Outbreak investigations and molecular characterization of foot-and-mouth disease viruses circulating in south-west Niger

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Summary
In Niger, the epidemiological situation regarding foot-and-mouth disease is unclear as many outbreaks are unreported. This study aimed (i) to identify Foot-and-mouth disease virus (FMDV) strains currently circulating in cattle herds, and (ii) to identify risk factors associated with Foot-and-mouth disease (FMD)-seropositive animals in clinical outbreaks. Epithelial tissues (n = 25) and sera (n = 227) were collected from cattle in eight districts of the south-western part of Niger. Testing of clinical material revealed the presence of FMDV serotype O that was characterized within the O/WEST AFRICA topotype. The antigenic relationship between one of the FMDV isolates from Niger (O/NGR/4/2015) and three reference vaccine strains was determined by the two-dimensional virus neutralization test (2dmVNT), revealing a close antigenic match between the field isolate from Niger and three FMDV serotype O vaccine strains. Serological analyses using a non-structural protein (NSP) test provided evidence for previous FMDV infection in 70% (158/227) of the sera tested. Multivariate logistic regression analysis revealed that only the herd composition (presence of both cattle and small ruminants) was significantly associated with FMDV seropositivity as defined by NSP-positive results (p-value = .006). Of these positive sera, subsequent testing by liquid-phase blocking ELISA (LPBE) showed that 86% (136/158) were positive for one (or more) of four FMDV serotypes (A, O, Southern African Territories (SAT) 1 and SAT 2). This study provides epidemiological information about FMD in the south-western part of Niger and highlights the complex transboundary nature of FMD in Africa. These findings may help to develop effective control and preventive strategies for FMD in Niger as well, as other countries in West Africa.

KEYWORDS
foot-and-mouth disease virus, identification, molecular characterization, risk factors, serology, south-western Niger
Foot-and-mouth disease (FMD) is a highly contagious transboundary disease of cloven-hoofed domestic and wild animals caused by Foot-and-mouth disease virus (FMDV) belonging to the Aphthovirus genus within the Picornaviridae family. FMDV is a small, non-enveloped, icosahedral virus that has a positive-sense, single-stranded RNA genome of approximately 8.5 kb that encodes a single polyprotein which is cleaved into four structural proteins (SP) and 10 non-structural proteins (NSPs) by virus-encoded proteases (Belsham, 1993). FMDV exists in seven immunologically distinct serotypes, O, A, C, Asia 1, SAT (Southern African Territories) 1, SAT 2 and SAT 3, each with a wide range of antigenically distinct subtypes. (Gleeson, 2002; Kasambula et al., 2012; Knowles & Samuel, 2003).

Foot-and-mouth disease is endemic in Niger where clinical disease has been reported mainly in cattle (Cousy-Hymann et al., 2006; Sangare, Bastos, Venter, & Vosloo, 2004; Sangare et al., 2001). FMD was first reported in Niger in 1945, when samples corresponding to serotype C were typed by the Laboratoire Central de Recherches Vétérinaires de Maisons-Alfort in France (Pagot, 1948). According to a retrospective study that reviewed FMD outbreaks occurring between 1971 and 2001 (Cousy-Hymann et al., 2006), four FMDV serotypes (namely O, A, SAT 1 and SAT 2) were suspected to be present in West African countries including Niger. Other published studies support the circulation of these four FMD serotypes in the region (Fasina et al., 2013; Gorna et al., 2014; Olabode, Kazeem, & Raji, 2014; Sangare, Bastos, Venter, & Vosloo, 2003; Sangare, Dungu, & Bastos, 2004; Sangare et al., 2001, 2004; Ularamu et al., 2016), although a comprehensive understanding of FMD epidemiology that can be used to inform disease control programmes is currently lacking. Unfortunately, livestock in Niger have never been vaccinated against FMD. Moreover, as the livestock production system is mostly characterized by transhumance, nomadism and trade with neighbouring countries, there are no restrictions on animal movements in the country or elsewhere in West Africa. Therefore, the objectives of this study were to identify FMDV strains responsible for outbreaks in the south-western part of Niger that occurred in cattle in 2014, as well as to describe risks factors associated with FMDV seropositivity in animals from these herds.

2 | MATERIALS AND METHODS

2.1 | Study area

In this article, sampling locations were defined at the district-level (Niger is administratively divided into eight regions, 63 departments and 265 districts). The study was conducted in eight districts in the south-western part of the country that included three regions namely Niamey (the capital), Tillabery and Dosso.

