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Experimental infection of sheep with Schmallenberg virus at days 45 and 60 of pregnancy

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Schmallenberg virus (SBV) is an *Orthobunyavirus* that emerged in Europe during summer 2011. SBV is responsible for an acute and unspecific syndrome in cattle. It also causes abortions, stillbirths and malformations in cattle, sheep and goat. To date the transmission of SBV from a pregnant ewe to its foetus stays poorly understood. In this study, the outcome of an inoculation of pregnant ewes at days 45 and 60 of pregnancy with SBV was evaluated.

Twenty-three virologically and serologically SBV negative "Mourerous" breed ewes of about 1 year-old were used. Eight and nine pregnant ewes were subcutaneously inoculated with 1 ml of SBV infectious serum at days 45 and 60 of pregnancy, respectively. At each time-point, three other pregnant ewes were mock-infected with PBS and constituted the control group.

The ewes were clinically monitored and blood was collected daily during the first 12 days post infection (dpi), thereafter once a week. The lambs were born at term via caesarean section, were clinically examined and immediately after birth, blood was collected. Both ewes and lambs were then euthanatised and following samples were collected during necropsy: lung, spleen, gonads, lymph nodes (prescapular, mesenteric and mediastinic), placenta and cotyledon; plus brain, cerebellum, brainstem, spinal cord, meconium, adrenal gland, cartilage, muscle, thymus and liver in lambs. All samples collected from ewes and lambs were tested for the presence of SBV S-segment RNA with qRT-PCR and the presence of SBV neutralising antibodies was evaluated by seroneutralisation tests (SNT).

No visible clinical impact was noticed and all inoculated ewes showed an RNAemia and seroconverted shortly after. Meanwhile, the overall rate of living lambs was 88.9% and 62.5% in the group inoculated at day 45 and 60 of pregnancy, respectively and 66.7% and 33.3% in the control groups mock-infected at days 45 and 60 of pregnancy, respectively. No malformations suggestive of SBV infection were noticed in any of the lambs. All living lambs were able to stand, had a good suction reflex and no obvious nervous disorders were observed. No viral RNA was detected in blood and no anti-SBV antibodies were found in any of the lambs except for a premature lamb, which had the opportunity to drink colostrum. With about 80% of the samples tested, viral RNA was found in the umbilical cord of 1 ewe and the placenta of another ewe in the group inoculated at day 45 of pregnancy. In the group inoculated at day 60 of pregnancy, viral RNA was found in 6 ewes. The SBV positive organs were the placenta, the cotyledon and the umbilical cord for the first ewe, the cotyledon and the umbilical cord for the second ewe, the placenta and the cotyledon for a third ewe, the umbilical cord for the fourth ewe and the placenta for the two last ewes. Viral RNA was also found in the prescapular lymph node and in the cartilage of one lamb and in the brainstem of a second lamb.

In conclusion, the inoculation of 'Mourerous' pregnant ewes at 45 and 60 days of pregnancy could not reproduce the teratogenic effects of SBV observed in the field. Although, the higher number of PCR positive samples in the group inoculated at 60 days of pregnancy suggests a time-dependent effect.

Impact of Schmallenberg virus on productive and reproductive performances of dairy cattle herds in Italy

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Schmallenberg virus (SBV) has emerged and spread across Europe since the second half of 2011, causing unspecific and transitory symptoms (fever, diarrhoea and milk drop) in dairy cattle and congenital malformations in calves. Evidence for the impact of SBV emergence is generally scarce and constrained by the high level of case under-ascertainment. Using a comprehensive data set from a SBV-affected province in North-eastern Italy, we aimed at assessing the impact of SBV emergence on 11 productive and reproductive performance indicators of dairy cattle herds, accounting for weather conditions and other herd-level factors that could have also influenced these indicators. A total of 127 farms with an average of 71 cows per farm (range 29-496) were monitored monthly from January 2009 to July 2012. Mixed effects linear models for longitudinal data were used to assess the average variation in herds' performance indicators over semesters (Jan-Jun 2009, Jul-Dec 2009, Jan-Jun 2010, Jul-Dec 2010, Jan-Jun 2011, Jul-Dec 2011, Jan-Jul 2012). A significant decrease in the average lactation length (–6 days, on average) and calving-to-conception interval (–4 days, on average) was observed in the second semester of 2011. However, such a decrease actually represents a positive outcome for farm (re)productivity and is not imputable to SBV emergence per se, but rather reflects other beneficial changes in farm management practices. None of the other indicators, including milk production, number of newborns and number of inseminations per pregnancy, showed significant differences, the lack of which mirrors the relatively mild expression of SBV infection in cattle. We concluded that, although the impact of SBV might have been substantial in some individual dairy cattle farms, overall at the province level their productive and reproductive performances do not seem to have been significantly affected by the emergence of SBV, at least not in a way that there would have been significant negative effects on farm profitability.

