First detection and genetic characterization of bovine viral diarrhea viruses (BVDV) types 1 and 2 in Tunisia

F. THABTI1,2, L. BAKKALI KASSIMI3, A. M’ZAH4, S. BEN ROMDANE4, P. RUSSO1, M.S. BEN SAID2, S. HAMMAMI2 and M. PEPIN1

1 AFSSA Sophia Antipolis, Laboratoire d’Etudes et de Recherches sur les Petits Ruminants et les Abeilles, BP 111, 06902 Sophia Antipolis Cedex, France
2 Institut de la Recherche Vétérinaire de Tunisie, Laboratoire de Virologie, Rue Djebel Lakhdar, La Rabta, 1006 Tunis, Tunisia
3 AFSSA site de Maisons Alfort, Laboratoire d’Etudes et de Recherches en Pathologies Animales et Zoonoses, BP 67, 94703 Maisons Alfort Cedex, France
4 Ecole Nationale de Médecine Vétérinaire de Sidi Thabet, Tunis, Tunisia

SUMMARY

Two outbreaks associated with bovine viral diarrhoea infections (BVD) are reported in Tunisia. Their origin was determined by serological and virological studies. In farm A, out of 188 (58%) sera analyzed by an ELISA test, 164 were positive for BVDV antibodies and 5 blood samples were positive by ELISA antigen and RT-PCR. Isolation of the virus on cell culture was successful in one animal. In farm B, out of 820 sera tested, 82% were positive for BVDV antibodies. Two BVDV isolates were recovered from blood of two persistently infected (PI) animals in cell culture. Genomic sequences of the 5’UTR and Npro regions of BVDV responsible for these infections were compared to other sequences of pestivirus strains. Phylogenetic analysis showed that the two outbreaks were independent: analysis of sequences from farm A showed that they belonged to BVDV type 2, whereas isolates from the second outbreak belonged to BVDV type 1. This report demonstrates the presence of the two types of BVDV in Tunisia. The detection of BVDV1 and BVDV2 associated with severe losses in two farms strongly suggests a need to implement a control scheme for the surveillance of BVD in Tunisia.

Keywords: BVDV - genetic characterization - Tunisia.

RÉSUMÉ

Premier isolement et caractérisation génétique du virus de la diarrhée virale bovine (BVDV) type 1 et 2 en Tunisie. Par F. THABTI, L. BAKKALI KASSIMI, A. M’ZAH, D. BEN ROMDANE, P. RUSSO, M. S. BEN SAID, S. HAMMAMI et M. PEPIN.

Deux épisodes de diarrhée virale bovine (BVDV) apparus en Tunisie sont rapportés dans cette étude. Afin de déterminer l’origine de ces infections, des analyses sérologiques et virologiques ont été conduites. Dans la ferme A, 164 séums sur 188 ont été positifs dans un test ELISA utilisé pour la recherche des anticorps dirigés contre le virus de la BVDV, et 5 prélèvements étaient positifs pour la recherche d’anticorps par ELISA. L’isolement du virus en culture cellulaire a été possible que pour 1 animal. Dans la ferme B, sur 820 séums analysés, 82% étaient positifs pour la recherche d’anticorps. Deux virus ont été isolés en culture cellulaire à partir des prélèvements sanguins réalisés sur deux animaux infectés permanents (PI). Les séquences des régions 5’UTR et Npro des virus responsables de ces épisodes ont été comparées aux séquences des autres pestiviruses. L’analyse des séquences des échantillons de la ferme A a montré qu’elles appartenaient au type 1. Cette étude démontre pour la première fois la co-existence des deux types de BVDV en Tunisie. Cette détection, associée au fait que les deux épisodes ont entraîné de lourdes pertes, suggère fortement la mise en place de mesures de contrôle de la BVD en Tunisie.

Mots-clés: BVDV - caractérisation génétique - Tunisie.