It is in the region of Tillabery that the largest numbers of samples were obtained in four districts: Kollo located 35 km from Niamey, Makalondi, Tamou and Alambaré bordering with Burkina Faso. In addition, Tamou and Alambaré are located near the W Regional Park, which is a major national park in West Africa (Niger, Burkina Faso and Benin) around a meander in the River Niger shaped like a "W." In the Dosso region, three districts were involved in the study, including Dole, Tanda and Gaya, which share a common border with Nigeria and Benin. In Niamey, one district (called the fourth Arrondissement) was involved. Except for Niamey’s district, these localities are located either on the transhumance route towards Benin and Nigeria (districts of Tanda, Dole and Gaya), or towards Burkina Faso and Benin (districts Tamou, Alambaré and Makalondi). This zone covers an area of more than 29,000 km² with a cattle population of about 500,000 animals (representing 5% of the cattle population at national level) based on the latest livestock census in 2007. Agriculture and livestock are the main activities of the resident population. The study area is depicted in Figure 1.

2.2 | Sampling design and disease investigation

In this study, an outbreak was defined as a district from which one or more clinical cases of FMD were reported by the district animal health service and/or by the farmers themselves. During September to October 2014, all reported outbreak sites were visited as soon as possible after notification; epithelium and serum samples were collected from cattle in the described study area.

As far as we are aware, no FMDV vaccination or other control measures were implemented at the study sites as in other parts of the country. The animals were first examined for evidence of salivation and lameness. Salivating and/or lame animals were restrained in a crush pen for thorough examination and sampling. The oral cavity of salivating animals was examined for evidence of intact and/or ruptured vesicles, erosions and ulcers on the tongue, dental pad and mucosa. The hooves of lame animals were thoroughly washed with water and carefully examined for lesions, particularly on the coronary bands and inter-digital spaces of the hooves. The epithelium samples were taken from sick animals showing suspected clinical signs and lesions of FMD, while the sera were taken from all examined animals during the herd visit, including those on which epithelium samples were collected (Table 1).

2.3 | Sample and data collection

Twenty-five epithelium tissues were collected from oral and foot lesions from suspected FMD-infected cattle in seven separate districts: Makalondi (n = 7), Gaya (n = 2), Dolé (n = 1), Tanda (n = 1), Kollo (n = 7), Alambaré (n = 5) and Tamou (n = 2). After collection, the tissues were immediately placed in a virus transport media composed of equal amount of sterile glycerol (50% v/v) and 0.04 M phosphate-buffered saline (PBS) at a pH between 7.2 and 7.6.

At the same time, 227 blood samples were collected from apparently healthy and from clinically affected cattle. Sera were collected in eight districts (seven mentioned above and in one of the districts of Niamey): Makalondi (n = 38), Gaya (n = 5), Dolé (n = 19), Tanda (n = 17), Kollo (n = 48), Alambaré (n = 14), Tamou (n = 10) and Niamey (n = 51). In the last district, Niamey, the FMD outbreak was
notified at least three weeks after the occurrence of the active outbreak, and at the time of the visit, there were neither clinical signs nor lesions in affected cattle. The samples (serum and epithelium) were transported to the National Veterinary Laboratory of Niamey (LABOCEL) on dry ice. At LABOCEL, samples were stored at /C0 20 °C (serum) and at /C0 80 °C (epithelium) until their shipment to the Bots-


FIGURE 1  Geographical locations of FMD outbreaks described in this study. Administrative regions: 1: Agadez, 2: Diffa, 3: Dosso, 4: Maradi, 5: Tahoua, 6: Tillabery, 7: Zinder and Niamey (capital city). Study area (Eight sampling districts described in this study): Niamey, Kollo, MK: Makalondi, Gaya (that covers administratively the district of Tanda, Dole) and Tamou (covering administratively Alambaré).

TABLE 1  Overview of the sampling strategy

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Number of herds visited</th>
<th>Number of sick animals</th>
<th>Number of apparently healthy animals</th>
<th>Number of Samples collected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Epithelium</td>
</tr>
<tr>
<td>Makalondi</td>
<td>6</td>
<td>32</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Gaya</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Dolé</td>
<td>4</td>
<td>8</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>Tanda</td>
<td>2</td>
<td>9</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Alambaré</td>
<td>2</td>
<td>11</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Tamou</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Kollo</td>
<td>5</td>
<td>26</td>
<td>29</td>
<td>7</td>
</tr>
<tr>
<td>4e Arrd (Niamey)</td>
<td>5</td>
<td>27</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>119</td>
<td>108</td>
<td>25</td>
</tr>
</tbody>
</table>

*Epithelium samples collected from sick animals with existing oral and foot lesions, bSera collected from all examined animals during the herd visit, including those on which epithelium samples were collected. i + j = total number of sampled animals during the herd visit that correspond to the total number of sera.

2.4  Serological analysis

2.4.1  Detection of antibodies against FMDV non-structural proteins (NSP ELISA)

Serological diagnostics were performed at the Botswana Vaccine Institute (BVI) in accordance with the established standards and practices of this OIE reference laboratory for sub-Saharan Africa. Sera were initially screened for antibodies against the highly conserved NSP of FMDV using the PrioCHECK® FMDV NS Enzyme-Linked Immunosorbent Assay (ELISA) test kit (Prionics AG, Switzerland), following the manufacturer’s protocol. The Optical Density at 450 nm (OD450) values of all samples were expressed as Percentage of Inhibition (PI) relative to the OD450 max. Positive results were defined as samples that generated a PI value of ≥50, whereas a strong positive result was set at a PI value of ≥70.