Replication and Genetic Characterization of Schmallenberg Virus Vertically Transmitted to the Sheep Fetus.

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Seronegative pregnant ewes were inoculated via the subcutaneous route with Schmallenberg virus (SBV) at 6 or 7 weeks of gestation. Seven days after inoculation ewes were euthanized and uteri were immediately excised and amnion fluid, umbilical cord blood, placentome and CNS tissue were collected from the fetuses and analyzed using a diagnostic S segment rRT-PCR. To characterize SBV present in fetal organs and amnion fluids, samples from three placentomes/fetuses were selected which scored a Ct-value of 25 or lower. Using Illumina-MiSeq high-coverage "next generation sequencing" (NGS), genetic variation in the M segment of SBV present in selected placentome and CNS samples was assessed. MiSeq sequencing of cDNA libraries produced from 2 regions in M, coding for parts of the hyper-variable region (HVR [1,2]), detected a considerable degree of sequence variation in the HVR of SBV present in fetal CNS tissue, especially around the amino acid motifs coding for the atypical IRL triplet within the HVR of SBV isolated from the brain of a malformed lamb (HL1 isolate [3]). In addition, a strand-specific RT-PCR was developed in order to separately quantify amounts of positive-sense messenger RNA (mRNA) and negative-sense viral RNA of the L segment in clinical samples. This strand-specific RT-PCR test detected a relative high amount of mRNA in placentome samples compared to "cell-free" clinical samples (serum and Vero cell culture medium), indicative for placentomes as a replication site of SBV. Results are discussed in relation to the pathogenesis of SBV in sheep fetuses.

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Innocuity and immunogenicity of EHDV-2 vaccine in pregnant cows

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Epizootic haemorrhagic disease (EHD) is an infectious viral disease affecting both wild and domestic ruminants. Eight serotypes have been described so far worldwide. Amongst them, the serotype 2 (EHDV-2) is a serotype particularly virulent for cattle. In Japan, modified live vaccines (MLV) have been developed to protect against EHDV-2 infection. The present study aimed to test the innocuity and immunogenicity of an EHDV-2 MLV strain (vEHDV-2) (Ibaraki virus, Kyoto Biken, Laboratories, Kyoto, Japan) in pregnant cows and, in particular, the capability of the attenuated virus to cross the placental barrier and infect foetuses. Five late-term pregnant cows were subcutaneously administered with 10^6 $TCID_{50}$ vEHDV-2. Two cows were sham-inoculated and served as control. EDTA-blood samples were collected three times a week and tested for the presence of EHDV RNA and infectious virus by RT-PCR and virus isolation, respectively. Serum samples were collected once a week and tested for EHDV-2 antibodies by serum neutralisation.
At calving, EDTA-blood and serum samples from calves (before colostral intake) as well as colostrum samples from dams were collected, and tested serologically and virologically. Whenever possible, placenta was collected and tested for the presence of EHDV RNA and infectious virus. Control cows and calves tested negative throughout the experiment. Neither fever nor signs of disease were observed in the vaccinated cows and their blood samples tested negative by RT-PCR and virus isolation, except for one blood sample collected from a cow 11 days post vaccination (pv), from which infectious virus was isolated. Four vaccinated cows seroconverted on day 17 pv, and one on day 25 pv. Antibodies were detected throughout the entire experimental period (107 days pv). The antibody titre peaked on day 65 pv. The cows calved between 25 and 52 days pv. All pre-colostrum samples tested negative. Colostral antibody titre ranged from 1:40 to 1:1280; nevertheless, only one calf reached an antibody titre of 1:10. This study showed that vEHDV-2 is innocuous in cattle. It did not cause either clinical signs or abortions in vaccinated cows. As one animal only evidenced detectable viraemia for one day following vaccination, the product could be considered safe in terms of spreading the vaccine virus in the environment. Conversely, vEHDV-2 strain was immunogenic. It was able to elicit a strong and long-lasting immune response in the vaccinated animals. Regarding the capability to cross the placental barrier phenomenon commonly observed for the MLV strains of Bluetongue, the vaccinal product tested in this study did not cross the placental barrier and infect foetal tissues.

