Detection and genetic characterization of foot-and-mouth disease viruses in samples from clinically healthy animals in endemic settings

S. M. Jamal1,2,3, G. Ferrari4, M. Hussain5, A. H. Nawroz6, A. A. Aslami6, E. Khan5, S. Murvatulloev7, S. Ahmed2 and G. J. Belsham3

1 National Veterinary Laboratory, Islamabad, Pakistan
2 Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan
3 National Veterinary Institute, Technical University of Denmark, Lindholm, Kalvehave, Denmark
4 Food and Agriculture Organization of the United Nations, Rome, Italy
5 FAO Project, GTFS/INT/907/ITA, National Veterinary Laboratory, Islamabad, Pakistan
6 FAO Project, GTFS/INT/907/ITA, Kabul, Afghanistan
7 FAO Project, GTFS/INT/907/ITA, Dushanbe, Tajikistan

Abstract

A total of 1501 oral swab samples from Pakistan, Afghanistan and Tajikistan were collected from clinically healthy animals between July 2008 and August 2009 and assayed for the presence of foot-and-mouth disease virus (FMDV) RNA. The oral swab samples from two (of four) live animal markets in Pakistan (n = 245), one (of three) live animal market in Afghanistan (n = 61) and both the live animal markets in Tajikistan (n = 120) all tested negative. However, 2 of 129 (2%) samples from Gondal and 11 of 123 (9%) from Chichawatni markets in Pakistan were positive for FMDV RNA. Similarly, 12 of 81 (15%) samples from Kabul and 10 of 20 (50%) from Badakhshan in Afghanistan were found to be positive. Serotypes A and O of FMDV were identified within these samples. Oral swab samples were also collected from dairy colonies in Harbanspura, Lahore (n = 232) and Nagori, Karachi (n = 136), but all tested negative for FMDV. In the Landhi dairy colony, Pakistan, a cohort of 179 apparently healthy animals was studied. On their arrival within the colony, thirty-nine (22%) of these animals were found positive for FMDV RNA (serotype A was identified), while 130 (72.6%) had antibodies to FMDV non-structural proteins. Thus, newly introduced animals may be a significant source of the disease in the colony. Only two animals from the cohort were detected as becoming positive for FMDV RNA during a follow-up period of 4 months; however, only 10 animals remained negative for anti-NSP antibodies during this period.

Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease affecting domesticated and wild ungulates including cattle, buffalo, sheep and pigs. The causative agent, foot-and-mouth disease virus (FMDV), belongs to the genus Aphthovirus within the family Picornaviridae. The disease can easily be transmitted from infected to in-contact animals. Typically, in susceptible animals, the disease causes a high level of morbidity (characterized by fever, drooling and lesions around the mouth and on the feet) but low levels of mortality (except in young animals). In some domestic species (e.g. sheep and goats) or in vaccinated animals and in certain wildlife species, for example African buffalo, a subclinical or inapparent infection can occur.

Many FMDV-infected animals clear the virus within 7–14 days. However, 50% of cattle can carry the virus asymptotically and intermittently in oropharyngeal...
Epidemiology of FMD in Pakistan and Afghanistan

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Livestock are an important component of the economy in rural areas of Afghanistan, Pakistan and Tajikistan. Infectious diseases represent one of the main constraints to livestock production and threaten food security by causing mortality and production losses. FMD is endemic in these countries (Schumann et al., 2008; Jamal et al., 2010, 2011a,b,c), and there is a need to have a better understanding of the epidemiological situation of this disease. For this purpose, a surveillance programme for FMD was initiated to (i) identify the risk of being infected among different subgroups on the basis of age, area of origin and production sector; (ii) characterize the FMDV strains circulating in the region; and (iii) assess, if possible, the extent of virus circulation independently from the clinical manifestations of the infection.