2.4.2  Detection of serotype-specific antibodies against FMDV liquid-phase blocking ELISA (LPBE)

Non-structural protein ELISA-positive reactive sera were further assessed using the liquid-phase blocking ELISA (LPBE) modified from the World Reference Laboratory for FMD (WRLFMD) at The Pirbright Institute, UK.

Data were collected using a questionnaire (see Appendix S1), which was used to interview farmers responsible for 28 herds (with a total of 227 sampled animals) selected on the basis of FMD outbreak notification. The recorded data included animal age, sex and location, and the presence or absence of clinical signs and lesions in cattle. In addition, the interview collected information regarding FMD risk factors such as the number of animals in the herd, the herd composition, the grazing and watering habits, the herd management (transhumance nomadic or sedentary) and the potential contact with wildlife.
Hamblin, Barnett, and Crowther (1986). Briefly, ELISA plates NUNC Maxisorp (Gibco, Cat#4-3945A) were coated with FMDV serotype-specific rabbit hyperimmune sera (serotypes O, A, SAT1 and SAT2 suspected to be present in Niger), and left overnight in a humid chamber at room temperature. In carrier plates, 2-fold series of each test serum were prepared, from 1/16 to 1/128. Control sera (strong and weak positive, and negative) were diluted at 1/16. To each well of the carrier plate, 50 μl the different FMDV serotype viral antigen was added at a pre-determined working dilution, resulted in a ratio of sera with FMD antigen starting from 1/32 to 1/256. The following day, the rabbit anti-serum-coated ELISA plates were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBST) (pH 7.4), and serum/antigen mixtures were transferred from the carrier plates to the rabbit-serum-coated ELISA plates and incubated at 37°C for 1 hr on a rotary shaker. The plates were then washed three times as previously, and FMDV serotype-specific guinea pig anti-serum was added to each well at a predetermined working concentration and incubated at 37°C for 1 hr on a rotary shaker. After incubation and washing step as previously, rabbit anti-guinea pig immunoglobulin conjugated to horseradish peroxidase was added to each well at a pre-determined working concentration. The plates were washed after 1 hr of incubation, and substrate solution (orthophenylene diamine [OPD] + 0.05% H2O2) was added to each well. The reaction was stopped by adding 50 μl of 1 M sulphuric acid. The plates were read at 492 nm on a Thermo Scientific™ Multiskan™ FC Microplate Photometer, and antibody titres were expressed as the final dilution of the tested serum giving 50% of the mean absorbance value in the virus control wells where test serum was absent. Titres of less than 1/40 (or 1.6 in reciprocal log10 form) were considered as negative, while titres more than 1/40 were considered positive (Hamblin et al., 1986).

2.5 Analysis of epithelium tissues

2.5.1 Virus isolation

The epithelium tissues were processed by the standard WRLFMD/World Organisation for Animal Health (OIE) procedure for virus isolation (OIE, 2012). The composition of the media used for virus isolation and culture of cells is as follows: 10% Minimum Essential Medium 10× (MEM 10×), 10% Lactalbumin Hydrolysate 10×, 4.5% Sodium Bicarbonate, 1% Negative Calf serum, 0.2% Penicillin and top up to 100 ml with sterile distilled water. The epithelium samples were first taken from the PBS/glycerol, and blotted dry on absorbent paper. A suspension was prepared by grinding 1 gram of the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium. Medium was added until a final volume of nine times that of added epithelial sample was reached, giving a 10% suspension. The suspension was clarified on a bench centrifuge at 2,000 g for 10 min at 4°C. The clarified suspensions suspected to contain FMDV were inoculated onto primary lamb kidney cell cultures (Rein de Mouton [RM]; at BVI) or primary bovine thyroid cell cultures (BTy; at WRLFMD) and incubated for 1 hr at 37°C. Fresh cell culture medium was then added (15 ml); the cultures were incubated at 37°C and monitored for cytopathic effect (CPE) for 48 hr. If no CPE was observed after 48 hr, the sample was considered as “no virus detected” and the culture was frozen at –70°C, then thawed and centrifuged at 2,000 g for 10 min at 4°C to collect supernatant for second passage (P2); this was repeated for third passage (P3); and if no CPE was observed at 48 hr, then the sample was considered negative for FMDV. The first passage (P1) and the second passage (P2) were subject to one freeze–thaw cycle. If CPE was observed, the culture medium was pooled and cleared by centrifugation at 2,000 g for 10 min at 4°C. A sample of supernatant was tested by reverse transcription-polymerase chain reaction (RT-PCR) following RNA extraction. However, it should be noted that the samples were examined for virus isolation nine months after they had been collected in the field.