Arbovirus monitoring in mosquitoes collected from horse stables during 2012-2013 in Korea

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West Nile Fever (WNF), Japanese Encephalitis (JE), Eastern Equine Encephalitis (EEE), Western Equine Encephalitis (WEE) and Venezuelan Equine Encephalitis (VEE) are caused by arboviruses that result in encephalitis in humans and horses. Although WNV, EEE, WEE and EEE have not been reported in Korea, climate change has raised the risk of a new introduction of these diseases. Also, because JE is endemic in Korea, diseases caused by introduction of these viruses could be mistaken for JE. In Korea, annual surveillance for WNV and JEV is conducted in horses including differential diagnosis of JE suspect horses, but few studies have been conducted to look for arboviruses in mosquitoes targeted to areas where horses are being raised. Because WN, JE, EEE, WEE and VEE are all vector-borne diseases, monitoring and testing of mosquitoes where there are high densities of horses could allow for these viruses to be quickly identified and proactive measures to be implemented to prevent transmission to horses. For this purpose, arbovirus monitoring in mosquitoes was conducted at Korean Racing Authority (KRA) operated stables from 2012 to 2013. Mosquito magnet traps were operated during the summer and autumn season, and samples were collected every two weeks. The collected mosquitoes were first identified to species and tested for arboviruses by Reverse Transcription polymerase chain reaction (RT-PCR). A total of 10,689 mosquitoes comprising of 15 species in 5 genera were collected during the study. The most frequently identified species were *Culex pipiens* (80.87%, n=8,644). Total of 2,709 mosquitoes were pooled into 303 samples, which were all tested negative for WNV, EEE, WEE and VEE. One pooled sample was positive for JEV as confirmed by sequencing of the envelope protein coding genes.

The significance of entomological studies to support the control of mosquito-borne diseases in north-eastern Italy

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After the emergence of West Nile Virus (WNV) in 2008 in north-eastern Italy, long- and short-term entomological studies and retrospective studies were implemented within regional surveillance plans. For long-term studies more than two million of mosquitoes were captured bimontly by CDC-CO2 traps from May to October (2009-2013). The long term studies assessed the mosquito species composition, seasonality, distribution, abundance and pathogen rates of infection. Cx.pipiens (Cxp) was the most abundant species (80%) and the main vector of WNV and USUV. Short-term studies targeting specific aspects included: a) diel activity of mosquitoes through captures every 2hr for 24hr; b) efficacy of disinfestation in reducing Cxp density; c) PCR blood meal analysis on fed Cxp. These studies showed that Cxp changed the host searching activity according to the season and that the efficacy of disinfestation varied depending to the methods used. Cxp preferred to feed on birds (76%), mainly blackbird, sparrow, magpie and collared dove, indicating bird targets for surveillance. In retrospective studies mosquitoes were screened also for other pathogens assessing the presence of Batai and Tahyna viruses, Dirofilaria immitis and D.repens. Mapping, modelling and spatial analyses were done using the output of the monitoring to identify correlations with climate, landscape, animal and human infections. The contribution of density-dependence in regulating Cxp population growth resulted greater than the most significant environmental factors, i.e. length of daylight and temperature in the 15 days prior to sampling. Other factors were the relative humidity from March to July, the hydroclimatic balance in April-July, and the rainfall in June-July. Modelling indicated the need to incorporate density dependence in combination with key environmental factors for robust prediction of Cxp population expansion, helping to identify when and where an increase in WNV transmission risk should be expected. Linear models detected significant relationships between WNV in humans and mosquitoes and spatial analysis detected clusters of WNV occurrences for animal and human hosts, identifying an area where to focus surveillance and promptly detect WNV re-activation. The integration of entomological monitoring into surveillance plans proved to be valuable for early detecting viral circulation, giving pivotal indications on when and where to start the control of blood donors, even in absence of human cases.

Web-based information system for the surveillance of canine Leishmaniasis in Emilia-Romagna public kennels.

