First cases of Schmallenberg virus in Italy: surveillance strategies

Federica Monaco¹, Maria Goffredo¹, Valentina Federici¹, Andrea Carvelli¹, Andrea Capobianco Dondona¹, Andrea Polci¹, Chiara Pinoni¹, Maria Luisa Danzetta¹, Lucia Selli², Michela Bonci², Michela Quaglia¹ & Paolo Calistri*¹

¹Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Campo Boario, 64100 Teramo, Italy
²Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro (PD), Italy

* Corresponding author at: Epidemiology Unit, National Reference Centre for Veterinary Epidemiology, Programming, Information and Risk Analysis, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Campo Boario, 64100 Teramo, Italy.
Tel.: +39 0861 332241, e-mail: p.calistri@izs.it

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Summary
Following the first report of Schmallenberg virus (SBV) in the brain of a dystocic goat foetus in 2012 in Northern Italy, immediate response actions were adopted to avoid the virus circulation. The brain tested positive by 2 different one-step real-time RT-PCR protocols; these results were also confirmed by partial sequencing of the viral genome. At that time this was the first detection of the new Orthobunyavirus genus within the Bunyaviridae family in Italy. An epidemiological investigation in the involved farm was carried out in collaboration with the CESME - National Reference Centre for the study and verification of Foreign Animal Diseases (Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Italy). Epidemiological information on the flock was provided and analysed, whole blood and serum samples were also collected from all animals in the farm for both virological and serological investigations. All blood samples tested negative for SBV, whereas serological positive results were obtained by virus-neutralization (VN). Epidemiological investigations indicated the possible virus circulation in the area. The subsequent surveillance actions were mainly based on the standardization and re-enforcement of passive surveillance protocols, a risk-based serological surveillance programme through VN and an entomological surveillance programme in the involved geographical areas were also put in place. Eventually SBV local circulation was confirmed by real time RT-PCR in 6 Culicoides pools, collected between September and November 2011 in 3 farms in the surroundings of the area of SBV outbreak.
Introduction

Between the end of 2011 and the beginning of 2012, Schmallenberg virus (SBV) has been reported in ruminants (cattle, sheep, goats and bison) in Germany, the Netherlands, Belgium, United Kingdom, France, Italy, Spain and Luxembourg (8, 9). Preliminary studies on its genome suggested the virus affiliation to the Simbu serogroup, belonging to the genus Orthobunyavirus within the Bunyaviridae family. Adult animals infected with SBV show mild clinical signs persisting for approximately one week and characterized by fever, loss of appetite, up to 50% reduction in milk yield and, sometimes, severe diarrhoea (14). SBV infection is also associated to foetal malformation and stillbirths (16).

After the first confirmed cases in several European countries, the Italian Ministry of Health provided indications to enforce passive surveillance in all farms with ruminants, particularly in those that had introduced live animals from affected countries. These provisions, coupled with the information disseminated through the website of the CESME (http://www.izs.it/IZS/Engine/RAServePG.php/P/357410010300/M/250010010303), the Italian National Reference Centre for the study and verification for Foreign Animal Diseases (Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise ‘G. Caporale’), arose the concern of veterinary services.

The detection of this new infection in the Italian territory posed new and pressing questions. Due to the lack of information on virus epidemiology, a broad-spectrum approach was chosen to quickly define the area that may have been potentially involved by virus circulation. In this respect, it turned out to be highly important to have in place a valuable entomological surveillance program, as it has been argued by Goffredo (12). This article describes the surveillance approaches endorsed by the Italian Ministry of Health to promptly investigate the impact of the infection and to coordinate the subsequent actions.

