

Experimental model of Border Disease Virus infection in lambs: comparative pathogenicity of *pestiviruses* isolated in France and Tunisia

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Abstract – *Pestiviruses* have been isolated from live sheep pox Tunisian vaccines. Vaccination with these vaccines caused outbreaks of Border Disease in Tunisia. In order to study more precisely the pathogenicity of these isolates, three groups of eight four month old lambs from a *pestivirus*-free flock were infected by the intratracheal route with a French strain (AV) and two Tunisian isolates (SN3G and Lot21). Clinical, hematological, immunological and virological parameters were evaluated. The three groups developed mild fever and leucopaenia by day 3 to 6 post infection (pi). The differences in the weight curves were not significant. Viruses were isolated from the peripheral blood buffy coat cells by day 4 to 9 pi. Antibodies were present on day 16 pi following infection by the French strain and on day 21 pi with the Tunisian isolates. The results demonstrated that SN3G and Lot21 are almost similar to the French strain used as the reference strain. In field conditions, they could induce economical losses in naive flocks, alone or in association with other pathogens.

***Pestivirus* / ovine / sheep pox vaccine / pathogenicity**

Résumé – **Mise au point d'un modèle expérimental d'infection par un virus de la Border Disease chez les petits ruminants et étude comparée du pouvoir pathogène de deux isolats tunisiens et d'une souche française.** Des *pestivirus* ont été isolés à partir de vaccins vivants anti-clavelée tunisiens. L'utilisation de ces vaccins a causé des foyers de Border Disease en Tunisie. Afin d'étudier plus précisément le pouvoir pathogène de ces isolats, 3 groupes de

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8 agneaux de 4 mois issus d'un troupeau indemne de *pestivirus* ont été infectés par voie intratrachéale avec une souche française de référence (AV) et 2 isolats tunisiens (SN3G et Lot21). Les données cliniques, hématologiques, immunologiques et virologiques ont été suivies. Les 3 groupes ont développé une légère hyperthermie et une leucopénie entre le 3^e et le 6^e jour post inoculation (pi). Les courbes de poids ne montraient pas de différence significative. Des *pestivirus* ont été isolés à partir des cellules blanches du sang périphérique entre le 4^e et le 9^e jour pi. Des anticorps ont été mis en évidence 16 jours pi avec la souche française et 21 jours pi avec les isolats tunisiens. L'ensemble des résultats a montré que les 2 isolats tunisiens ont un pouvoir pathogène presque similaire à celui de la souche française; ils pourraient provoquer, dans les conditions du terrain, des pertes économiques, seuls ou en association avec d'autres agents pathogènes.

***Pestivirus* / ovin / vaccins anti-clavelée / pouvoir pathogène**

1. INTRODUCTION

Border Disease (BD) is a transmissible congenital disease caused by a *pestivirus*, which affects sheep and occasionally goats [15, 21]. Clinically, the disease is characterized in sheep by abortion, stillbirth and the birth of weak lambs with nervous disorders [1, 17, 22, 27]. The Border Disease Virus (BDV) induces, under some conditions, a severe hemorrhagic syndrome, which has some features analogous to the experimentally produced mucosal disease-like syndrome. This hemorrhagic disease was reported in 1983 in the Aveyron department in France [7, 8, 21].

Border Disease Virus belongs to the genus *Pestivirus*, which also includes bovine viral diarrhoea virus (BVDV) and classical swine fever virus (CSFV). The genera *Pestivirus*, *Flavivirus* and the *hepatitis C* virus are included in the family *Flaviviridae*. Two biotypes of BDV are distinguished by their effects in cell cultures: the cytopathic biotype (cp) and the non cytopathic one (ncp). Most of the field isolates of BDV are non cytopathic whereas some isolates include the two virus biotypes [18].

Pestiviruses are enveloped particles, spherical in shape approximately 40–60 nm in diameter. The genome is a positive single-

stranded RNA molecule with a length of 12.3 kb which encodes a polyprotein of about 4000 amino acids [2]. Many advances have been realized in the molecular structure of BDV [20, 32], and genetic diversity of *pestiviruses* and the relationship between isolates have been extensively studied [3, 10, 18].