Introduction

Infections by two pestiviruses, Bovine viral diarrhea virus (BVDV) and Border disease virus (BDV), are enzootic in most livestock-producing countries and cause major economic losses in cattle and sheep throughout the world [11]. Infections with BVDV have a variable outcome. Acute BVDV infections frequently cause mild disease characterized by transient fever and leukopenia, with respiratory symptoms and/or diarrhoea [3]. Acute infection of pregnant animals can result in abortion, congenital malformations, stillbirth, or generation of immunotolerant persistently infected (PI) calves, which shed the virus throughout their lives. PI animals are infected by a non-cytopathogenic (ncp) virus; when they are super-infected with a cytopathogenic (cp) BVDV antigenically related to the ncp strain, they develop the mucosal disease (MD) [4, 9, 12, 17, 21]. There is significant antigenic and genetic diversity among BVDV isolates. Based on serological and sequence relatedness, BVDV isolates have been classified in two main genotypes, namely types 1 and 2 [19]. The BVDV1 genotype includes most viruses commonly used for vaccine production and laboratory diagnosis. Viruses belonging to the BVDV2 genotype were originally identified in severe outbreaks of acute haemorrhagic disease in Canada and the USA [6, 18]. Several authors since have reported their presence in Europe [13], South America and Japan [10, 16, 24]. In contrast, no BVDV type 2 has yet been detected in Africa [1]. Likewise, BVDV type 2 does not always correlate with high virulence as BVDV2 isolates of low virulence have been detected.

The presence of pestiviruses in Tunisia was first revealed in sheep by clinical reports and serological studies conducted in 1991, but without any virus isolation [28]. A serological survey carried out in 1998 in Tunisia to evaluate the incidence of abortive diseases in small ruminants showed that BDV was the most frequent cause (95% of the sampled flocks) [7]. A recent study has classified Tunisian BD isolates as a separate subgroup in the BD group of viruses [23]. In contrast, there has been no report on BVDV incidence in cattle populations, with the exception of one serological
study carried out in 2000 on 772 cattle showing a serological prevalence of 58% and indicating the presence of BVDV in Tunisia [2].

In this study, two outbreaks of BVD in two Tunisian cattle farms are described. A phylogenetic analysis was performed in order to characterize isolates responsible for these outbreaks. Sequences alignment from the 5'UTR and Npro regions of three positive BVDV samples of farm A showed that they belonged to genotype BVDV2. In farm B, BVDV type 1 was identified in two animals.

Materials and methods

ANIMALS

All samples analyzed in this study came from two Tunisian farms with a BVD clinical history.

Farm A

The first outbreak took place in a closed dairy bovine farm in North Tunisia. The farm was composed of 325 animals among which 188 cows, 61 heifers, 21 young calves and 55 young bulls. In 2001, a pestivirus outbreak was declared in this farm. Respiratory and digestive clinical signs were observed in cows and young calves. A morbidity of 20% was observed in cows and a mortality rate of 4% and 90% were detected in cows and calves respectively. Furthermore, mild haemorrhagic signs were seen in the intestinal tract after autopsy. A total of 7 abortions were declared in the period from April to June 2001.

Farm B

This second farm, with a closed herd, is also located in North Tunisia. Six hundred heifers were imported in 1988 from the USA. The farm was composed of 960 cattle of which 450 cows (> 2 years old), 360 heifers (< 2 years old), and 150 young females (< 1 year old). Artificial insemination was used in this farm with semen also imported from the USA. Many abortion problems, stillbirths and births of calves with malformations were observed in a period between August 2001 and March 2002. An abortion rate of 16% was declared in 2001 (0% in 2000). The main clinical signs observed were chronic diarrhoea, an acute gastroenteritis and respiratory signs characterized by a bronchopneumonia. At autopsy, lesions were mainly observed in the intestinal tract and in the lungs.

No prophylactic measures against BVD were taken in the two farms; only suspect animals with malformations or with respiratory signs or diarrhoea were quarantined until confirmation of their infection or not.