2.5.2 Conventional RT-PCR assay for VP1 analysis

RNA was extracted from the ground tissue suspension samples using ZR Viral RNA kit (ZymoResearch, USA) following the manufacturer’s instructions. Extracted nucleic acid samples were analysed for FMDV RNA using conventional reverse transcription-polymerase chain reaction (RT-PCR) using oligonucleotide forward primer O-1C244F (5’-GCAGCAAAACACATGTCAAACACCTT-3) and reverse primer EUR 2B-52R (5’-GACATGTCCCTCCTGATCGGTTCGAT-3’) targeting the VP1 gene within the FMDV RNA genome (Knowles, Wadsworth, Bachanek-Bankowska, & King, 2016). At the BVI, the RT-PCR was set and ran as following: reverse transcription at 48°C for 30 min; the initial denaturation at 94°C for 1 min; 40 cycles (denaturation at 94°C for 15 s; annealing at 60°C for 30 s; extension at 68°C for 1 min); a final extension at 68°C for 5 min and then hold at 4°C. Amplification products were separated on a 1.5% agarose gel and visualized by Gel Red staining and UV irradiation. One-step RT-PCR at the WRLFMD was performed as previously described (Knowles et al., 2016).

2.5.3 Sequencing and phylogenetic analysis

The RT-PCR amplicons were sequenced on both strands as previously described (Knowles et al., 2016). The sequences were assembled and verified using SeqMan software (DNASTar, Lasergene v.8). VP1 nucleotide sequences were aligned using BioEdit version 7.2.5 (Hall, 1999) and Clustal W (Thompson, Higgins, & Gibson, 1994).

The comparison and mid-point-rooted Neighbor-joining trees of FMDV VP1 sequences from Niger with those from Africa available in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) were performed using MEGA 6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The robustness of tree topology was assessed with 1,000 bootstrap replicates using the model in MEGA 6.06. Bootstrap values of >70 are shown at the relevant major nodes. Sequences showing 100% nucleotide identity in VP1 were classified as a single genetic variant. The complete VP1 nucleotide sequences generated in this study corresponding to each genetic variant but also collected from a specific geographic location
were submitted to the NCBI GenBank database under the accession numbers (KX424677-KX424682).

2.6 Vaccine strain selection

Vaccine strain selection for serotype O isolates was performed at WRLFMD by two-dimensional virus neutralization test (2D-VNT). The vaccines used in this study were provided by international vaccine manufacturers (Merial Animal Health and Merck Animal Health). The 2D-VNT test was carried out using the pooled post-vaccination monovalent bovine vaccine sera (BVS) collected after 21 days post-vaccination of naïve animals. Briefly, the BVS was tested against both the homologous (vaccine strain) and the heterologous (field strain). Antibody titres of the reference serum against the homologous (reference) and heterologous (field) viruses for five virus doses were calculated, and a linear regression line was drawn (Minitab program) to allow the log10 reciprocal antibody dilution required for 50% neutralization of 100 tissue culture infective units (TCID50) of virus to be calculated. The antigenic relationship between the field strain and the reference strain was then expressed as an \( r^1 \) value based on the following equation: Reciprocal log10 of (heterologous titre – homologous titre) (Rweyemamu, Pay, & Parker, 1976). An \( r \) value of \( >.3 \) suggests that the vaccine virus may protect against the field strain (Paton et al., 2005).

2.7 Statistical analysis

In a first step, a multilevel mixed-effects model was used to take into account the possible herd and/or district levels as random effects. Because random effects were not observed, logistic regression was used to model the odds of being NSP positive as a function of investigated potential exposure risk factors. Initial screening of potential risk factors for FMD was performed by univariate regression (Hosmer & Lemeshow, 2000). Secondly, a multivariate logistic regression using backward stepwise analysis was used to check the relationship between NSP-positive results and explanatory variables (Petrie & Watson, 2006). The following explanatory variables and their respective reference classes were used: province of origin of the herd (4th Arrondissement as reference), herd type (nomadism or transhumance as reference), herd size (continuous variable), herd composition (only cattle as reference), contact with wildlife (rare as reference), transhumance destination (inside the country as reference), detection for FMD cases after the transhumance (yes as reference), gender (male as reference), age (<2 years as reference), animal origin (birth inside the herd as reference), clinical signs (presence as reference) and lesions (presence as reference). In addition, to assess the collinearity, a backward elimination of variables was performed (Preux, Odermatt, Perna, Marin, & Vergnenegre, 2005). If a variable induced a modification of the odds ratio of more than 20%, this variable was retained in the final model where the interaction was tested in case of biological relevance. Goodness of fit was assessed using the Hosmer–Lemeshow goodness-of-fit test. Statistical analyses were performed using STATA/SE Acad. 14 (Stata Corp., College Station, Texas, USA).