Pestiviruses are frequent contaminants of modified live virus vaccines produced on ovine cells [26, 29, 31]. In 1995, sheep pox vaccination of Tunisian state flocks were followed by several Border Disease outbreaks characterized by high rates of abortion (15 to 80%), still births, weak and hairy-shaker lambs. Whereas no obvious clinical signs were seen in lambs or adult sheep after vaccination, abortions occurred in ewes 7 to 10 days after vaccination in the last third of gestation. The contaminant was likely of Tunisian origin since the vaccine had been locally produced on cells from Tunisian lamb kidneys. Out of 14 sheep pox vaccine batches used during this vaccination campaign, 11 have been found to be contaminated with *Pestiviruses* [23]. The aim of this study was to evaluate the pathogenicity of two of these Tunisian isolates by comparison to a French *Pestivirus* strain (AV). Following experimental inoculation to young lambs, both Tunisian isolates were revealed to be as pathogenic as the French strain of BDV.

2. MATERIALS AND METHODS

2.1. Lambs and experimental design

Twenty-eight Préalpes × Merino lambs originated from the flock reared in the experimental station of AFSSA-Sophia Antipolis. This flock was free of the main sheep diseases: abortive diseases and Maedi-Visna as demonstrated by a regular negative serology. All lambs were tested negative to BD virus (see Sect. 2.4) and sero-antibodies (see Sect. 2.5). Twenty-eight four-month old lambs were randomly distributed into three experimental groups ($n = 8$) and one control group ($n = 4$); all of the four groups were housed in separate pens in security isolation facilities. After one week of adaptation, lambs of the experimental groups were inoculated with the French AV strain and the Tunisian SN3G or Lot21 isolates respectively for each group. Every lamb received 10^6 TCID₅₀ (5 mL) of BDV by the intratracheal route. Lambs of the control group were inoculated with 5 mL of BDV-free cell culture.

2.2. Viral strains

The AV strain (kindly provided by Dr G. Chappuis, Merial-France) was isolated from sheep during a haemorrhagic syndrome, also called syndrome "X" or petega ovina that occurred in 1983 in the Aveyron department. This disease killed 1 500 ewes and 24 000 lambs in 1984 [7]. This isolate has been shown to produce profound leucopaenia and death in 50% of 3 to 5 month-old lambs [8]. The SN3G and Lot21 strains were isolated from two batches of live sheep pox Tunisian vaccines produced on Tunisian sheep kidney cells [23]. All viruses were produced on permissive cell lines (ETM52) established in our laboratory and derived from a whole sheep embryo. Briefly, viruses were inoculated at a m.o.i. of 1 on a confluent monolayer. These strains are not cytopathic, thus cultures were

frozen after 5 to 7 days of incubation. Before inoculation, one flask of each strain was thawed and titrated on sterile 96-well microplates by the immuno-fluorescence method (see below). The original stock of the AV strain was passaged four times on permissive IRO4 cells (Merial) before its introduction in our laboratory. To ensure the best reproducibility between experiments, the three viruses were submitted to the same treatment since they were cloned by limit dilution; the AV and the two Tunisian strains were used respectively after 5 and 10 passages on ETM52.

2.3. Clinical examination and sample collection

Behaviour and clinical signs of infected and naive animals were observed at regular intervals before and every day after inoculation.

Rectal temperature was taken daily for 3 days before exposure and until 11-day pi, then on days 13, 16 and 22 pi. Body weight was measured and blood samples were taken every week. Serum samples were stored at a temperature ≤ -20 °C until the end of the experiment for titrating all sera in a single round. Additionally, for numeration of white blood cells and viremia, whole blood was sampled into tubes with heparin daily from day 0 to day 11 pi, then every second day.