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In 2007 a regional surveillance program for canine Leishmaniasis (CanL) has been implemented in all public kennels of Emilia-Romagna region (ER, Northern Italy). Serological and entomological monitoring activities - IFAT and sticky traps, respectively - have been performed annually to rank kennels in four risk classes, in order to apply specific risk-based control interventions.
According to this approach, all stray dogs are sampled for CanL at the moment of admittance to the kennel. Seropositive (IFAT titre ≥ 1/160) or inconclusive dogs (IFAT titre of 1/40 or 1/80) (1) are re-tested to follow up the health status and decide for appropriate therapy. Clinical suspects are sampled to confirm CanL diagnosis, and in kennels where the presence of the vector has already been proved, a sample of sentinel housed dogs, tested negative at least once, is checked yearly to monitor the spread of CanL infection.
Being monitoring activities and control measures carried out by the official veterinary service well-established, in 2014 we have developed a web-based information system (WIS) for the surveillance of CanL in public kennels, with the aim of enhancing quality and data collection.
The system, available online at http://seer.izsler.it/, currently collects data on more than 25,000 univocally identified dogs, tested in the 73 kennels of ER.
The results of serological testing, which are updated daily, can be consulted online by the official veterinary service, accessing the WIS by signing in with a user name and password. The system displays the microchip number or tattoo of positive and inconclusive dogs to facilitate the planning of (i) further serological testing, (ii) an appropriate therapy and (iii) the adoption of preventive measures. Moreover, the WIS permits the integration of anamnestic information over time (symptoms, therapy, etc.), as well as the registration of the date of adoption or death.
Finally, possible inconsistencies of the dog identification or mistakes while filling in the sampling forms are automatically highlighted, providing timely and consistent information which are a key for the success of a surveillance program.
The online informative system, released in May 2014, could be further developed by adding a risk map on the spread of CanL infection in ER. This would provide physicians and veterinary practitioners with updated information on the epidemiology of CanL in their territory of competence.

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Characterization of *Leishmania infantum* strains circulating in dogs and humans in Emilia-Romagna Region (Northern Italy) by using the Multi Locus Microsatellite Typing (MLMT)

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Introduction. Zoonotic Visceral Leishmaniasis (ZVL) caused by *Leishmania infantum* is a public-health concern in various Mediterranean countries. Dogs affected with Canine Leishmaniasis (CanL) are considered the main reservoir for human infection. The northward diffusion of CanL and the increasing number of ZVL cases observed in the last years in Emilia Romagna (Northern Italy) led health authorities to focus the attention on this re-emerging parasitic disease. The understanding of *L. infantum* epidemiology in humans and in reservoir species is essential for the development of control strategies. In order to investigate the population structure of human and canine *L. infantum* strains circulating in Northern Italy by using a molecular approach, a research project started in 2013. In this work the preliminary results of the project are presented.

Materials and methods. *Leishmania infantum* isolates, from presumably autochthonous CanL cases (IFAT≥160) in 14 dog-shelters of 8 Emilia-Romagna Provinces, and from human VL were collected. Each isolate was identified as *L. infantum* with a specific PCR and submitted to the K26-PCR assay in order to discriminate MON-1 from other *L. infantum* zymodemes according to the length polymorphism of the K26 gene. Subsequently genotyping was performed by a Multi Locus Microsatellite Typing (MLMT) scheme based on the amplification of 15 loci: Li21-34, Li 22-35, Li 23-41, Li41-56, Li 45-24, Li 46-67, Li 71-5/2, Li 71-7, Li 71-33, Lm2TG, Lm4TA, TubCA, CS20, kLIST 7031 and kLIST 7039.

Results. Between February 2013 and May 2014, 21 canine *L. infantum* isolates were collected. A K26 amplicon typical for *L. infantum* MON-1 was presented by 18 isolates, whereas 3 isolates showed a K26 amplicon related to non-MON-1 zymodeme. The MLMT detected 16 different profiles. In dog-shelters where multiple strains were isolated, MLMT typing showed the circulation of the same or different types.

Three isolates of human origin were collected from VL cases occurred in the first months of 2014 in a defined area of Modena Province. All the 3 isolates showed a K26 amplicon related to non-MON-1 zymodeme and presented MLMT profiles different from the available canine isolates. Two human isolates shared the same MLMT profile, instead the third one differed in Li23-41 locus.

In 4 loci (Li21-34, Li41-56, Li46-67 and TubCA) all canine isolates showed unique alleles differing from human VL isolates.

Discussion. The K26-PCR results were consistent with *L. infantum* MON-1 being the prevalent zymodeme in the Mediterranean basin. The circulation of *L. infantum* non-MON-1 strains was also observed. The MLMT revealed an intra-zymodeme (MON-1) genetic heterogeneity providing evidence that this method could be a useful tool for epidemiological studies. The interesting difference in genotypes involved in ZVL cases needs further investigations. To this purpose genomic data sharing could contribute to better understand the *L. infantum* epidemiology and the role of dog as reservoir species for other than *L. infantum* MON-1 zymodemes. Finally interdisciplinary teams of medical, veterinary and environmental scientists are crucial for integrated control strategies against leishmaniasis.

