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Sheep pestivirus in Morocco: sero-epidemiological and molecular study

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ABSTRACT

The present study is the first to investigate Border disease caused by the sheep pestivirus (SPV) in sheep herds in Morocco. Sero-epidemiological investigations were carried out in six regions of the Kingdom, known as important in terms of sheep breeding. A total of 760 blood samples were collected including aborted ewes from 28 randomly selected farms. The samples were analysed, for the determination of anti-pestivirus antibodies, using indirect ELISA technique. Next, reverse transcriptase PCR (RT-PCR) was conducted on serologically negative samples to identify possible persistently infected (PI) animals, through detection of specific RNA fragment. The results revealed an overall SPV seroprevalence in studied areas of 28.9%. The difference in seroprevalence between the six investigated regions was not statistically significant ($p>0.05$) and varied slightly from 20.9% to 37.5%. Furthermore, 93% of investigated farms were affected with an average seroprevalence of 22.7% (with a variation of 1%–74%). RT-PCR results were all negative, indicating the absence of PI animals in the tested samples. Nevertheless, the present study revealed that SPV is endemic in Morocco.

INTRODUCTION

Sheep pestivirus (SPV), also known as Border disease (BD), is a disease of sheep caused by a cosmopolitan pestivirus which is closely related to classical swine fever (CSF) and bovine viral diarrhoea (BVD) viruses. BD was first described in 1959 in the border region of England and Wales.[1–4] BD is recorded in the World Organisation for Animal Health (OIE) list of notifiable animal diseases. It is responsible for serious socio-economic and health consequences along with substantial impact on international trade in animals and animal products.[3]

Knowledge about the clinical aspects of the disease and its epidemiological characteristics is essential to implement control and eradication programme.[5 6] To determine the disease prevalence, several worldwide sero-epidemiological surveys were conducted.[7–10] Overall, results showed average prevalences of 5%–50%.[3] In Morocco, a SPV epizootic has occurred in late 2007. Suspected cases originating from the Tadla region were presented to the Department of Internal Medicine, Surgery and Reproduction of the Hassan II Agronomy and Veterinary Institute. The disease was confirmed through clinical and pathological examinations. Further cases were subsequently confirmed in Oulmes, Gharb, Oriental, Middle Atlas and Fes regions.[11 12] Nonetheless, epidemiological situation in Morocco is still unknown. Epidemiological investigations are thus essential to determine the disease sero-prevalence and to identify principal circulating virus strains. Within this framework, the present study has two objectives: (1) to confirm the BD circulation in Morocco through serological screening based on ELISA tests, (2) to identify persistently infected (PI) animals, the most significant source of disease transmission, through a reverse transcriptase PCR (RT-PCR) analysis carried out on the negative ELISA sera.

MATERIALS AND METHODS

Study area and sampling

The present work involves six Moroccan areas covering the northern half of the country, including the Gharb-Chrarda-Benihssen, Rabat-Sale-Zemmour-Zaër, Doukkala-Abda, Meknes-Tafilalet, Marakech-Tansift-Alhaouz and Oriental (figure 1). The survey was conducted in 28 randomly selected sheep farms, where blood samples (n=760) were collected from jugular vein of randomly selected sheep and aborted ewes. The sample size within each farm varied from 5.2% to 41.3% (an average of 11.8%). The majority of the studied farms belonged to semi-intensive and intensive production system of sheep and goat, with the presence of cattle in some of the sampled farms. The herds' size varied from 70 to 800 individuals. Males and females of different ages were reared in the same sheepfold, which is often not adapted for good husbandry conditions (dirty litter, ammonia odour, etc).

Samples pre-processing

From each farm, an average of 27 blood samples was collected in Ethylenediaminetetraacetic