Necropsies were performed as follows: one lamb per infected group at day 7 pi, two lambs per group and one control lamb at day 9 pi; two lambs per group at day 21 pi and three lambs per group at day 28. Virus isolation attempts were performed from pooled tissues: lung, spleen, mesenteric lymph nodes and small intestine.

2.4. Virological examination

After collection, each fresh blood sample was diluted with an equal volume of

sterile ammonium chloride (8.7 g/L of distilled water). After incubation at room temperature for 10 to 15 minutes, the mixture was centrifuged at $800 \times g$ for 10 min. The pellet was washed twice with Eagle medium (HMEM Stabilix Biomedica-France), suspended in 1 mL and stored at -85°C . All samples were tested at the end of the experiment on a permissive ETM52 cell line on sterile 24-well microplates. Three or four days later, the microplates were freeze-thawed one time and a second passage was performed in 96-well microplates. After three or four days of incubation, the cells were fixed and examined by the immunofluorescence method.

Briefly the cells were fixed with cold acetone-water (85%–15%) at $+4^\circ\text{C}$ for 30 min. A monoclonal antibody anti-pestivirus (Monoclonal Ab Polypesti, Synbiotics Europe, Lyon, France) was incubated on monolayers for 30 min at 37°C . After three washes in phosphate buffer saline pH 7.2 (PBS), an antimouse IgG (H+L) FITC conjugated (PARIS, Compiègne, France) was added and incubated for 30 min at 37°C . After three washes in PBS and one wash in distilled water, the microplate was immediately examined under a Carl Zeiss Axioskop 2 by epifluorescence.

2.5. Serological tests

The serological examination was performed using a commercial enzyme linked immunosorbent assay kit (ELISA BVD/MD P80 Institut Pourquier, Montpellier, France) according to the procedure described by the manufacturer and by a micro-seroneutralisation test against the cytopathic BPII strain [30] of *Pestivirus* (Moredun Institute, Edinburgh UK). A fixed dose of virus per well (100 TCID₅₀ of BPII) was incubated for 1 h at 37°C with four-fold serum dilutions in growth medium. Cells (ETM52) were added and the cultures were incubated in microtitre plates for 4 to 5 days at 37°C in a CO₂ incubator.

Growth or neutralisation of the cytopathic viruses at the end of the incubation was determined by microscopic examination of the cytopathic effects. Seroneutralising titres were determined by the Karber method and were considered significant when above 0.9 (Log₁₀ of dilution).

2.6. Statistical analysis

Differences between the groups of lambs were analysed by ANOVA; the paired t-test was used. A *P* value of less than 0.05 was considered significant. Differences over time were tested by a repeated measure analysis of variance using an ANOVA repeated measure (Statview®, Abacus Concepts, Berkeley, USA).

3. RESULTS

3.1. Clinical observations

All lambs remained clinically normal during the adaptation period before inoculation. No obvious clinical symptoms were observed in the control or the experimentally infected lambs after the adaptation period. Daily temperatures are described in Figure 1. There were day to day variations in rectal temperatures in the control group and BDV-infected lambs. However, the daily variation in the control group was not significant as individual temperatures remained within the 95% confidence interval of temperatures measured during the observation period. In contrast, experimental infection with the three viruses induced a significant increase in body temperature from day 4 to day 9 pi for all lambs of the AV group and from day 6 to day 9 pi for 6 lambs out of 8 for the SN3G and Lot21 groups. Lambs of the AV group experienced the longest period with high temperatures compared to the other infected groups. The temperatures of inoculated groups were significantly higher than those