3 RESULTS

3.1 Characteristics of sampled animal

A total of 227 cattle including 93 males (41%) and 134 females (59%) belonging to 28 herds (20 transhumant or nomadic herds and 8 sedentary herds) were sampled during the period between 4 September 2014 and 16 October 2014. Most of the sampled animals were relatively young as the age of 58% (\( n = 132 \)) was estimated between 0 and 2 years, while 42% (\( n = 95 \)) had an estimated age between 3 and 4 years or more. Only 15% (\( n = 33 \)) of the sampled animals were introduced into their respective herds from outside, via purchase from livestock markets. With respect to animal species composition, 7 of the 28 of the sampled herds were composed only of cattle, while the 21 of the other herds were mixed (8 herds with cattle and small ruminants and 13 herds with cattle, small ruminants and other animals such as poultry, camels and horses). In Makalondi District, a single mixed herd included pigs. All the sampled animals of the selected herds mixed with animals of other herds of neighbouring districts during grazing and access to water points. According to herdsmen, in more than half of the selected herds (54%, \( n = 15 \)), clinical cases of FMD were reported when the cattle came back from transhumance. Of the total of 227 animals tested, 38 animals (17%) exhibited both clinical signs and lesions of FMD. Accordingly, it was among these 38 animals that sufficient epithelium samples were taken from 25 sick cattle.

3.2 Serological analysis

Using the NSP ELISA test, 70% (158/227) of sera were positive for the presence of antibodies against FMDV. There was random distribution of positive animals among age classes (\( \chi^2(3 \ df) = 6.12; p = .11 \)). The seroprevalence of animals of the age group between 3 and 4 years (83%) was not significantly higher than the prevalence of animals of other age categories (70%, 62% and 65% for \( \leq 2 \) years, \( >2 \) and \( \leq 3 \) years and \( >4 \) years respectively) Table 2.

Among the NSP ELISA-positive sera tested by LPBE, 86% (136/158) were positive for one or more serotypes (A, O, SAT 1 and SAT 2). Based on the distribution of seroprevalence by sampling site, the highest serological prevalence was for serotype O observed in 7/8 districts (except the district of Tamou) (Figure 2).

In addition, either as single or as multiple serological reactions, there was a clear dominance of serotype O followed by serotypes A and SAT1. However, only 11.3% (\( n = 18 \)) of NSP ELISA-positive samples yielded positive results for a single serotype: against serotypes A (5 samples), SAT1 (4 samples) or O (9 samples), while 86.1% (\( n = 136 \)) were positive for 2 or more serotypes, and only 2.5% (\( n = 4 \)) generated negative results with the LPBE (Figure 3 and Appendix S2).
3.3 Factors associated with FMDV seropositivity based on a logistic regression analysis

The results of univariate regression analysis for odds of being NSP ELISA positive as a function of investigated potential exposure risk factors showed that only the herd composition (presence of both cattle and small ruminants) was highly significantly associated with FMDV seropositivity (p = .002; Table 3). The remaining variables were not significantly associated with FMDV seropositivity at the 5% level, but those with a p-value ≤ .2 were considered as potential risk factors and therefore entered in the multivariable analysis model (herd composition, district of origin and age of animals).

Multivariate analysis including all variables (with a p-value less than .20 after univariate analysis) exploited a final model that included district and herd composition as variables. Herd composition was significantly associated with FMDV positivity (p = .006). The Hosmer–Lemeshow test showed that this final model fitted the data well ($\chi^2 = 1.81; df = 6, p$-value = .94). The interaction between the two retained variables was not tested because of the lack of biological relevance (Table 4).

### TABLE 2 NSP ELISA-positive animals by age class

<table>
<thead>
<tr>
<th>Age category</th>
<th>Number of tested animal</th>
<th>Number of NSP ELISA positive</th>
<th>Seroprevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤2 years</td>
<td>74</td>
<td>52</td>
<td>70</td>
</tr>
<tr>
<td>&gt;2 and ≤3 years</td>
<td>58</td>
<td>36</td>
<td>62</td>
</tr>
<tr>
<td>&gt;3 and ≥4 years</td>
<td>47</td>
<td>39</td>
<td>83</td>
</tr>
<tr>
<td>&gt;4 years</td>
<td>48</td>
<td>31</td>
<td>65</td>
</tr>
<tr>
<td>Total</td>
<td>227</td>
<td>158</td>
<td>70</td>
</tr>
</tbody>
</table>

NSP, non-structural proteins. Sampled cattle were classified into four age groups, this table shows the seroprevalence of animals of each age class, and 70% represent the overall seroprevalence yielded by NSP ELISA.