Materials and methods

First case finding

At the beginning of February 2012 suspicions of infection were notified in 3 Italian regions. Eventually, only 1 case was confirmed in a small herd of Treviso Province, Veneto Region (Figure 1). In this farm, where 1 calf and 6 goats were kept, a female goat suddenly died the day after parturition of a healthy kid. The carcass was submitted for post-mortem examination and necropsy revealed the retention of a dystocic foetus showing congenital malformations, namely: scoliosis, arthrogryposis and ankylosis of some of the limb joints. Brain and spleen samples of the foetus were submitted for virus detection.
cells (BHK21, ATCC-CCL 10). Serial ten-fold dilutions of antigen were made. After 5 days the titre was determined by using the Reed and Muench (1938) formula (15). Serum samples were inactivated at 56°C for 30 min. Starting from 1:2, serial two-fold dilutions were made from serum samples in microtitre plates, and 100 TCID₅₀ units of antigen were added to each dilution. Thereafter, the mixtures were incubated at 37°C for 1h and 10⁵ BHK21 cells were added to the wells. Plates were read after 5 days of incubation at 37°C. The antibody titre was defined as the reciprocal of the highest dilution of the test serum sample, which showed at least 90% neutralization. Positive and negative control sera were included in each plate.

Total RNA was extracted from 200 µl of cell culture supernatant from the brain of the positive foetus with High Pure Viral Nucleic Acid kit (Roche, Nutley, NJ, USA) and eluted with 50 µl of Elution buffer according to the manufacturer instruction. Viral RNA was reverse transcribed and the small (S) segment amplified. Amplification and sequencing were repeated twice to avoid introduction of artificial substitutions. Raw sequence data were assembled using Contig Express (Vector NTI® suite 9.1, Invitrogen, Carlsbad, CA, USA) and the consensus sequence was aligned with the German SBV strain isolated in 2012 (HE649914) to evaluate the variation within the strains.

Surveillance activities

A wide spectrum of surveillance actions was put in place to verify the SBV circulation in the area and possibly define the geographical extension of infection. The choice of an appropriate surveillance strategy was hampered by the absence of a serological assay (i.e. ELISA) for processing a large number of sera when first cases occurred at the beginning of 2012 in Italy.

The surveillance programme was mainly based on:

- standardization and re-enforcement of passive surveillance protocols;
- risk-based serological surveillance through VN;
- entomological surveillance in the involved geographical areas.

As a first step for the re-enforcement of passive surveillance actions, the Italian Ministry of Health adopted clear definitions of suspected and confirmed cases (Table I). The official publication and dissemination of these definitions, together with the preparation of specific forms to be filled by veterinarians in case of suspicion, allowed for standardizing the veterinary services actions and for a better harmonization of the collected data (4).

Given the unavailability at that time of a serological
assay for processing large number of sera, a risk-based approach was chosen for the serological surveillance. In case of suspicion, local veterinary services collected blood samples from all animals in the herd/flock, which were then tested by VN at the CESME laboratory. Additional clinical examinations in epidemiologically linked farms and in all animals living within 4 km of radius were also mandatory. A sample of animals was also tested in order to determine the serological prevalence of infection within suspected herds/flocks. The sample size was calculated for an expected within-herd prevalence of 10% and a level of confidence of 95%. This size was chosen according to previous experiences with bluetongue infection in Italy (2).

As for the entomological surveillance, a retrospective survey was carried out on 6 selected sites, located in Veneto and Friuli Venezia Giulia regions: in particular 2 in Treviso province, 1 in Belluno province and 3 in Pordenone province. The selected sites were located in a radius of about 50 km from the outbreak.

Between June the 1st and November the 30th 2011, 87 Culicoides collections were made within the framework of the national surveillance plan for bluetongue. The samples were stored in ethanol 70% and were selected to be tested for SBV. A total of 20,380 Culicoides were identified, according to Delécolle (5), Campbell & Pelham-Clinton (3), and Goffredo & Meiswinkel (11).

The Obsoletus Complex (including Culicoides obsoletus and Culicoides scoticus) alone represented 94% of the collected midges (n = 19,272), followed by the Nubeculosus and Pulicaris complexes (3% and 1%, respectively). Other species, considered responsible for the transmission of arboviruses, were very low abundant (i.e. few specimens of Culicoides dewulfi) or absent (i.e. Culicoides chiopterus).