SEROLOGICAL AND VIROLOGICAL ANALYSIS

Blood samples were taken from 188 animals (186 cows and 2 calves) in farm A and from 820 animals (450 cows, 230 heifers and 140 young females) in farm B (Table I). The serological examination was performed by using a commercial enzyme-linked immunosorbent assay kit (ELISA AcBVD/MD Symbiotics Europe, Lyon, France), according to the procedure described by the manufacturer. Seronegative samples were also tested for the presence of BVD antigens in whole blood using an ELISA antigen test (Serelisa BVD/BD Ag, Symbiotics Europe, Lyon, France). Furthermore, blood samples from seronegative animals were used for analysis by RT-PCR and examined for the presence of live virus on a bovine turbinate (BT) cell line by an indirect immunofluorescence method (IF) as previously described [22]. Cells and foetal calf sera (FCS) were regularly tested for the absence of pestiviruses by RT-PCR and immunofluorescence. For the FCS, the absence of anti-pestivirus antibodies was shown by lack of virus neutralization.

RT-PCR AND SEQUENCING

To perform PCR, total RNA was extracted directly from whole blood or serum by means of a QIAamp viral RNA extraction kit according to the manufacturer’s procedures (QIAamp viral RNA purification kit, QIAGEN, France). Two viral sequences were amplified and analyzed in this study: 5’UTR (288 bp) and Npro (738 bp) regions were amplified according to the protocols described respectively by Vilcek et al. (1994, 1997) [25, 26]. DNA was sequenced on both strands (Qiagen Sequencing Service, Germany) and the nucleotide sequences obtained were compared with those of representative pestivirus strains present in GenBank database (NCBI, USA). Eight nucleotide sequences of the Tunisian isolates were submitted to the GenBank database under accession numbers AF462003 to AF462005 (5’UTR region: isolates 119, 98 and 63), AF462016 to AF42018 (Npro region : isolates 119, 98 and 63), AY453631 (5’UTR region : isolate 294) and AY452486 (Npro region : isolate 294).

Results

In farm A, the serological study determined that 164 out of 188 sera were positive to BVDV antibodies by the ELISA (Table I). Of the 24 seronegative animals, 5 were positive for BVDV by ELISA antigen and by RT-PCR. Isolation of the virus by cell culture was only successful in one animal. In farm B, of the 820 sera tested, 82% were seropositive to BVDV antibodies: cows represented 55.3% of seropositive results and heifers 26.7%. Two pestivirus isolates were reco-
vered from whole blood of two infected animals in cell culture; these animals were also positive in RT-PCR.

To characterize these Tunisian pestiviruses, the 5'UTR and the Npro fragments were amplified and sequenced; sequences were compared to reference strains representing groups and subgroups of pestiviruses. From farm A, three sequences, named 63, 119 and 098, were analyzed, and showed 100% identity to each other in both regions. Sequence similarities in the 5'UTR region (224 bp in length) were 84% with the BVDV2 reference strain 890, 65% with the BVDV1 reference strain NADL, and 57% with the reference strain X818 for BDV. When the 5'UTR sequences were compared to other BVDV2 sequences available from the GenBank database, two subgroups were distinguished (Figure 1): subgroup BVDV2a and subgroup BVDV2b. The Tunisian isolates from farm A were clustered in the BVDV2a subgroup with isolates from Japan, Italy and the USA. The origin and the dates of introduction of these pestiviruses into the Tunisian cattle industry cannot be determined by the present study; a large retrospective study performed throughout the country would be necessary to address these questions. Lacking these data, several hypotheses can be put forward. It is likely that the type 1 has been present in Tunisia for a long time, as reported in many other countries. Introduction of type 2 may be more recent: importation of cattle and/or semen from other countries could be one explanation among several for the introduction of the new genotype. The other hypotheses could be the pre-existence of this particular genotype as for the type 1, or its introduction via contaminated vaccines. Several well-documented examples of bovine vaccines contaminated by pestiviruses suggest that this possibility is likely [8, 23]. Other goals of a larger survey about bovine pestiviruses in Tunisia should be (i) to perform a serological study both by ELISA and seroneutralization with representative field strains (types 1 and 2), (ii) to isolate more viral strains in order to type them by cross-neutralization and molecular profiling, and (iii) to define the real importance of BVD in Tunisia and its consequences in the cattle industry. Although acute BVDV infections can present with fulminating and fatal disease, the majority of these infections are subclinical.