3.4 Isolation and identification of FMDV

Thirteen of the 25 epithelial samples produced CPE during one, two or three passages on primary lamb kidney cell cultures at BVI. These samples were from the districts of Tamou (3), Gaya (2), Makalondi (2) and Kollo (6). By antigen ELISA (performed at the WRLFMD), FMDV serotype O was identified in cell culture harvests from seven epithelia collected in Gaya (n = 1), Makalondi (n = 2) and Kollo (n = 4) districts. Based on the sequence comparison using BLAST, the serotype identification of these samples was in concordance with the Ag-ELISA results. The other six samples (from the 13 CPE-positive samples) were detected negative by both antigen ELISA and PCR tests. Sequences were obtained for six of the seven isolates of FMDV serotype O, and these are included in the phylogenetic analysis and listed in Table 5.
Foot-and-mouth disease virus (O/West Africa (WA)). Those isolated from Kollo district (NGR/4/2015) had pairwise alignment (nt) identities of 99.3%-99.7% with each other while the viruses isolated from Makalondi (NGR/15/2015 and NGR/16/2015) had 100% nt identity with each other. The FMDV isolate from Gaya (NGR/11/2015) had pairwise nt identity of 99.0%-99.4% with other isolates from other districts. The VP1 sequences from Niger were compared to those available in the GenBank database (Figure 4). The analysis revealed that the Niger isolates are mostly related to the FMDV from Benin [O/BEN/40/2010 (KC832986) with 95.2%-95.8% nt identity and O/BEN/26/2010 (KC832981) with 94.2%-95.8% nt identity], Togo [O/TOG/1/2004 (KX258038) with 90.3%-92.3% nt identity and O/TOG/1/2005 (KX258039) with 92.1% nt identity] and from Ghana [O/Lam/GHA/2012 (KF305227) with 90.3%-90.9% nt identity] all being classified within the type O/WA topotype. However, the Niger FMDV isolates show lower relationship values with other earlier West African FMDV serotype O isolates from Côte d’Ivoire [O/CIV/8/99 (AJ303485) with 88.9%-90.4% nt identity] and from Ghana [O/GHA/5/93 (AJ303488) with 85.8%-87.4% nt identity] (Figure 4).

### 3.5 Phylogenetic analysis

From FMDV isolates collected in 2014, amplicons corresponding to the complete VP1 coding region were generated by RT-PCR and sequenced for six of the virus isolates. These sequences were compared with others from NCBI, GenBank and results from phylogenetic analyses revealed that they all belonged to the topotype O/West Africa (WA). Those isolated from Kollo district (NGR/4/2015, NGR/21/2015 and NGR/24/2015) had pairwise alignment (nt) identities of 99.3%-99.7% with each other while the viruses isolated from Makalondi (NGR/15/2015 and NGR/16/2015) had 100% nt identity with each other. The FMDV isolate from Gaya (NGR/11/2015) had pairwise nt identity of 99.0%-99.4% with other isolates from other districts. The VP1 sequences from Niger were compared to those available in the GenBank database (Figure 4). The analysis revealed that the Niger isolates are mostly related to the FMDV from Benin [O/BEN/40/2010 (KC832986) with 95.2%-95.8% nt identity and O/BEN/26/2010 (KC832981) with 94.2%-95.8% nt identity], Togo [O/TOG/1/2004 (KX258038) with 90.3%-92.3% nt identity and O/TOG/1/2005 (KX258039) with 92.1% nt identity] and from Ghana [O/Lam/GHA/2012 (KF305227) with 90.3%-90.9% nt identity] all being classified within the type O/WA topotype. However, the Niger FMDV isolates show lower relationship values with other earlier West African FMDV serotype O isolates from Côte d’Ivoire [O/CIV/8/99 (AJ303485) with 88.9%-90.4% nt identity] and from Ghana [O/GHA/5/93 (AJ303488) with 85.8%-87.4% nt identity] (Figure 4).

### 3.6 Vaccine strain selection

The antigenic relationship between one of the FMDV isolates from Niger (O/NGR/4/2015) and three reference vaccine strains were determined by the two-dimensional virus neutralization test (2D-VNT). The results presented (Table 6) revealed that there is a close antigenic relationship between the three FMDV serotype O vaccine strains and Niger’s FMDV serotype O field isolate. The calculated “r1” value was greater than the minimum requirement (>0.3) for especially the two vaccine strains (O3039 and O/TUR/5/2009).
TABLE 5 Diagnostic results on epithelium samples collected in Niger in 2014 and the GenBank accession numbers of VP1 sequences

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>BVI code</th>
<th>WRLFMD code</th>
<th>Outbreak Location</th>
<th>Animal age class</th>
<th>Cell passage</th>
<th>Serotyping by Ag-ELISA</th>
<th>PCR</th>
<th>GenBank accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GY7</td>
<td>NGR/11/2015</td>
<td>Gaya</td>
<td>1</td>
<td>3rd P</td>
<td>O</td>
<td>FMDV-GD</td>
<td></td>
<td>KX424677</td>
</tr>
<tr>
<td>MK7</td>
<td>NGR/15/2015</td>
<td>Makalondi</td>
<td>0</td>
<td>1st P</td>
<td>O</td>
<td>FMDV-GD</td>
<td></td>
<td>KX424678</td>
</tr>
<tr>
<td>MK17</td>
<td>NGR/16/2015</td>
<td>Makalondi</td>
<td>0</td>
<td>2nd P</td>
<td>O</td>
<td>FMDV-GD</td>
<td></td>
<td>KX424679</td>
</tr>
<tr>
<td>KL2</td>
<td>NGR/20/2015</td>
<td>NGR/4/2015</td>
<td>Kollo</td>
<td>0</td>
<td>1st P</td>
<td>O</td>
<td></td>
<td>KX424680</td>
</tr>
<tr>
<td>KL44</td>
<td>NGR/21/2015</td>
<td>Kollo</td>
<td>2</td>
<td>1st P</td>
<td>O</td>
<td>FMDV-GD</td>
<td></td>
<td>KX424681</td>
</tr>
<tr>
<td>KL3</td>
<td>NGR/24/2015</td>
<td>Kollo</td>
<td>3</td>
<td>1st P</td>
<td>O</td>
<td>FMDV-GD</td>
<td></td>
<td>KX424682</td>
</tr>
</tbody>
</table>