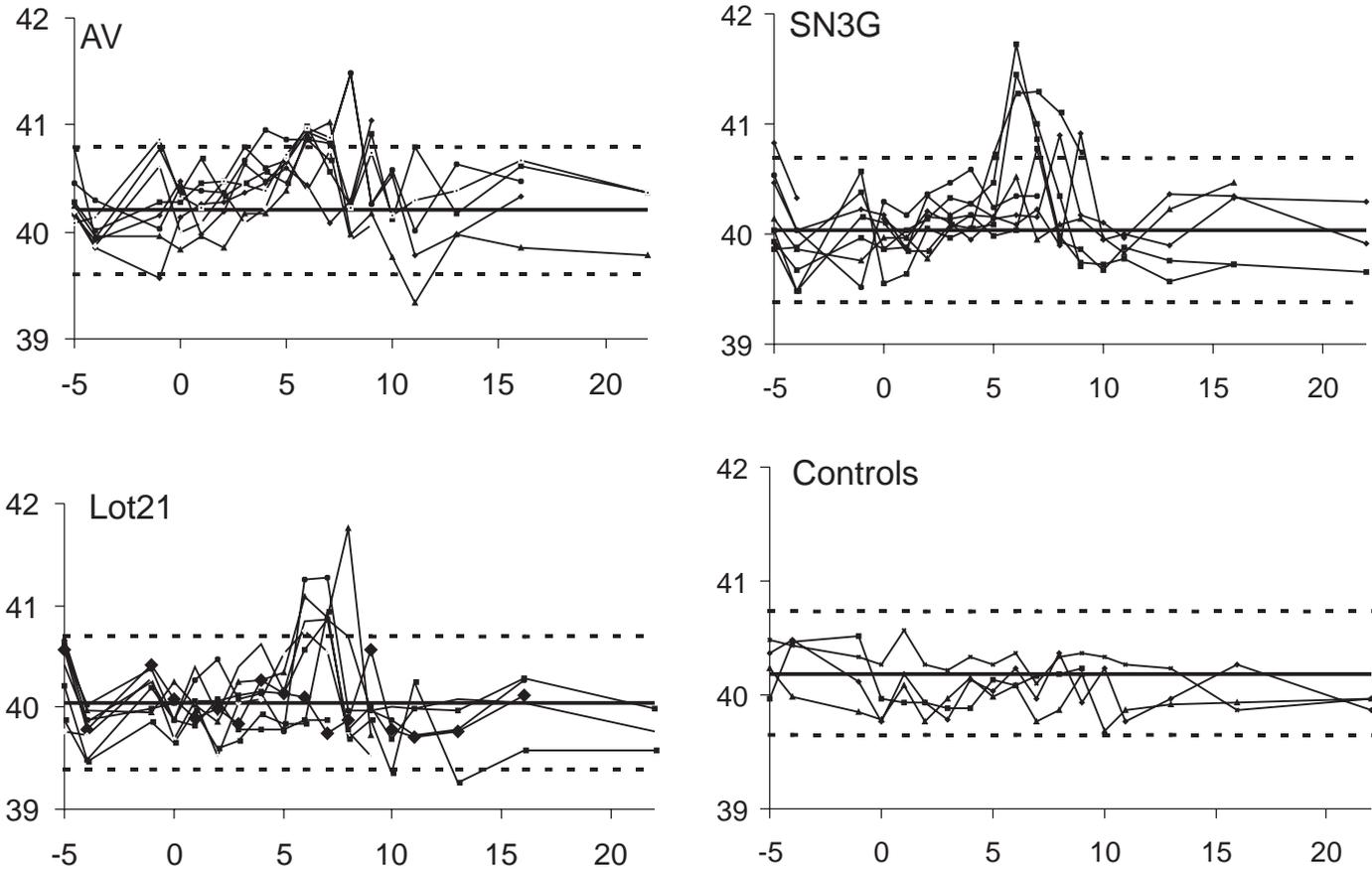


Figure 1. Individual body temperatures of lambs before and following inoculation for each experimental group. The bold line represents the mean individual temperature recorded during the observation period (from D_{-5} to D_0) and the dusted lines encompass the confidence interval (95%) of this mean temperature.

of the control group from day 2 to day 7 pi for the AV group ($p = 0.04, 0.0006, 0.02, 0.0007, 0.05, \text{ and } 0.01$ respectively) and for the SN3G and Lot21 groups at day 7 pi ($p = 0.03$ or 0.02).

The weight curves (data not shown) shown no significant differences between infected and control lambs.

