.

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Recent advances on avian influenza ecology

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Summary - The ecology of influenza A viruses, beginning with the Zoogeographic Region, is always related to the biological characteristics of the reservoirs and the eventual role of the epiphenomena. The ecology of influenza viruses is a very dynamic system in which certain structural constants (reservoir migrations) and other important environmental variables coexist. This system influences both the biology of reservoirs and that of epiphenomena, and in turn their interactions with the virus. High viral loads in feces of infected birds allow fecal-oral transmission. However, this route does not fully account for the efficiency of avian influenza virus (AIV) spread since dilution of infectious feces in water progressively decreases the chances of virus/host interaction. Recent studies showed that preen oil gland secretion, by which all aquatic birds make their feathers waterproof, could support a natural concentration mechanism of AIVs from water to birds' bodies, thus favouring virus spread and persistence in the aquatic environment. We demonstrate a novel viral transmission route that adds to, and possibly contributes to explain the knowledge of long-distance movements and long-term infectivity of lowly and highly pathogenic AIVs in nature.

Key words: Avian influenza virus, Influenza A, reservoir of AIV

Wild waterbirds are considered to be the natural reservoir of influenza viruses (Webster *et al.*, 1992). Millions of years of coevolution during the speciation of *Anseriformes*, which took place between 8 and 13 million years ago, led to an equilibrium between wild ducks and influenza viruses. This virus-host equilibrium ensures that viral population has a chance to exist for a certain period of time and that wild ducks becomes infected without showing clinical signs. This "intimate" relationship is the basis for maintaining the gene pool of influenza A viruses (AIVs) which provides all the genetic diversity required for the emergence of new influenza viruses in humans and animals. Nowadays, the natural setting that has allowed a relative evolutionary stability for so long, has profoundly changed. In our planet, as consequence of a rapidly expanding population, humans and domesticated species have provided an enormous opportunity for replication and spread of influenza viruses, with more frequent jumps of species or classes (Guan *et al.*, 2002; Stallknecht and Shane, 1988). Never before in the history of this disease, influenza viruses have so many opportunities to replicate and a broad "domestic" substrate on which they can reproduce and evolve. The ecological role of these viruses in the

micro world is to quantitatively control the expanding population sensitive to them, as well as in the macro world the preys' numbers and movements influence predators populations. In a natural habitat, relationships and interactions between organisms are often governed by complex mechanisms that enable both groups to survive in a evolution dynamic. In the predator-prey interaction the predator species usually loose energy every time step, and can gain energy by predation. Whereas the prey's advantage consists of a natural selection that acts to favor those individuals with characters increasing fitness. In the world of micro-predators of cells such as viruses, it has tried to understand what is the molecular mechanism of cell predation while the encounter between a virus such as influenza and a potential host was considered a random event. Looking at small flocks of wild ducks stopping during migration in the aquatic environment, how is it possible that viruses measuring just a few hundred nanometers, dispersed in million cubic meters of *water*, can meet and infect ducks? The virus takes maximum advantage of the meeting, increasing its opportunity to reproduce. This chance is reduced day by day due to many environmental factors that limit from a few months to a few days the virus's ability to survive in the

environment, once the virus leaves the body's host. Environmental factors including air and water temperature, exposure to ultraviolet rays of the sun, pH of the environment, can influence virus survival. Why a so specialized predator is limited to passively waiting for a meeting with the species 'prey' before die? A further difficulty for the influenza virus is due to phylogenetically distance among waterfowl species. From an evolutionary perspective, the structures and the cell receptor affinities to the viruses are very different, then it is natural to ask what have in common wild ducks, herons, gulls, waders, rails to contribute to influenza transmission and circulation.

The ecology of influenza A viruses is always related to the biological characteristics of the bird species implicated as reservoirs or, eventually, epiphenomena. The ecology of influenza viruses is a very dynamic system in which certain structural constants (reservoir migrations) and other important environmental variables coexist within zoogeographic regions. This system influences both the biology of reservoirs and that of epiphenomena, and in turn their interactions with the virus. In wild ducks population parameters such as sex and age classes could affect the isolation prevalences of influenza viruses in ducks trapped in a Mediterranean area. For example cloacal swabs taken from 146 dabbling ducks trapped from October 2005 to September 2006 in Laguna di Orbetello WWF Oasis (Central Italy) showed significant differences in viral isolation prevalences related to ducks' age, ducks' sex, during an yearly sampling periods (fall migration, wintering period, post-reproductive period). AIV RT-PCR and isolation prevalences were 26% (38/146) and 9.6% (14/146), respectively. During the overall sampling period no age- and sex-related differences in virus isolation prevalences were detected. Considering each sampling period, significant differences were only found during the wintering season January-March 2006, when the juvenile ducks showed higher isolation prevalence than the adult ones. The very low temperatures recorded in north-eastern Europe during winter 2005-2006 could explain erratic movements of juvenile ducks, thus increasing opportunities of interaction among different birds' subpopulations and viral subtypes. These results confirm that the juvenile ducks play a key role in the movement of AIVs. Moreover, climatic variations may induce changes in the host species' behavior affecting viral population's ecology.