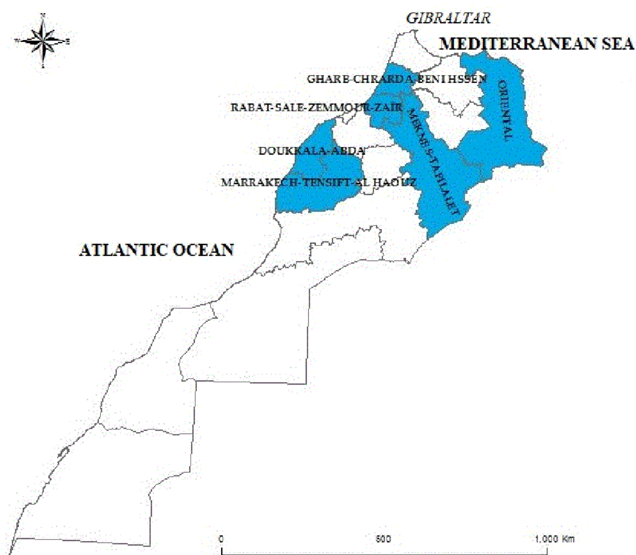

Figure 1 Repartition of sampled areas in Morocco.

acid (EDTA) tubes. After centrifugation and plasma collection, samples were stored at +4°C or at −20°C, for, respectively, samples destined to serology and to RT-PCR. The samples were collected by a qualified veterinarian. Restraint and handling of animals were carried out by the breeder or another person with whom animals are used to. The owner was always advised of the aims and the type of analyses to be done.

Assays

Analyses undertaken on collected plasma samples were:

- Blocking ELISA to detect antiviral antibodies using the SERELISA BVD p80 Ab Mono Blocking test kit, following the manufacturer's instructions.
- RT-PCR to detect viral gene in negative ELISA samples aiming to differentiate between PI animals and genuine non-exposed animals. This test was conducted on pools of five samples (giving a total of 102 pools). In case of positive results, individual test was planned to be carried out. The technical has taken place as follows:

Viral RNA extraction

200 µL volume of plasma was extracted with 300 µL of Trizol (Invitrogen). The solution was subjected to vortexes for 30 s every 2 min during 10 min. The solution was submitted to centrifugation at 12 000×g for 12 min at 4°C and the supernatant was removed and extracted with 200 µL volume of chloroform. Following a 5 min of incubation at room temperature, the supernatant was collected by centrifugation at 12 000×g for 15 min. Viral RNA in the supernatant was precipitated with an equal volume of isopropanol and collected using centrifugation at 12 000×g for 15 min at 4°C. The RNA pellet was rinsed once adding 700 µL with 75% ethanol and suspended in 60 µL of diethylpyrocarbonate until used.

Real-time RT-PCR

A one-step RT-PCR was carried out using ADIAVET BVDV REAL TIME kit (Reference: ADI105-100). The reaction volume contained 12.5 µL 2×RT-PCR buffer mix, 0.5 µL Rox (25 mM), 5.5 µL nuclease-free water, 0.25 µL E3 reverse transcriptase enzyme (200 U), 1 µL primers to a final concentration of 10 µM, 0.5 µL probe to a final concentration of 10 µM and 2 µL RNA template. The reaction was carried out in Applied Biosystem 7500 Real-Time PCR system at 95°C for 10–15 min for the initialisation step, and 20–35 cycles of 94°C–98°C for 20–30 s for the denaturation step, 50°C–60°C for 2–60 s for the hybridisation step and 72°C for 4–120 s for the elongation step. Amplifications were recorded, analysed, and the threshold cycle (Ct) determined with the Applied Biosystem software.

RESULTS

SPV seroprevalence in the study areas

Obtained seroprevalence values are shown in table 1. The overall SPV seroprevalence in the studied areas was 28.9% (95% CI: 22.0% to 35.8%). The difference in seroprevalence between the six investigated regions was not statistically significant (χ^2=7.76; p>0.05), and it varied slightly from 20.9% to 37.5%, respectively. The lowest seroprevalence was observed in the region of Doukkala-Abda, and the highest was obtained in the region of Gharb-Chrarda-Benihssen.

SPV seroprevalence within flocks

SPV seroprevalence results in the 28 sampled sheep flocks revealed an average seroprevalence of 22.7%.