P, passage, FMDV-GD, FMDV genome detected, age class: 0: ≤2 years; 1: >2 and ≤3 years; 2: >2 and ≤4 years; 3: >4 years. Epithelium tissues (n = 25) were obtained from clinical FMD cattle originating from seven districts of south-western of Niger. This table indicates the positive diagnostic (virus isolation, Ag-ELISA and PCR) results with high-quality sequences (n = 6). These positives samples were from the following districts: Gaya (GY), Makalondi (MK) and Kollo (KL).

4 | DISCUSSION

This study reports on serological and molecular information for FMD outbreaks in south-west Niger based on samples collected from cattle in September and October 2014. FMD is endemic in most parts of Africa and only few countries in the south of the continent have managed to control the disease (Brito, Rodriguez, Hammond, Pinto, & Perez, 2017; Vosloo, Bastos, Sangare, Hargreaves, & Thomson, 2002; Vosloo, Boshoff, Dwarka, & Bastos, 2002), while only sporadic cases of FMD are regularly reported (Brito et al., 2017; Tekleghiorghis, Moormann, Weerdmeester, & Dekker, 2016). Niger with an area of 1.267,000 km² is one of the largest West African countries. Based on the general census of agriculture and livestock in 2007, the cattle population was estimated at more than 7 million of heads. However, despite the important role of the livestock sector in Niger (La Rovere, Hiernaux, Van Keulen, Schiere, & Szonyi, 2005; Turner & Williams, 2002), this industry is continuously challenged with multiple constraints such as the persistence of animal diseases, including FMD. Although FMD outbreaks have been reported every year, the veterinary authorities and farmers have placed little emphasis on FMD. Hence, even though FMD is on the list of monitored animal diseases in epidemic-surveillance networks, there is still an under-reporting of FMD outbreaks. The main purpose of this study was to characterize FMD viruses responsible for clinical cases and additionally to have an overview of circulating FMDV antibodies in livestock associated with risk factors analysis. This was only the justification of the adopted sampling method that can be designated as a "convenience sampling" consisting therefore to sample suspected sick animals (for epithelial tissues) and both the suspected sick animals and apparently healthy animals (for sera) in the all reported infected herd (as soon as possible after the rare notification of outbreaks). However, despite the limited nature of sampling, this study could certainly have the value to update data on FMD in a country where the epidemiological status of the disease is poorly understood.

The serological results indicate that FMDV is endemic within the livestock population in the study area, suggesting that multiple FMDV serotypes (such as A, O, SAT 1 and SAT 2) may be involved as has been shown elsewhere in the West African region (Brito et al., 2017; Di Nardo, Knowles, & Paton, 2011; Ehizibolo et al., 2014; Fasina et al., 2013; Gorna et al., 2014). Using the budget available for this study, serological testing (by LPBE) was designed to detect four different FMDV serotypes (A, O, SAT 1 and SAT 2) suspected to be present in Niger. Further studies may be warranted to also include serotypes C and SAT 3, although serotype C has not been detected in any country since 2004. SAT 3-specific antibodies have been recorded in sera from west and central Africa (Ehizibolo et al., 2014; Ludi et al., 2016) and from eastern Africa (Ayebazibwe et al., 2010; Dhikusooka et al., 2015; Mwino et al., 2010; Namatovu et al., 2015), although this serotype has not previously been detected in Niger. Although the sampling strategy is different to that implemented by Ludi et al. (2016), our results appear to be similar regarding the presence of different serotypes in unvaccinated animals. Serological tests also reveal that antibodies to four FMDV serotypes were present among the animals sampled although only one FMDV serotype (O) was detected by viral isolation and sequencing. The presence of animals with single serological reactivity to serotypes A and SAT 1 (Figure 3) may indicate either past exposure to these FMDV or may arise as a result of cross-reactivity among serotypes in the LPBE (Hedger, Barnett, & Gradwell, 1982; Jackson et al., 2007). Future serological studies are warranted to these results.

