First cases of Schmallenberg virus in Italy: surveillance strategies

Federica Monaco[1], Maria Goffredo[1], Valentina Federici[1], Andrea Carvelli[1], Andrea Capobianco Dondona[1], Andrea Polci[1], Chiara Pinoni[1], Maria Luisa Danzetta[1], Lucia Selli[2], Michela Bonci[2], Michela Quaglia[1] & Paolo Calistri[1*]

[1] *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Campo Boario, 64100 Teramo, Italy*
[2] *Istituto 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*

Veterinaria Italiana 2013, **49** (3), 269-275. doi: 10.12834/VetIt.1101.11
Accepted: 02.09.2013 | Available on line: 24.09.2013

Keywords
Control strategies, Epidemiological analysis, Schmallenberg virus (SBV), Surveillance, Italy.

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.

Primi casi di infezione da virus Schmallenberg in Italia: strategie di sorveglianza

Parole chiave
Analisi epidemiologica, Italia, Sorveglianza, Strategie di controllo, Virus Schmallenberg (SBV).

Riassunto
Lo studio descrive le strategie di soveglianza implementate in seguito al primo rinvenimento in Italia del virus Schmallenberg (SBV) appartenente al genere Orthobunyavirus. Il virus è stato rilevato nel 2012 nel cervello di un feto di capra nato da un parto distocico nel Nord Italia. Il cervello del feto è risultato positivo a due differenti protocolli di real time RT-PCR one-step, gli stessi risultati sono stati confermati anche mediante sequenziamento parziale del genoma virale. A seguito del rilevamento del virus sono state adottate azioni di risposta immediata per impedirne la diffusione. Un'indagine epidemiologica è stata condotta nell'azienda interessata in collaborazione con il CESME, Centro di Referenza Nazionale per lo studio e l'accertamento delle malattie esotiche degli animali (National Reference Centre for the study and verification of Foreign Animal Diseases - Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale"). L'indagine ha permesso di raccogliere e analizzare informazioni di interesse epidemiologico sul gregge e sottoporre ad analisi sierologiche e virologiche i campioni di siero e sangue intero di tutti gli animali presenti nell'azienda. Gli accertamenti sierologici mediante siero-neutralizzazione hanno dato risultati positivi, a differenza dei campioni di sangue intero risultati negativi a SBV. Le indagini epidemiologiche hanno indicato la possibile circolazione del virus nell'area in esame. Le azioni di sorveglianza seguite all'indagine sono state basate principalmente su: standardizzazione e applicazione di protocolli di sorveglianza passiva, sorveglianza sierologica (basata sul rischio mediante l'impiego di siero-neutralizzazione) e sorveglianza entomologica nelle aree geografiche coinvolte. La circolazione locale di SBV è stata, in seguito, confermata dal real time RT-PCR in sei pool di *Culicoides* raccolti, tra settembre e novembre 2011, in tre aziende ubicate nelle vicinanze del focolaio di SBV.

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 on 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.

Veterinaria Italiana 2013, **49** (3), 269-275. doi: 10.12834/Vetlt.1101.11

Figure 1. *Geographical area in which confirmed and not confirmed first cases of SBV occurred.*

After RNA extraction, samples were tested by 2 one-step real-time RT-PCR protocols, developed by the Friedrich Loeffler Institut (FLI) (Insel Riems, Germany), respectively targeting the L1 and S3 genomic fragments (13). Brain tested positive to both protocols, whereas spleen was negative. Both samples were sent to CESME, which confirmed the presence of SBV in the brain by qRT-PCR and partial sequence of the viral genome.

Virus neutralization and sequencing

After the outbreak confirmation, an epidemiological investigation in the farm was immediately carried out in collaboration with CESME's personnel. Epidemiological information on the flock was collected as well as whole blood and serum samples from all animals. The samples were then sent to the CESME's laboratories for serological and virological analyses. Blood was tested by both qRT-PCRs, whereas the detection of the presence of SBV specific antibodies was assessed by virus-neutralization (VN).