3.2. Haematological parameters (Fig. 2)

Experimental infection with the three pestivirus strains resulted in a significant decrease in the total number of leucocytes, from day 2 pi to day 6 pi.

For lambs infected with the AV strain, the total leucocyte counts were significantly reduced as early as two days pi reaching the lowest value of 3 500 cells/mm³ at day 5 pi, from an initial value of 9000 cells/mm³. This decrease was statistically significant from day 2 to day 6 pi ($0.002 \leq p \leq 0.01$). The AV group showed a second significant decrease in leucocyte counts at day 10 pi ($p = 0.01$) and the counts returned to pre-inoculation values on day 13 pi.

The duration of leucopaenia was longer in the AV group than in SN3G and Lot21 groups that showed a significant decrease only at day 2 pi ($p = 0.01$ or $p = 0.04$) in comparison to the initial values before inoculation. There was also a significant difference between the AV group and the two Tunisian groups at day 5 and 6 pi ($p < 0.0001$), and also between the control group and each one of the infected groups at the same period ($p = 0.004$).

3.3. Gross lesions (Tab. I)

The necropsy of one lamb per group at day 7 pi, showed multifocal areas of pneumonia of the apical lobes of both lungs. Lambs from the AV group had the more severe lesions. Very mild intestinal lesions (petechiae, hypertrophy of mesenteric lymph nodes) were observed in the lambs of the AV and Lot21 groups.

The necropsy of 2 lambs per group and one control lamb at day 9 pi revealed a partial cicatrization of pneumonia in apical lobes in AV and SN3G groups, but no healing in the

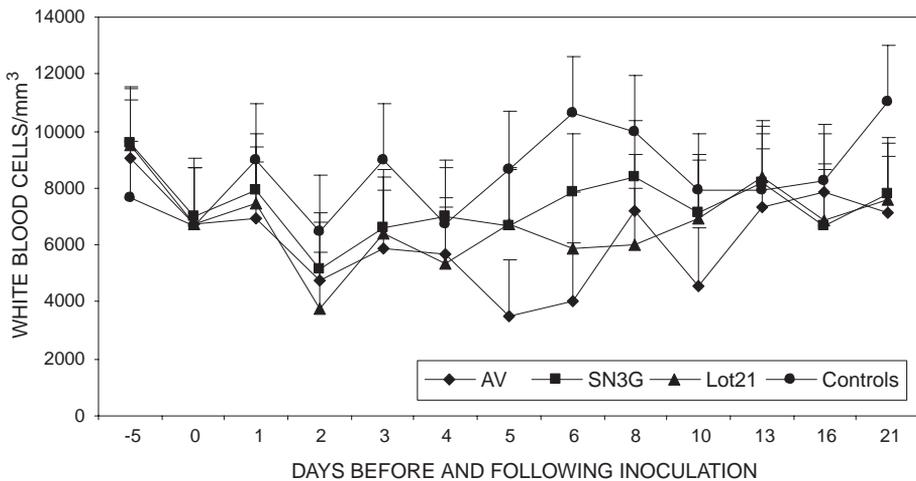


Figure 2. Mean number of leukocyte cells in peripheral blood of control and experimentally-infected lambs with three different strains of Border Disease Virus.

Table I. Pathological findings in organs after necropsy of lambs experimentally infected by the intratracheal route with 10^6 TCID₅₀ of three different strains of the Border Disease Virus.

Strains	Organs	D7	D9	D21	D28
AV	Lungs	+++ ^a	Slight cicatrisation	Total cicatrisation	Total cicatrisation
	Intestine	+/-	+/-	-	-
SN3G	Lungs	++	Slight cicatrisation	Total cicatrisation	Total cicatrisation
	Intestine	-	+/-	-	-
Lot21	Lungs	++	++	Total cicatrisation	Total cicatrisation
	Intestine	+/-	+	-	-

^a Grading of lesions: +/- slight lesions; ++ important lesions; +++ severe lesions.

Table II. Immunofluorescence detection of the *pestivirus* in white blood cells from lambs infected by the intratracheal route with 10^6 TCID₅₀ of three different strains of the Border Disease Virus.