Table 1 Sheep pestivirus seroprevalence in the study area

Areas	Number of farms	Livestock inventories averages	Number of tested sheep (sampling %)	Number of positive sheep	Seroprevalence (%)
Rabat-Sale-Zemmour-Zaër	5	126	126 (15.4%)	33	26.2
Gharb-Chrarda-Benihssen	4	112	112 (11.9%)	42	37.5
Doukkala-Abda	5	182	182 (10.2%)	38	20.9
Meknes-Tafilalet	4	127	127 (9.3%)	43	33.9
Marakech-Tansift-Alhaouz	3	130	130 (11.5%)	42	32.3
Oriental	7	118	83 (16.4)	19	22.9
Total			760	217	28.9±6.9 (95% CI)

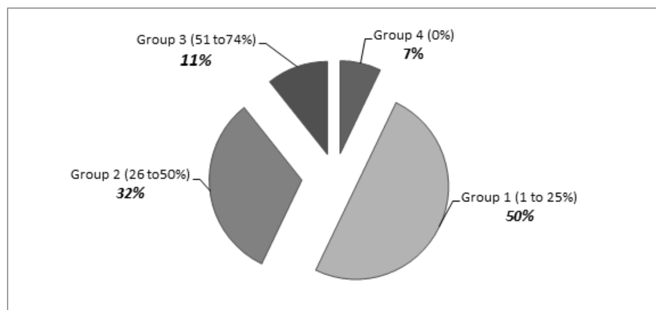

Figure 2 Distribution of farms' groups according to their sheep pestivirus seropositivity.

To more elucidate these results, surveyed farms were divided in four groups according to their obtained seroprevalence (figure 2). The results showed that 50% of the farms were holding seropositive animals with prevalences of 1%–25% (Group 1), 32% were holding moderately seropositive animals (26%–50%) (Group 2), 11% were holding strongly seropositive animals (51%–74%) (Group 3). However, only 7% of the farms were not holding any seropositive animals (Group 4).

RT-PCR prevalence

To detect the possible existence of PI animals among those revealed negative by ELISA test, all the seronegative samples were tested by RT-PCR. The results showed that they were all negative; thus, the presence of the viral genome could not be detected.

To ensure that there is no dilution effect that could have masked the presence of the viral genome in the analysed samples, two further confirmatory tests were performed:

- Among the 102 pools that were mentioned above, 150 samples of 30 pools were individually retested by RT-PCR.
- The same samples were tested by direct ELISA (using SERELISA BVD p80 Ag Mono Indirect (ELISA) test kit) to detect the possible presence of SPV antigens.

Negative results, in the both cases, have confirmed RT-PCR initial results.

DISCUSSION

Regarding its contagiousness, socio-economic and sanitary consequences alongside the important impact on international trade in livestock and animal products, BD is recorded in the OIE list of notifiable animal diseases.[3]

In Morocco, considering the outbreak declared in the Tadla region in 2007[11] and the subsequent cases detected in the regions of Oulmes, Gharb, Oriental, Middle Atlas and Fes,[11 12] the present study is the SPV first systematical investigation in Moroccan sheep population. Sero-epidemiological and molecular studies were conducted in 760 sheep belonging to 28 farms across different regions of the kingdom.

The results confirmed the above-mentioned studies and revealed the presence of the disease in Morocco with national average seroprevalence of 28.9% with no statistically significant difference in seroprevalence between the six investigated regions. In fact, 93% of sampled farms were affected with a seroprevalence of 1%–75%. These findings are in line with those cited in the OIE Terrestrial Manual, reporting that the SPV seroprevalences vary moderately from 5% to 50% depending on countries and areas of the same country.[3]

The present study also suggested that the SPV is moderately endemic in Morocco and the recorded seroprevalence was almost similar to that observed in Austria (29.4%),[13] but lower than that reported in some other countries such as Netherlands (45%),[14] Turkey (63.6%, 78.5% and 60.0%–82.5%)[9 15 16] and the UK (90%).[17 18] On the other side, the current Moroccan seroprevalence is higher than that recorded in France, Spain and Canada accounting for 6%–22%,[1] 17.6%[10] and 43%,[7] respectively.