Both the strain and the positive control used during this study were kindly supplied by the FLI. Prior to the VN test, SBV was titrated in a cytopathic effect (CPE) $TCID_{50}$ assay using Baby Hamster Kidney 21 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 $TCID_{50}$ units of antigen were added to each dilution. Thereafter, the mixtures were incubated at 37°C for 1h and 10^5 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

Table I. *Definitions of suspected and confirmed SBV cases adopted by the Italian Ministry of Health.*

	Suspected case	Confirmed case
Foetuses and neonates	Arthrogryposis hydranencephaly syndrome (AHS), mummified foetuses, ataxia, paralysed limbs, muscle atrophy, torticollis, brachygnatia, nervous system failure or stillbirths, not attributed to a known cause.	Virus detection or viral genome detection by direct (RT-PCR or virus isolation) or indirect (serological assays) in animal samples or virus detection or viral genome detection (RT-PCR) in midges.
Adult animals	Ruminants with diarrhoea or milk production drop, not attributed to a known cause.	
Midges		

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 radium 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 abuntant (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).

Table II. *Suspected and not confirmed cases notified until May the 2nd 2012.*

Case	Region	Province	Species	Test
1	Veneto	Venezia	Bovine	RT-PCR
2	Veneto	Venezia	Bovine	VN
3	Veneto	Treviso	Bovine	RT-PCR
4	Piemonte	Torino	Bovine	RT-PCR
5	Sardegna	Cagliari	Bovine	RT-PCR
6	Repubblica di San Marino		Bovine	RT-PCR
7	Piemonte	Cuneo	Sheep	RT-PCR
8	Sardegna	Cagliari	Bovine	RT-PCR
9	Sardegna	Cagliari	Sheep	RT-PCR
10	Sardegna	Cagliari	Sheep	RT-PCR
11	Marche	Ascoli Piceno	Goat	RT-PCR
12	Piemonte	Torino	Bovine	RT-PCR
13	Lazio	Frosinone	Water buffalo	RT-PCR
14	Sardegna	Oristano	Sheep	RT-PCR
15	Repubblica di San Marino		Bovine	RT-PCR
16	Repubblica di San Marino		Bovine	VN
17	Repubblica di San Marino		Bovine	VN
18	Repubblica di San Marino		Bovine	VN
19	Repubblica di San Marino		Bovine	VN

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

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.*

Region	Province	Farm	Longitude	Latitude	Species	Number of positive animals	Test
Veneto	Treviso	Farm #1	12.401932	45.983328	Goat	1	RT-PCR*
					Bovine	1	VN**
		Farm #2	12.014148	45.914440	Goat	4	VN
					Bovine	1	VN

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*.

Region	Province	Farm	Longitude	Latitude	RT-PCR positive / analysed pools (n. midges)	Collection date of positive pools
Friuli Venezia Giulia	Pordenone	Farm #1	12.480959	45.980202	1/16 (45)	04/10/2011
Friuli Venezia Giulia	Pordenone	Farm #4	12.84439	46.15498	0/42	-
Friuli Venezia Giulia	Pordenone	Farm #5	12.661484	46.145069	0/23	-
Veneto	Belluno	Farm #2	11.893233	46.016153	4/34 (1, 28, 42, 47)	06/09/2011
						21/10/2011
						25/10/ 2011
						03/11/2011
Veneto	Treviso	Farm #3	12.093435	45.712662	1/46 (5)	7/11/2011
Veneto	Treviso	Farm #6	12.113165	45.784651	0/14	-