Strains	Days following inoculation						
	D0	D4	D5	D6	D8	D9	D10
AV	0/8 ^a	2/8	7/8	7/8	4/7	1/7	0/5
SN3G	0/8	0/8	0/8	3/8	2/7	0/7	0/5
Lot21	0/8	0/8	4/8	6/8	2/7	0/7	0/5
Controls	0/4	0/4	0/4	0/4	0/4	0/4	0/3

^a Number of positive lambs/Total number under test.

Lot21 group. Slight intestinal lesions were noted in the SN3G group.

The necropsy at day 21 pi showed a total recovery of pulmonary lesions in all infected lambs. No lesions were ever observed in the control lambs.

3.4. Virus distribution (Tab. II)

BD viruses were detected in leucocytes from day 4 pi to day 9 pi in the AV group. In this group, 7 lambs out of 8 were positive at days 5 and 6 pi. The SN3G and the Lot21 isolates were detected less frequently in white blood cells: from day 6 to 8 pi and from day 5 to 8 pi respectively. The virus was also identified from lungs in the AV group at day 7 pi and at day 9 pi with a weak

fluorescence; in contrast, no fluorescence was detected in pooled tissues from SN3G and Lot21 groups and from control lambs.

3.5. Antibody response (Fig. 3)

All infected groups showed a significant neutralising antibody response starting at day 16 pi for the AV group, and at day 21 for the SN3G and Lot21 groups. Maximum titres ranging between 1.7 and 1.8 were reached at day 28 pi for all groups. In contrast, the ELISA test detected significant antibody titres only from day 28 pi. No BDV-specific antibodies were detected in control lambs.

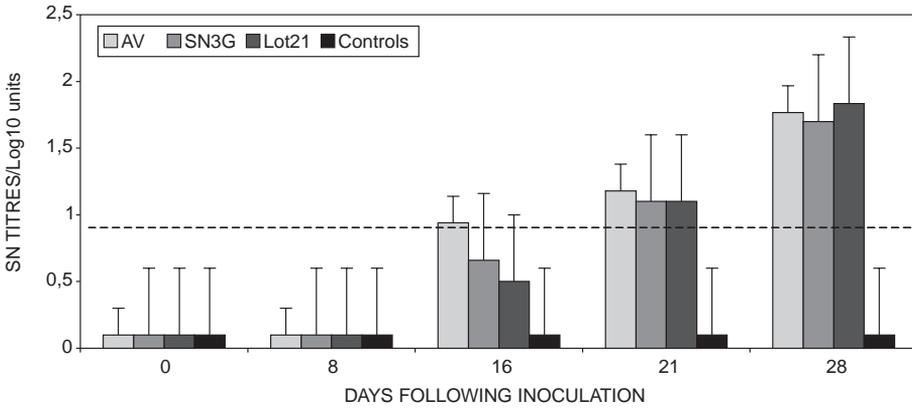


Figure 3. Kinetics of BDV-specific neutralising antibody titres of three groups of lambs inoculated by three different strains of BDV. The discontinuous line represents the significant seroneutralising titre (above 0.9 Log₁₀ of dilution).

4. DISCUSSION

There are many studies on the experimental reproduction of the Border Disease in cattle, sheep and pigs [4, 5, 11, 14, 16, 25, 28] with BVDV; there is, however, less information in the literature on the events associated with a postnatal infection of lambs with ovine *pestiviruses* [12, 13, 19].

The present results obtained by experimental infection of lambs with one French or two Tunisian isolates of the Border Disease virus allow to conclude that the experimental model we developed allows: (a) to reproduce with all of the three strains, three major symptoms of the disease: increase in body temperature, leucopaenia, and specific seroconversion, and (b) to distinguish the virus strains on the basis of frequency, delay and duration of these symptoms in the inoculated groups of lambs. Indeed, compared to both Tunisian strains, the French AV strain more frequently induced all the above mentioned symptoms. With this strain, the period with high temperature was earlier, and lasted longer. Similarly, leucopaenia was more frequent and severe, the virus was more frequently isolated and

for a longer period. In this respect, the differences in pathogenicity of the French and Tunisian strains are in accordance with their different geographical origins.