The Western Palearctic is a zoogeographic region that includes Europe, the part of Asia to East Himalaya, a part of Arabia, and the part of Africa to the north of the Tropic of Cancer. This region, together with the Nearctic Region, makes up the largest zoogeographic region of the western hemisphere, known as the Holarctic Region. Italy is situated in the central-south-western area of the Palearctic Region and represents an ideal geographic

bridge between the northern limits (tundra, northern Europe) and the southern limits (northern Saharan Africa) of the Western Palearctic. Birds belonging to the Sub-Family Anatinae are the major reservoir of the influenza viruses. The following duck species are present in the Palearctic Region, though their distribution is holarctic: shelduck (*Tadorna tadorna*), wigeon (*Anas penelope*), gadwall (*Anas strepera*), teal (*Anas crecca*), pintail (*Anas acuta*), eider (*Somateria mollissima*), king eider (*Somateria spectabilis*), harlequin (*Histrionicus histrionicus*), longtailed duck (*Clangula hyemalis*), common scoter (*Melanitta nigra*), velvet scoter (*Melanitta fusca*), mallard (*Anas platyrhynchos*) and red-breasted merganser (*Mergus serrator*). In the Holarctic and Oriental regions we can find teal, mallard, pintail, tufted duck (*Aythya marila*) and goosander (*Mergus merganser*). However, the whiteheaded duck (*Oxyura leucocephala*) and marbled teal (*Marmaronetta angustirostris*) are found only in the Palearctic region, while the American wigeon (*Anas americana*) and blue-winged teal (*Anas discors*) are found only in the Nearctic Region. The ruddy shelduck (*Tadorna ferruginea*), mandarin duck (*Aix galericulata*), Baikal teal (*Anas formosa*) and redcrested pochard (*Netta rufina*) inhabit the Palearctic and Oriental Regions, while the mallard is the only species to be found in the Holarctic, Oriental and Australian Regions. The garganey (*Anas querquedula*), pochard (*Aythya ferina*), tufted duck (*Aythya fuligula*), and ferruginous duck (*Aythya nyroca*), can be found in the Palearctic, Ethiopic and Oriental Regions, while the shoveler (*Anas clypeata*) is found in the Ethiopic, Oriental and Holarctic Regions (Chelini, 1984; Scott and Rose, 1996). It therefore appears obvious that each species can frequent different zoogeographic areas, but for many the presence in Palearctic Region is a constant. The reasons for this distribution are given by both the habitat and the distribution of the reproductive (north) and wintering (south) zones. Wild ducks migrate to satisfy several biological needs vital to their survival; these factors vary from species to species, and within the species from population to population. In order to reach the various zones involved in their life cycle, the birds undertake seasonal migration. This migration takes them south in late summer and autumn in the quest for a mild climate, and north to reproductive areas at the end of winter-beginning of spring. During the migration south, the adult birds are accompanied by young birds born during the reproductive season, while, upon return north the entire bird population is potentially reproductive (Scott and Rose, 1996). Since the cyclic nature of the influenza infection is conditioned by the existence of young birds, this migrational behaviour is of great importance. Furthermore, the migration towards the south is much slower than that north and the birds make