All the midges were age-graded according to Dyce (6). The whole bodies of parous and, when present, engorged females were separately sorted out in pools according to species, place and date of collection. All the pools were tested by qRT-PCR for the presence of SBV and by RT-PCR according to the technique describe by Bilk (1). A total of 175 pools were prepared (ranging from 1 to 50 specimens): 138 composed by midges belonging to the Obsoletus Complex (number of midges = 4,062), 17 to the Nubeculosus Complex (number of midges = 100) and 20 to the Pulicaris Complex (number of midges = 52).

Results

After the molecular detection of the first Italian case of SBV, 19 suspected cases have been submitted to CESME laboratory for confirmation (Table II).
The presence of antibodies against the SBV was confirmed in 4 goats and 2 calves, with titres ranging from 1:16 to 1:1280. All the goats and 1 of the calves belonged to the farm where infection was first detected by RT-PCR, whereas a second positive calf was identified thanks to the serological monitoring in a farm located 30 km from the previous outbreak (Table III).

Once compared with the sequence of SBV isolated in Germany, the Italian strain showed 100% similarity of the small genome segment.

The epidemiological investigation carried out in the first positive farm excluded the introduction of animals from the EU infected countries, hence supporting the hypothesis of a local virus circulation. A total of 6 pools resulted positive to SBV. 4 pools were collected in a farm located 40 km from the outbreak on September the 6th, in October from the 21st to the 25th and on November the 3rd, 1 pool was collected on October the 4th about 8 km away and another pool was collected on November the 7th about 35 km away from the first SBV outbreak (Table IV). All the positive pools consisted of species of the Obsoletus Complex, 5 of them were composed by parous females (ranging from 5 to 47) and 1 by a single engorged midge collected in Feltre on September the 6th.

**Discussion**

The detection of SBV in Central Europe and, eventually, in Italy posed new and complex problems, which needed a tempestive solution. In particular, the lack of knowledge on disease epidemiology and its impact and the availability of limited diagnostic resources forced the Italian Ministry of Health to evaluate different surveillance approaches.

After SBV occurrence, the most pressing issues to be addressed were:

- verify whether SBV had actively circulated within the populations of competent vectors in Italy;
- define the geographical extension of SBV transmission;
- collect information for assessing the possible impact of the disease on Italian ruminant population.

The chosen surveillance approach considered 3 main pillars:

- standardization and re-enforcement of passive surveillance protocols;
- risk-based serological surveillance through VN;
- entomological surveillance in the involved geographical areas.

### Table III. Confirmed cases until May the 2nd 2012.

<table>
<thead>
<tr>
<th>Region</th>
<th>Province</th>
<th>Farm</th>
<th>Longitude</th>
<th>Latitude</th>
<th>Species</th>
<th>Number of positive animals</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veneto</td>
<td>Treviso</td>
<td>Farm #1</td>
<td>12.401932</td>
<td>45.983328</td>
<td>Goat</td>
<td>1</td>
<td>RT-PCR*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Farm #2</td>
<td>12.014148</td>
<td>45.914440</td>
<td>Bovine</td>
<td>1</td>
<td>VN**</td>
</tr>
</tbody>
</table>

*RT-PCR = real-time RT-PCR assays targeting the L1 and S3 genomic fragments; VN = virus neutralization.

### Table IV. Pools of Culicoides collected within the bluetongue surveillance from June the 1st to November the 30th 2011 and tested for SBV. All the positive pools belong to the Obsoletus Complex, including *C. obsoletus* and *C. scoticus*.