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

1. Bilk S., Schulze C., Fischer M., Beer M., Hlinak A. & Hoffmann B. 2012. Organ distribution of Schmallenberg virus RNA in malformed newborns. *Vet Microbiol*, **159**(1-2), 236-238.
2. Calistri P., Giovannini A., Conte A., Nannini D., Santucci U., Patta C., Rolesu S. & Caporale V. 2004. Bluetongue in Italy: Part I. *Vet Ital*, **40**(3), 243-251.
3. Campbell J.A. & Pelham-Clinton E.C. 1960. A taxonomic review of the British species Culicoides Latreille (Diptera, Ceratopogonidae). *Proceedings of the Royal Society of Edinburgh (B)*, **67**(3), 181-302.
4. Colangeli P., Iannetti S., Cerella A., Ippoliti C., Di Lorenzo A., Santucci U., Simonetti P., Calistri P. & Lelli R. 2011. The national information system for the notification of animal diseases in Italy. *Vet Ital*, **47**(3), 303-312.
5. Delécolle J.C. 1985. Nouvelle contibution à l'étude systématique et iconographique des espéces du genre *Culicoides* (Diptera: Ceratopogonidae) du Nord-Est de la France. Thesis, U.E.R sciences, Vie et Terre, Université Louis Pasteur de Strasbourg, 238 p.
6. Dyce A.L. 1969. The recognition of nulliparous and parous *Culicoides* (Diptera: Ceratopogonidae) without dissection. *J Austr Entomol Soc*, **8**(1), 11-15.
7. Elbers A., Loeffen W.L.A., Els S.Q., Quak S., de Boer-Luijtze E., van der Spek A.N., Bouwstra R., Maas R., Spierenburg M.A.H., de Kluijver E.P., van Schaik G. & van der Poel W.H.M. 2012. Seroprevalence of Schmallenberg Virus Antibodies among Dairy Cattle, the Netherlands, Winter 2011-2012. *Emerg Infect Dis*, **18**(7),1065-1071.
8. European Food Safety Authority (EFSA). 2012. "Schmallenberg" virus: analysis of the epidemiological data. Supporting Publications 2012. EN-261. 30 p. (http://www.efsa.europa.eu/it/search/doc/261e.pdf accessed on 06/04/2012).
9. Gibbens N. 2012. Schmallenberg virus: a novel viral disease in northern Europe. *Vet Rec*, **170**(2), 58.
10. Giovannini A., Calistri P., Conte A., Savini L., Nannini D., Patta C., Santucci U. & Caporale V. 2004. Bluetongue virus surveillance in a newly infected area. *Vet Ital*, **40**(3), 188-197.
11. Goffredo M., Conte A. & Meiswinkel R. 2004. Distribution and abundance of *Culicoides imicola*, Obsoletus Complex and Pulicaris Complex (Diptera: Ceratopogonidae) in Italy. *Vet Ital*, **40** (3), 270-273.
12. Goffredo M., Monaco F., Capelli G., Quaglia M., Federici V., Catalani M., Montarsi F., Polci A., Pinoni C., Calistri P. & Savini G. 2013. Schmallenberg virus in Italy: a retrospective survey in *Culicoides* stored during the bluetongue Italian surveillance program. *Prev Med Vet*, **111**(3-4), 230-236.
13. Hoffmann B., Scheuch M., Höper D., Jungblut R., Holsteg M., Schirrmeier H., Eschbaumer M., Goller K.V., Wernike K., Fischer M., Breithaupt A., Mettenleiter T.C. & Beer M. 2012. Novel orthobunyavirus in Cattle, Europe, 2011. *Emerg Infect Dis*, **18**(3) 469-472.
14. Muskens J., Smolenaars A.J., van der Poel W.H., Mars M.H., van Wuijckhuise L., Holzhauer M., van Weering H. & Kock P. 2012. Diarrhea and loss of production on Dutch dairy farms caused by the Schmallenberg virus. *Tijdschr Diergeneeskd*, **137**(2), 112-115.
15. Reed, L.J. & H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am J Hyg*, **2**, 493-497.
16. Van den Brom R., Luttikholt S.J., Lievaart-Peterson K., Peperkamp N.H., Mars M.H., van der Poel W.H. & Vellema P. 2012. Epizootic of ovine congenital malformations associated with Schmallenberg virus infection. *Tijdschr Diergeneeskd*, **137**(2), 106-111.
17. World Organization for Animal Health (Office International des Épizooties: OIE). 2012. Schmallenberg virus. Recommendations endorsed by the OIE Scientific Commission on 16 February 2012. (http://www.oie.int/en/our-scientific-expertise/specific-information-and-recommendations/schmallenberg-virus/ accessed on 16/04/2012).