numerous stops along the way. The Palearctic Anatidae populations have three different behavioural characteristics: (a) the sedentary species (e.g. ruddy shelduck, white-headed duck and marbled duck) remain for their entire life in the same geographic region); (b) the completely migratory species (smew (*Mergus albellus*), garganey), make a complete migrations between north and south; (c) the partially migratory species (e.g. mallard) constitute the major part of the Anatidae. In the species belonging to the last group, only a part of the population migrate (Chelini, 1984). The various flyways used by waterfowl to cross the Western Palearctic in order to migrate south are: (1) from Sweden, across France and Gibraltar to Africa; (2) from Finland, across Holland, north-western Italy and Sardinia to Africa (Rose and Scott, 1997); (3) from central Europe across the Balkans, south Italy and Sicily to North Africa. Other routes, which cover more easterly zones are (4) from central Europe, over the Balkans and Greece to North Africa; (5) from Central Europe across the Black Sea and Turkey to East Africa (Scott and Rose, 1996). Except for garganeys, which winter in western Africa and migrate over the Sahara desert, all other species winter in the Mediterranean wetlands. The wintering areas of most of the waterfowl that migrate in spring and autumn are found in Italy (Baccetti *et al.* 2002); these same areas are also the breeding sites for some partially migratory species such as mallards. Waterfowl migrations are studied by catching and ringing wild birds and by verifying the sites of recapture. From the data obtained, it is evident that the duck populations wintering in Italy come from north-eastern Europe (Chelini, 1984; Scott and Rose, 1996). Numerous and varied wintering areas can be found in Italy, representing the various ecological habitats of each species. The diving ducks usually winter in the deep water of the most important Italian lakes and ponds; while dabbling ducks are mainly concentrated in marshes. A great number of different species of waterfowl crowd the highly productive wintering areas, and these areas are shared for a while with many other heterogeneous sedentary or migratory bird species. Thus, homospecific and heterospecific aggregations are formed and are favoured by feed availability and the absence of hunting. This is a favourable situation for the transmission of influenza viruses both between allopatric homospecific populations, and between heterospecific groups (De Marco *et al.*, 1999; 2000). During this aggregation, which takes place in autumn and winter, the high population density may allow virus transmission during this period characterized by a low prevalence of influenza infection. The pH, salinity and temperature of the water may facilitate survival of the virus (Stallknecht *et al.*, 1990a, 1990b). By drinking water or filtering it in the quest for food, birds may acquire the virus. The depth and turnover of the water are the main factors

that influence the ecological interaction between host and virus. The virus can spread easily in the small highly frequented areas where direct bird-to-bird contact is facilitated by the high population density. Interactions can also be enhanced during the winter season by particularly adverse climatic conditions (ice). The water should be considered as a means of virus preservation as well as of virus transmission, allowing the spread of infection without direct contact between birds. Numerous bird species drink the same water; thus, species that are normally separated because of ethological and ecological limits come into contact when they drink the same water and the virus can circulate freely both in reservoirs and in epiphenomena. The latter are hosts that are generally able to harbour the virus for a limited period but are not able to maintain the disease in the wild. The influenza virus is characterized by a moderate host-specificity and this provides many epidemiological possibilities: the virus can be transmitted by water to a wide range of birds and mammals. The infection may or may not cause disease. Therefore, most of the more than 400 bird species that constitute the Italian avifauna (Chelini, 1984) could potentially assume the role of an epiphenomenon.

The host/viruses/ environment interaction

Recent research (Delogu *et al.*, 2010) shows that it is possible to activate an interaction between influenza virus dispersed in the water of rivers, lakes and marshes and the surface of the plumage of aquatic birds in hundreds of species including in this group. Water birds during an evolutionary convergence process produce a gland secretion (preen oil) to waterproof their plumage. This oil is secreted by a gland called uropygial gland and used by birds to coat their surface. This is certainly the first point of interaction between water and the body of these birds (Fig. 1). At each stop of migration, during the day, aquatic birds spend hours of their time in groom activities. Birds perform grooming on them-

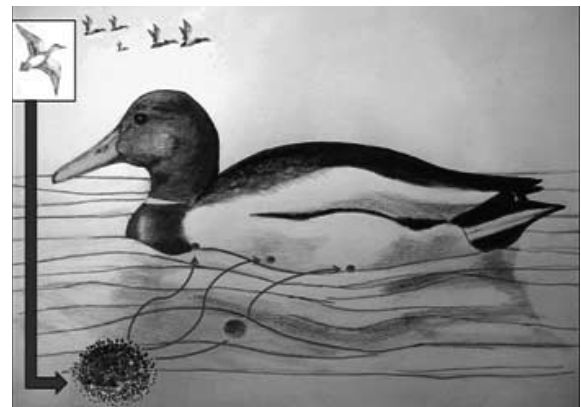


Figure 1 - In water, the preen oil gland secretions by which all aquatic birds make their feathers waterproof, could concentrate avian influenza viruses from water onto birds' bodies feathered surface.

selves (self-preening) and on their partners in the same flock (allopreening). The same fat that makes up the uropygial secretion is replaced by new secretion on the feathers removed while it is ingested as a supply of vitamin D.