<table>
<thead>
<tr>
<th>Region</th>
<th>Province</th>
<th>Farm</th>
<th>Longitude</th>
<th>Latitude</th>
<th>RT-PCR positive / analysed pools (n. midges)</th>
<th>Collection date of positive pools</th>
</tr>
</thead>
<tbody>
<tr>
<td>Friuli Venezia Giulia</td>
<td>Pordenone</td>
<td>Farm #1</td>
<td>12.408059</td>
<td>45.980202</td>
<td>1/16 (45)</td>
<td>04/10/2011</td>
</tr>
<tr>
<td>Friuli Venezia Giulia</td>
<td>Pordenone</td>
<td>Farm #4</td>
<td>12.84439</td>
<td>46.15498</td>
<td>0/42</td>
<td>-</td>
</tr>
<tr>
<td>Friuli Venezia Giulia</td>
<td>Pordenone</td>
<td>Farm #5</td>
<td>12.661484</td>
<td>46.145069</td>
<td>0/23</td>
<td>-</td>
</tr>
<tr>
<td>Veneto</td>
<td>Belluno</td>
<td>Farm #2</td>
<td>11.893233</td>
<td>46.016153</td>
<td>4/34 (1, 28, 42, 47)</td>
<td>06/09/2011</td>
</tr>
<tr>
<td></td>
<td>Pordenone</td>
<td>Farm #3</td>
<td>12.093435</td>
<td>45.712662</td>
<td>1/46 (5)</td>
<td>21/10/2011</td>
</tr>
<tr>
<td></td>
<td>Pordenone</td>
<td>Farm #6</td>
<td>12.113165</td>
<td>45.784651</td>
<td>0/14</td>
<td>25/10/2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>03/11/2011</td>
</tr>
<tr>
<td>Veneto</td>
<td>Treviso</td>
<td>Farm #3</td>
<td>12.093435</td>
<td>45.712662</td>
<td>1/46 (5)</td>
<td>07/11/2011</td>
</tr>
<tr>
<td></td>
<td>Treviso</td>
<td>Farm #6</td>
<td>12.113165</td>
<td>45.784651</td>
<td>0/14</td>
<td>-</td>
</tr>
</tbody>
</table>
Besides the laboratory data, clear definitions of suspected and confirmed cases were developed in accordance with the suggestion of the European Food Safety Authority (8), in order to obtain more accurate temporal and spatial information on the occurrence of SBV infection. The harmonization of case definitions and the collection of standardised epidemiological data at the European level aimed also at assessing the impact of the disease and at providing useful data on the epidemiology of infection.

Under this surveillance approach, the activities carried out over few weeks (from February to April 2012) permitted to verify the existence of SBV active transmission in a relatively limited part of Veneto region.

Although bluetongue virus and SBV are considered vector borne diseases, SBV seems to have a lower pathogenicity but a much greater spreading capacity. Elbers (7) reported about 70% antibody prevalence for SBV in dairy cattle population in the Netherlands; a within-herd serological prevalence between 70% and 95% in sheep flocks; and prevalence between 70% and 100% in dairy herds. The main differences with bluetongue in relation to the surveillance approaches are related to SBV capacity of infecting foetuses and causing malformations in offspring at parturition. In fact, during the vector season serological and virological surveillance activities in hosts or vectors may be performed (10), but passive surveillance on clinical signs should also consider the length of the gestation periods in the different animal species and be concentrated during the Winter and Spring seasons.

Although the epidemiological role of vertical transmission in ruminants is currently not clear, nowadays the birth of viraemic calves or kids cannot be excluded suggesting, therefore, the existence of a possible important overwintering and spreading mechanism, influencing also live animal trade. The recommendations of the World Organisation for Animal Health (17) take into account this hypothesis and suggest precautions in moving live pregnant animals and newborns.

As already described by Goffredo (12), the presence in Italy of a pre-existing surveillance system for bluetongue permitted, retrospectively, to get critical information on SBV infection in Italy. Looking for SBV through the Culicoides collected within the bluetongue surveillance programme allowed for showing that SBV was circulating in at least 3 Italian provinces since early September, nearly 5 months prior the outbreak and at least 40 km away from the first reported case. It was also confirmed that species of the Obsoletus Complex, including C. obsoletus and C. scoticus, play a role in transmitting this virus.

Up to this date Italian Ministry of Health has not activated a wider serological monitoring in other Italian regions and the surveillance on SBV is actually based on the sole notification of suspected clinical cases. This decision is actually hampering the possibility of drawing a more precise picture of SBV distribution in Italy, which remains unknown.
References