The differences in seroprevalences through endemic countries and regions are probably due to the farming system. In fact, risk transmission of SPV is higher in intensive farming system which would facilitate the virus rapid spread among sheep herds given the close contact between animals. Otherwise, several studies reported that the disease may be caused in sheep by bovine viral diarrhoea virus (BVDV), transmitted from PI cattle to sheep which will present similar symptoms of BD.[17 19–24] This actually occurs frequently and there are even countries where BD is caused exclusively by the BVDV.[25] Moreover, some studies[26 27] even suggested that the classical host-species-based terminology of pestiviruses is confusing and should be reviewed. Since these viruses are not strictly host-specific. Thus, they proposed that the four pestivirus genotypes would be described in a 'non-speciesist' manner, that is, BVDV-I as PEST-1, CSFV as PEST-2, BDV as PEST-3 and BVDV-II as PEST-4. Cohabitation between species may thus lead to bovine virus transmission to sheep and vice-versa. Investigated regions in the present

Table 2 Comparison between SPV seroprevalences revealed in this study and those of BVD recorded in the same regions by other authors

	Seroprevalences (%)		
Regions	SPV (present study)	BVD (Aiyar, 2006)[28]	BVD (El Ghourdaf, 2013)[29]
Doukkala-Abda	20.9	59.4	45.7
Gharb-Chrarda-Benihssen	37.5	48.8	45.2
Oriental	22.9	50.2	16.7

BVD, bovine viral diarrhoea; SPV, sheep pestivirus.

study are mostly based on semi-intensive and intensive production system, where cohabitation between cattle and sheep was recorded. Furthermore, the ELISA kit (SYNBIOTICS SERELISABVD p80, Ab Monoblocking and Ag Mono Indirect) used is not specific since it does not allow distinction between BDV and BVDV. These three factors may explain the relatively high SPV seroprevalence levels observed in this study, especially if we consider the high BVD prevalence described in Morocco.[28 29] Actually, several BVD sero-epidemiological studies were conducted in Morocco, table 2 summarises obtained seroprevalences in the same explored regions as our study.

Table 2 shows that the prevalence of BVD in Morocco is significantly higher than the obtained SPV prevalence in our study. This finding supports our previously mentioned deduction regarding the possible BVDV transmission, in Moroccan context, from cattle to sheep; insofar as reported that cattle are less sensitive, even though receptive, to the infection by BDV. Infected animals usually have no symptoms and do not transmit the virus.[25]So an eventual BDV sheep infection could barely have a bovine origin.

Indeed, infection transmission from cattle to sheep has been described in several studies. Giangaspero *et al*[30] detected in 41.5% of sheep suffering from BD, the BVDV-1 or BVDV-2 strains. Another research regarding 42 SPV collected during 18 years in Great Britain, Sweden and New Zeeland, confirmed the co-circulation of BVDV-1 and BVDV-2 in sampled sheep herds.[27] Moreover, BVDV-1 genotype was detected in sheep in Germany, Sweden and Norway,[27] in the UK,[31] in Italy[32] and in the USA.[33 34] BVDV-2 isolates from sheep are less frequent; this genotype was detected in USA,[34] Italy[30 32] and India.[23]

On another note, pestiviruses, as reported in many studies, could be frequent contaminants of modified live virus vaccines produced on pestivirus primary infected ovine and bovine cells and may thus play a role in the maintenance and dissemination of these viruses in a given region.[35 36] This had been the case with outbreaks of BD in herds of goats, vaccinated with an experimental Orf vaccine, in Norway,[37] and following a vaccination campaign against sheep pox in Tunisia in 1995.[35 38] This leads to suggest a possible sheep pox vaccine contamination in Morocco, which perhaps would have been prepared on pestiviruses contaminated cells, as the BD outbreak observed in the country, occurred after the 2007 national vaccination campaign against sheep pox.

On the other hand, RT-PCR and ELISA-Ag results reporting the genome and the antigen absence in our study's samples could be explained by partial or total virus protein destruction during samples handling, shipment or storage. These findings could also be attributed to the early death of PI animals, they are thus rarely encountered. These results are in line with previous studies conducted in different countries such as Tunisia, Canada, Tanzania,[38] which also failed to detect the viral genome and the PI animals in herds even if they succeeded in detecting virus antibodies. However, studies undertaken in Spain revealed the PI animals presence with prevalences of 0.5%–0.7%[39] and 0.3%–0.6%.[31]

CONCLUSION

It appears from this first viro-serological study of the SPV in different regions of Morocco, despite its failure in detecting PI individuals, that the SPV is endemic in the country. Furthermore, this study suggests that in the Moroccan context, there are high chances of BVDV transmission to sheep, and may be confused as sheep BD; since in this species the two diseases have the similar symptoms, BVDV circulates with high prevalences in the country and as the diagnostic kit used cannot differentiate between them.

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