Molecular detection of Toscana virus (*Bunyaviridae: Phlebovirus*) in sandflies (*Diptera: Psycodidae*) from Reggio Emilia province

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Toscana virus (Bunyaviridae: Phlebovirus) (TOSV) is recognized as one of first cause of summer meningitis in Mediterranean countries, particularly in Italy. This virus is transmitted by phlebotomine sandflies and the vertical transmission in vectors, reported at high rate in experimental studies, has been suggested as a possible mechanism of environmental persistence (Maroli et al.,1993, Med Vet Entomol. 1993 Jul;7(3):283-6.), but its ecology is still largely unknown.

The entomological surveillance was conducted in hilly areas (between 65 and 500 a.s.l.) of Reggio Emilia province. We placed carbon dioxide baited trap, working overnight, in 21 georeferenced sites; 13 sites were sampled for two years, while, in 2013, 8 more sites were sampled in different locations.

Sandflies male and female were separated. Males were identified at optic microscope after chlorolactophenol clarification by morphology keys, in particular by the shape of aedeagus (Romi e tal., 1994, Quad ISS: 114 p). Females were divided in groups with a maximum of 200 individual. and ground in 1.5 ml eppendorf vial with 500 ul of PBS by pellet pestle, and then submitted to biomolecular analysis.

Phlebotomus RNA were extracted using TrizolHLS Reagent (Invitrogen, Carlsbad, CA); cDNA synthesis was achieved using random hexamers (Roche Diagnostics, Mannheim, D) and SuperScriptH II Reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and then submitted to specific Real Time PCR for TOSV detection (Pérez-Ruiz et al. ,2007, J Clin Virol, 39(4), 276-281)

A total of 3388 sandflies were collected in 2012 and 10164 sandflies in 2013. Most of sandflies male identified belong to *Ph. perfiliewi* species (n= 3784; 97%) , only 3% of males belong to *Ph. perniciosus* species (n=109). Females represented 71% of sandflies catched by our traps (n=9656).

We examined by Real-time PCR 78 pools in 2012 and 91 pools in 2013.

Sandflies abundance was similar in the two years of sampling, with one peak of activity (almost 1000 sandfly per trap/night) in the second half of July both in 2012 and 2013. For sites monitored only in 2013 there was a peak in one trap at the end of August, catching about 3000 exemplars; in this site we found TOSV RNA in five pools; other one pool results positive in the same week but in other site.

Two species of sandflies were collected during the survey, *Ph. perfiliewi* and *Ph. pernicious* . Both species are considered efficient vector of TOSV (Charrel et al., 2005, Emerg Infect Dis Nov;11(11):1657-63), but due to the overwhelming abundance *Ph. perfiliewi* seems to be the main vector of TOSV in Emilia-Romagna. The different detection of TOSV between years may be due to different population dynamics of the vector: TOSV RNA was found the in the more abundant samples obtained during survey, that group over than 3000 specimens. These results seem to imply a strong correlation between the presence of the virus and sandflies density, according to previous field evidence that link the peak in human cases with periods of highest density of the vector. If confirmed, this strong correlation would be a very useful tool for assessing the risk of infection of TOSV.

Further studies are needed to identify possible TOSV reservoirs, particularly in study areas in which positive pools were sampled, characterized by agricultural fields, woods and hedges.

Serological Investigation of Akabane Infection in Cattle and Sheep in the Marmara Region of Turkey

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Akabane virus is the aetiological agent of Akabane disease which is a vector-borne viral infection of cattle, sheep and goats causing reproductive disorders, congenital deaths, arthrogryposis-hydranencephalus (AH), abortions and mumified fetuses. It is an economically important disease and has been frequently reported in countries populated with *Aedes*, *Culex* and *Culicoides* flies including Turkey. The aim of this study was to investigate presence of antibodies to Akabane virus in cattle and sheep sera in the Marmara region of Turkey which borders European Union. For this, sera were collected from 125 cattle and 50 sheep in the past two years and analysed by using a commercial ELISA kit. Antibodies to Akabane virus were detected in 17 cattle while none of the sheep was found to be positive for antibodies to Akabane virus.

The results of this study indicate that Akabane disease exist in the Marmara region of Turkey with a considerable frequency. Therefore, preventive measurements like fly control should be carried out to protect cattle, sheep and goats. Further investigations are necessary to determine the genetic characterisation of Akabane virus circulating in Turkey.