The experimental model of infection described here has been improved compared to the preliminary trial we performed with the same three virus strains on small groups of 9 month old lambs and three pregnant ewes (data not shown). Following inoculation with 10⁵ TCID₅₀ only of *pestivirus*, lambs developed a very slight leucopaenia and no increase in body temperature. Virus isolation in white blood cells was only obtained at day 6 pi with one virus strain (AV) and no viruses were isolated in the organs of any lamb whatever the BDV strain used for inoculation. The three ewes aborted. By keeping the same route of inoculation and using a higher titre (10⁶ TCID₅₀) and younger lambs (4 months instead of 9 months), we obtained a significant panel of clinical, hematological or virological observations.

On the contrary to the results obtained by Chappuis et al. [7, 8], the infection model we developed did not allow to reproduce the more severe symptoms that were observed in the field with the AV strains:

hemorrhagic symptoms, distress and death. Several reasons may explain these discrepancies. Chappuis et al. [8] used a different race of lamb that could be more sensitive than the "Préalpes × Merino" we used. Our protocol may not have appropriately reproduced the conditions that prevailed during the natural outbreaks: the zootechnical and environmental conditions. The breed and the health status of lambs as well as the coinfection with other pathogens has been shown to enhance the severity of BDV infection [24]. Other reasons may be related to the passage histories and the culture conditions of viruses. Intra-strain comparisons of BDV from different laboratories showed that strains known under the same name and having varying passages, can be antigenically different [6]. On the contrary, it has been shown that different virus clones prepared from the same stock can have a widely different pathogenicity. The use of crude contaminated vaccines does not exclude the possible presence of other agents, principally other viruses which could have altered the pathogenicity of the different inocula. We selected the conventional virological approach of cloning our virus stocks at limiting dilution to exclude the possibility of other contaminants, even though this increased the risk of virus attenuation by selecting the virus growing to the highest titre in cell culture. We felt this virus stock prepared according to a similar protocol allowed the most significant comparison of BD viruses from the two countries, and would ensure a good reproducibility of experiments. As a result, the cloned stocks may not have been completely representative of the field virus populations from which they were derived: this may be the case with the AV strain whose severe symptoms observed in the field [7, 8] were not reproduced in our experiment while the two Tunisian isolates tested in this experiment did not provoke more severe clinical signs than those observed in the field. Indeed, the BD outbreaks that followed the use of the sheep pox vaccines in Tunisia were not as

severe as the "petega ovina" experienced in France.

In Tunisia very little is known about the Border Disease and the local virus strains. The presence of BDV infection was first revealed by a clinical report and serological studies conducted in 1991 but with no virus isolation [33]. A serological survey carried out in 1998 in the country that compared the incidence of abortive diseases in small ruminants showed that BD was the most frequent (95% of the sampled flocks) [9].

Following the BD outbreaks that were directly related to the use of contaminated sheep pox vaccine batches [23], 11 BD virus isolates from vaccine batches became available. The first origin of our Tunisian isolates will probably never be ascertained considering that it was impossible to exclude the possibility that the vaccine had been contaminated by the bovine sera used for cell cultivation or by the sheep poxvirus seed. Anyhow since the contaminated vaccine batches have been used on Tunisian sheep, the viruses isolated from the vaccines should be now circulating in Tunisia. Additionally one local strain of BDV has been isolated from one Tunisian sheep (F. Thabti, unpublished data). Genetic and antigenic studies will be carried out in order to determine the relationship between this Tunisian field isolate and the sheep pox vaccine derived *pestivirus*. More BD virus isolates are expected from current field studies in Tunisia. Their pathogenicity will be compared to reference strains of BDV, which could indicate if local strains should be included in a candidate *pestivirus* vaccine that would be more adapted to the Tunisian epidemiological context.

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