The field research was conducted in a oasis where it was possible to follow continuously the movements of wild ducks and their relationship with avian influenza viruses in nature. The molecular studies performed on several hundred of feathers swabs collected from the waterproof plumage of ducks showed that on the plumage's surface were present very high concentrations of avian influenza viruses. In particular, we showed that, by RT-PCR, feather swabs were 2.5 times more often AIV positive than cloacal swabs. Whereas, virus isolation percentages were 2.3 times higher in cloacal swabs than in feather swabs.

These significant differences suggest partial inactivation of AIVs stuck on feathers, probably due to environmental factors such as UV rays or unsuitable temperatures.

The surprising element is that many of these ducks were negative to routine surveillance tests but strongly positive if checked on feathers.

If the proposed preening-mediated mechanism of infection is at play in nature, birds carrying viruses on their feathers but testing negative for virus in the cloaca and trachea by current surveillance programs might still play an active role in spreading AIV infection. These "false-negative" birds could include susceptible birds that are naive to AIV infection, as well as unsuitable birds that are naturally immunized to AIV infection. In the second case, this novel infection mechanism might escape the birds' immune system, such that unsuitable hosts might infect susceptible birds by allopreening.

Results from our field studies indicate that AIVs can be carried on the feather surface of infected ducks (i.e., those VI-positive from both cloacal and feathers swabs) and uninfected ones (i.e., those VI-positive from feathers only). For this reason, in routine surveillance programs, additional sampling methods could be necessary to detect AIVs on birds' bodies.

Moreover, to evaluate whether alternate mechanisms facilitate AIV transmission in aquatic bird populations, we investigated whether the preen oil gland secretions by which all aquatic birds make their feathers waterproof could support a natural mechanism that concentrates AIVs from water onto birds' bodies, thus, representing a possible source of infection by preening activity. To confirm the existence of these mechanisms we developed laboratory experiments designed to reproduce and explain what we observed in nature. Our findings indicate that a progressive virus "sticking" occurs because AIV-contaminated waters interact with the uropygial gland secretion, which covers body surfaces.

Our field and experimental results also suggest that during the time period between the virus adhesion to

the bird's body and the infection (possibly due to self- and/or allopreening), the virus could move in nature with the host by an undescribed circulation mechanism. In such a context, the epidemiologic status of uninfected birds carrying AIVs on their feathers certainly does not affect the fitness of the host, in contrast to what is reported for LPAIV, and HPAIV infections. With particular regard to the geographical spread of the Eurasian H5N1 HPAI virus in wild birds, the uninfected carrier hosts could have facilitate, by preening behaviour, the circulation of a virus able to kill the natural reservoir. The presence of Eurasian H5N1 HPAI virus on swan feathers, possibly due to the preen oil-virus interaction or fecal contamination, may also explain the only recorded human case of fatal infection passed from wild birds in February 2006. All infected humans were involved in defeathering of dead wild swans after a massive die-off of these aquatic birds occurred in Azerbaijan. Because women defeather birds more often than men do, their high exposure to infected feathers may explain their higher incidence of infection.

DISCUSSION

Our field and laboratory trials showed a concentration mechanism of AIVs on the bodies of aquatic birds and a potential preening-mediated route of infection. Virus adsorption on bird bodies appears to be a natural mechanism by which virus particles are captured by preened feathers and concentrated from the aquatic environment to bird bodies. By preening, birds spread preen oil all over their plumage, and this behaviour could facilitate a protracted ingestion of AIV particles, thus possibly promoting a preening-mediated infection in a large number of aquatic birds species.

The results of this research may identify a new natural cycle of influenza viruses, which explains many unknown aspects of the relationship between virus and host interaction.

Our results may explain some unrecognized mechanisms of transmission of the A/H5N1 virus in Asia and Europe, providing insights on how the highly pathogenic emerging A/H5N1 virus that can kill the natural reservoir may have circulated in wild bird populations of Asia and Europe. During migration stops, AIVs shed in the water via infected feces may interact with the body surface of susceptible birds (Fig. 2), and 1 of 3 scenarios can occur:

1. Susceptible birds become infected by the classical fecal-oral route (Figs. 2A, 2B)
2. Some susceptible birds become coated with AIVs whereas others stay uncoated: both types can migrate from infected environments to uninfected ones, where susceptible uncoated birds can infect themselves by preening of coated birds (Fig. 2C)
3. Coated susceptible birds (Fig. 2D) become infected by self- or allopreening at a migration stop (Fig. 2E), and delayed virus replication

cycles may perpetuate AIVs in nature. Our newly proposed mode of transmission of AIVs in aquatic birds adds to the knowledge of long-distance movements and long-term infectivity of AIVs in nature and integrates well with the recently proposed epidemiological approach that emphasizes

infection with avian influenza H5N1 virus have been reported from 4 countries: Bangladesh (2), Cambodia (8), Egypt (33), and Indonesia (11). Of these, 12 have been fatal in Egypt, 9 in Indonesia and 8 in Cambodia. The majority of these human cases infection have been associated with direct or

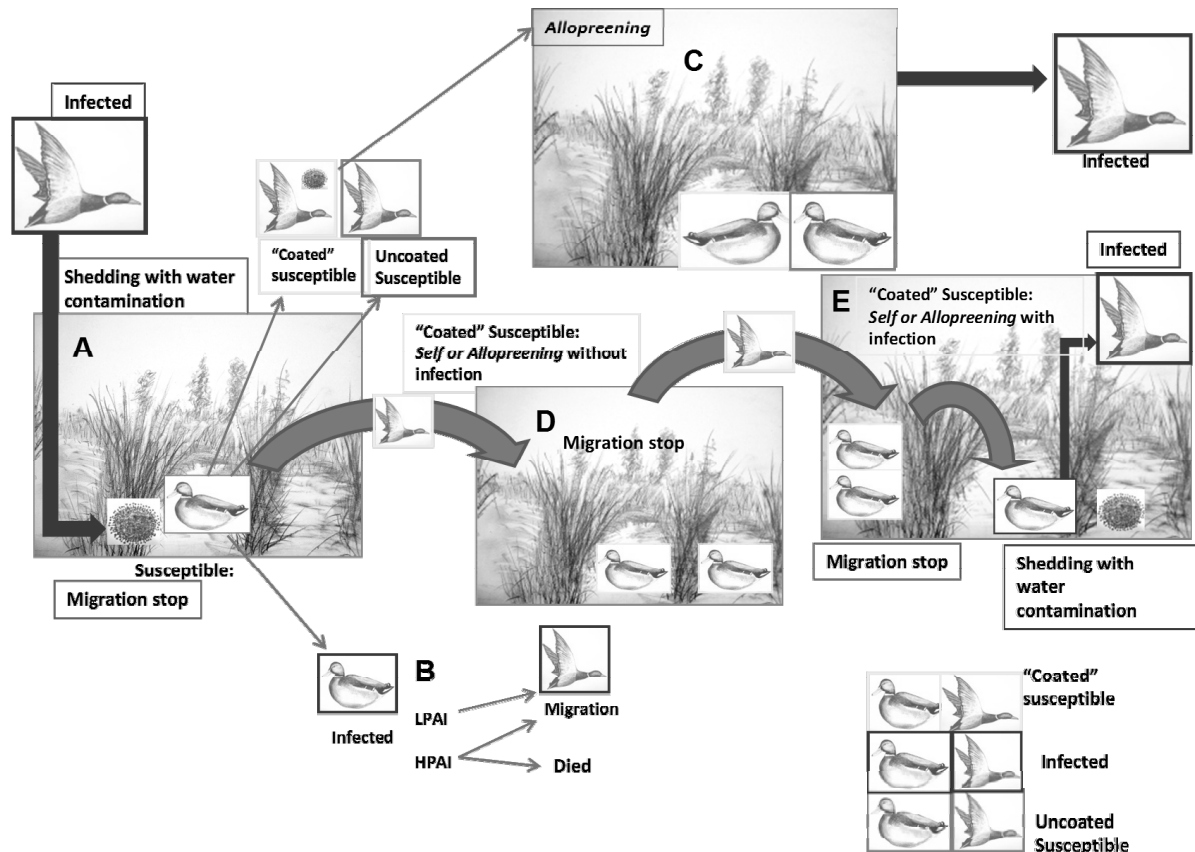


Figure 2 - Preen oil-mediated ecological link among AIVs, environment, and aquatic birds. (A) During migration stops, AIVs shed in the water by infected feces interact with the body surface of susceptible birds. (B) Susceptible birds can become infected by the classical fecal-oral route. (C) Some susceptible birds become covered with AIVs whereas the others stay uncoated; both can move from infected environments and migrate to uninfected ones (D), where susceptible uncoated birds may infect themselves by preening of coated birds. (E) Coated susceptible birds can become infected by self- or allopreening during a migration stop. It is possible that delayed virus replication cycles can aid perpetuation of AIVs in nature. The term “coated” indicates birds carrying AIVs stuck on their naturally preened feather surface. LPAIVs: lowly pathogenic avian influenza viruses; HPAIVs: highly pathogenic avian influenza viruses.