**FIGURE 1.** — Comparison of the 5'UTR region of pestivirus isolated from cattle in Tunisia. The tree was constructed by the comparison of 224 bp from 5 Tunisian bovine pestivirus sequences. Other field and reference strains included for comparison purposes were extracted from the GenBank database: BS95 II (AJ288903), Shinozaki (AB042676), MS-1 (AB019688), SE6444 (Z79777), Soldan (U94914), LL/Mi/97 (AJ293603), 713-2 (AF039177), Lees (U65051), V-FLL (AB019687), L373 (AF145967), NADL (M31182), Odessa (M96687), Oregon (AF091605), SD-1 (M96751), strain cp (AF220247), 890 (U18059), C413 (AF002227). The Tunisian isolates from farm A are shown in grey circles and isolates from farm B in white ones. Sequence alignments were done by the Clustal V multiple alignment method (DNASTAR Software, Lasergene Supplier, MD, USA). The phylogenetic tree was generated using the neighbor-joining method (MEGA, version 2.1) and subject to 1000 bootstrap samplings. The scale bar represents the number of substitutions per position.

The two genotypes of BVDV, types 1 and 2, have been detected in two distinct farms. If the genotype 1 is widely distributed, in contrast the BVDV type 2 was first described in the USA in a case of fatal haemorrhagic disease [6, 18] and was only recently identified in Europe [8, 13, 14, 27]. However, BVDV2 has not yet been isolated in Africa [1, 10, 16, 24]. The origin and the dates of introduction of these pestiviruses into the Tunisian cattle industry cannot be determined by the present study; a large retrospective study performed throughout the country would be necessary to address these questions. Lacking these data, several hypotheses can be put forward. It is likely that the type 1 has been present in Tunisia for a long time, as reported in many other countries. Introduction of type 2 may be more recent: importation of cattle and/or semen from other countries could be one explanation among several for the introduction of the new genotype. The other hypotheses could be the pre-existence of this particular genotype as for the type 1, or its introduction via contaminated vaccines. Several well-documented examples of bovine vaccines contaminated by pestiviruses suggest that this possibility is likely [8, 23]. Other goals of a larger survey about bovine pestiviruses in Tunisia should be (i) to perform a serological study both by ELISA and seroneutralization with representative field strains (types 1 and 2), (ii) to isolate more viral strains in order to type them by cross-neutralization and molecular profiling, and (iii) to define the real importance of BVD in Tunisia and its consequences in the cattle industry. Although acute BVDV infections can present with fulminating and fatal disease, the majority of these infections are subclinical.

**Discussion**

In this study, isolation and characterization of bovine field isolates of BVDV were reported for the first time in Tunisia.

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infections are inapparent or subclinical. In contrast, the profound immunosuppressive effects of BVDV infections have been well documented and are responsible for the potentiality of a variety of diseases in cattle including bovine respiratory diseases, salmonellosis, rotavirus and coronavirus in calves [5]. As a consequence for dairy and beef cattle industry, a majority of countries and regions have implemented measures to control and/or eradicate BVDV through a variety of measures such as vaccination, identification and removal of PI cattle from herds, preventive introduction of PI cattle in herds [15, 20]. In Tunisia, these measures could be implemented according to the assessment of the importance and impact of BVD in the cattle industry, and keeping into account the cost of the preventive or eradication measures.

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