Since 2005, only O and SAT 2 serotypes have been isolated in Niger, serotype A having been isolated for the last time in 1973 and SAT1 in 1976 (WRLFMD, 2016b). In this study, the highest serological prevalence was that of serotype O (89%), followed by serotypes SAT 1, A and SAT 2. Serotype O was detected in more than 80% of samples from all selected districts where FMD outbreaks occurred. Furthermore, for individual districts, serotype O was most frequently detected, except in Gaya and Tanda Districts where serotype A (at 33%) and serotype SAT1 (at 45%) were found, respectively. Interestingly, specific response to serotype O was obtained in cattle from 3 of 7 districts, namely Tamou, Kollo and Niamey. Additionally, in Niamey where the epithelium sampling was not possible due to the
delay in the notification of the FMD outbreak, five sera were specifically positive to serotype O. The serological results for serotype O could be interpreted as significant for this study because the serotype O was the only FMDV detected positive through viral isolation test. However, there is no evidence about any conclusion regarding the serological responses by the fact that the adopted sampling scheme is not consistent to make an accurate statement on statistical inference of results.

There was no association between seropositivity and age. Generally, keeping young animals around the homestead or in areas separated from adult animals helps to decrease their exposure to FMDV (Bayissa, Ayelet, Kyule, Jibril, & Gelaye, 2011; Bronsvoort et al.,...
An "r_t" value greater than 0.3 indicates the existence of close antigenic relationship between the vaccine strain and the field isolate. FMDV, Foot-and-mouth disease virus.

In epidemiological settings, such as Niger with the existing livestock management practices, all potential risk factors could contribute to FMD infection. However, the statistical analysis showed that only the herd composition (cattle mixed with small ruminants) was highly significantly associated with FMDV seropositivity in FMD outbreaks. Despite these results, the role of other factors should not be ignored. The role of transhumance in FMD spread has been shown to play an important role elsewhere in sub-Saharan Africa (Rweyemamu et al., 2008). Furthermore, significant buffalo populations exist in West and Central Africa, including the W park (trans-border area shared between Benin, Burkina Faso and Niger). Notably, two districts in the study area (Alambar and Tamou) are located at the interface zone between domestic animals and wildlife through the national park W of Niger. To what extent types of FMDV prevalent in domestic ruminants infect wildlife is unknown, and this important pattern of the FMD transmission dynamics remains to be explained (Anderson et al., 1993; Ayebazibwe et al., 2010; Di Nardo et al., 2015; Fevre, Bronsvoort, Hamilton, & Cleaveland, 2006; Vosloo et al., 2002). Furthermore, there are important rural livestock markets in the study area (for example Alambaré), where contact between animals increases by the absence of any quarantine measure and where subsequently the transmission of FMD virus and other animal diseases is enhanced (Dean et al., 2013; Garland & de Clercq, 2011). It is obvious that the effect of the potential risk factors would be more clearly reflected with a comprehensive random sampling in domestic animals as well as in wildlife.

Out of the total analysed epithelium samples (n = 25), only six VP1 sequences were obtained for phylogenetic analysis. This relatively low rate (6/25) of sequence recovery could be explained by several factors such as the insufficient quality of the samples with degradation of the genome, due to a long time of storage of samples—about 10 months—and to poor shipping conditions or, on the other hand, by the lower analytical sensitivity of the sequencing VP1 RT-PCRs or primer mismatches. Furthermore, the relative lower quality of epithelium tissue samples could likewise be the reason that one FMDV isolate was recovered among the four samples sent to the WRLFMD. The failure to isolate FMDV from more samples restricted the extent of vaccine-matching work that could be performed at the WRLFMD. Further work is urgently required to expand these vaccine-matching studies to more field isolates from the country. Furthermore, these in vitro results would benefit from results of in vivo pilot studies that evaluate the performance of the vaccine in the target host species.

During the last ten years, serotype O field isolates have been characterized in Burkina Faso, Togo, Nigeria, Ghana, Cameroon, Senegal, Mali and Niger. VP1 sequence analysis undertaken in this study indicates that these FMD viruses from Niger are closely related to strains previously isolated in West Africa. These isolates display the closest relationship with the strains from Benin (O/BEN/40/2010 and O/BEN/26/2010), Togo (O/TOG/1/2004 and O/TOG/1/2005), and from Ghana (O/Lam/GHA/2012). This close genetic relationship supports the role of cross-border animal movements as a major route by which FMD spreads in the region (Brito et al., 2017; Bronsvoort et al., 2004; Couacy-Hymann et al., 2006; Di Nardo et al., 2011; Ezhibolo et al., 2014; Fasina et al., 2013; Goma et al., 2014; Knowles & Samuel, 2003; WRLFMD, 2016a). In addition to the uncontrolled movement of animals along the border, to our knowledge, countries such as Benin and Togo do not practice mass vaccination against FMD.

In conclusion, the serological and molecular observations of this study urge for continuous surveillance of FMD enabling to monitor the infection status and the spread of FMDV serotypes in livestock as well as in wildlife populations in Niger. It is anticipated that the results of this study despite its limited sampling design will motivate further work to characterize FMDV from field outbreaks in the country where the epidemiological status of the disease is poorly understood. In addition, regarding to transboundary animal movements and international animal trade, an integrated control approach at regional or continental level is strongly recommended.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.
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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.