the epidemiological role of environmental transmission of AIVs to better understand influenza ecology. The presence of A/H5N1 virus on swan feathers, possibly due to the preen oil-virus interaction or fecal contamination, may also explain the only recorded human case of fatal infection passed from wild birds in February 2006. All infected humans were involved in defeathering of dead wild swans after a massive die-off of these aquatic birds occurred in Azerbaijan. As women more often defeather birds than men, their high exposure to infected feathers may explain the high incidence of infection in females.

To date, in 2011, 54 confirmed human cases of

indirect contact with infected live or dead poultry. In conclusion, ongoing circulation of H5N1 viruses in poultry, especially when endemic, continues to pose threats to public health, as these viruses have both the potential to cause serious disease in people and may have the potential to change into a form that is more transmissible among humans.

Our results strongly suggest that a preened body surface could be the common denominator that explains how AIV infection occurs in different taxonomic groups of aquatic birds. Future studies need to determine the role of this mechanism as a potential infection route for humans.

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All abbreviations and acronyms must be put *in extenso* the first time they are used.

Short notes and technical notes should not have an abstract and need not to be divided into sections.

Letters to the editor should not exceed 500 words; tables and figures are rarely accepted. References, if essential, should be given in the text.

FIRST PAGE

The first page of each manuscript should contain the title, the name(s) of author(s), the institution of the author(s), and e-mail address for the correspondence.

A running title of no more than 40 characters (including letters and spaces) should also be provided.

TABLES AND FIGURES

Each table should be typed on a separate sheet. The heading should be sufficiently clear so that the meaning of the data will be understandable without reference to the text.

Figures (drawings, graphs, photographs) should be of a size suitable for reduction to single column (75mm) or full page (160mm) width. Figures should be in .jpg or .tif format.

Figure legends should be sufficiently clear so that the figure is understandable without reference to the text.

Tables and figures should be numbered with Arabic numbers in a consecutive and independent way and must be referred to in the text.

REFERENCES

The list of references should include only those publications which are cited in the text and should be in alphabetical order at the end of the paper.

In the text, references should be cited thus: "it has been shown (BRUCE-CHWATT, 1986).

If there are two authors, both should be named; if more than two, only the first need to be named, followed by "*et al.*", in the text.

Each reference should include the following:

a) Paper published in periodicals: Author's surname with the initials of first name, year of publication in brackets, full title of paper, full journal title, volume number, first and last page numbers, e.g.:

PASTICCI M.B., MORETTI A., PAULUZZI S. (1991). Antibiotic resistance in methicillin-resistant staphylococci: a cause for concern. *Farmaci & Terapia*, 8: 203-204;

ROUGEMONT A., BRESLOW N., BRENNER E., MORET A.L., DOUMBO O., DOLO A. SOULA G. & PERRIN L. (1991). Epidemiological basis for clinical diagnosis of childhood malaria in an endemic zone in West Africa. *The Lancet*, 338: 1292-5.

b) Books: Author's surname with the initials of first name, year of publication in brackets, full title, edition, name and city of publisher, first and last page numbers e.g.

BRUCE-CHWATT L.J. (1985). *Essential Malariology* (2nd edition). W. Heinemann Medical Books, London. 452 pp.

CASSONE A., TOROSANTUCCI A. (1991). Immunological moieties of the cell wall. In: *The molecular biology of Candida albicans*. R. Prasad (Ed.). Berlin-Heidelberg, Springer Verlag, pp. 89-107

WORLD HEALTH ORGANIZATION (1985). *The control of Schistosomiasis*. Technical Report Series n. 728. World Health Organization, Geneva. 113 pp.

c) Abstracts in Proceedings of Congress: Author's surname with the initials of first names, year of publication in brackets, full title, name and city of publisher, page numbers, e.g.:

SOULE' C., FABIEN J. F., MAILLOT E. (1994). Animal Hydatidosis in France. In: *Abstracts of 8th International Congress of Parasitology*. Izmir, Turkey, 10-14 October 1994. Turkish Society for Parasitology. Izmir, Turkey, p. 348.

PROOFS

Proofs will be sent to Authors for correction and should be returned to the Editorial Office.