An insight into the first issue was achieved through the use of serological surveys, particularly through the detection of antibodies to non-structural proteins (NSP) of FMD virus, which can allow discrimination between infected and vaccinated animals if purified vaccines have been used (Hussain et al., 2009; Ferrari et al., 2010). The second objective was achieved through the collection of samples from animals suspected, on a clinical basis, of having FMD as such samples have a high likelihood of permitting identification and further characterization of the circulating virus strains (Jamal et al., 2011a,b,c). To address the third issue, specific production sectors (i.e. live animal markets and dairy colonies) were targeted because the constant mixing of animals, owing to their high turnover, may create suitable conditions for the maintenance of FMD. Oral swab samples were collected from apparently healthy animals and tested, using sensitive and specific assays, for the presence of FMDV RNA. The relationship between the viruses detected in apparently healthy and in clinically diseased animals was ascertained by comparing the sequences of the VP1 coding region of the FMDV genomes.

Materials and Methods

Sample collection

A total of 1501 oral swab samples (Table 1) were collected from clinically healthy animals (cattle and buffaloes) from the two different production systems (live animal markets and dairy colonies) in Pakistan (n = 1201), Afghanistan (n = 180) and Tajikistan (n = 120) as part of the FAO regional project, GTFS/INT/907/ITA. The minimum sample size for each live animal market selected was 20 oral swabs per visit. This is the number required to detect at least one positive sample (at a 95% confidence level) if the proportion of positive cases on the day of collection is ≥15%. In fact, the sample size in dairy colonies in Pakistan was 50 samples per visit. Animals in the markets or in the colonies were initially screened to determine that there were no suspected clinical cases of FMD and only then collection of the established number of samples was performed from randomly selected animals. The oral swab samples were placed directly into 1 ml RLT buffer (Qiagen, Hilden, Germany) as described previously (Klein et al., 2007) and shipped to the National Veterinary Institute, Technical University of Denmark (DTU Vet), Lindholm, Denmark.

In Pakistan, the swab samples were collected from four live animal markets (Gondal, Chichawatni, Lahore and TM Khan) and three dairy colonies (Landhi dairy colony, Karachi; Nagori colony, Karachi; and Harbanspura dairy colony, Lahore). These samples from Pakistan were collected between November 2008 and August 2009. Oral swab samples from Afghanistan were obtained between July 2008 and July 2009 from three different live animal markets located in Kabul, Nangarhar and Badakhshan. Samples from Tajikistan were collected between March and May 2009 from two different live animal market located in Rudaki and Gissar provinces. The locations of the live animal markets and dairy colonies are shown in Fig. 1.

In addition, a cohort of 179 animals entering the Landhi dairy colony was selected to determine the extent of their prior exposure to FMDV. This group of animals was purchased from different live animal markets located in Punjab and Sindh provinces. These animals were ear-tagged and kept on 15 different farms within the colony. In addition to oral swab samples, blood was also collected and then serum isolated. These samples were tested...

Table 1. Number of oral swab samples collected from live animal markets and dairy colonies in Pakistan, Afghanistan and Tajikistan

<table>
<thead>
<tr>
<th>Country</th>
<th>Origin of samples (production system)</th>
<th>No. of samples collected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cattle</td>
<td>Buffaloes</td>
</tr>
<tr>
<td>Pakistan</td>
<td>Live animal markets</td>
<td>210</td>
</tr>
<tr>
<td>Pakistan</td>
<td>Dairy colonies</td>
<td>202</td>
</tr>
<tr>
<td>Afghanistan</td>
<td>Live animal market</td>
<td>180</td>
</tr>
<tr>
<td>Tajikistan</td>
<td>Live animal market</td>
<td>120</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>712</td>
<td>789</td>
</tr>
</tbody>
</table>
for antibodies to the non-structural proteins (NSP) of FMDV using an indirect trapping ELISA (De Diego et al., 1997). A second round of sampling was performed on those animals that were negative for antibodies against FMDV NSPs in the initial assays, and thus 45 animals were sampled in April 2009, one month after the first sampling. A further 38 samples were collected each in May and June and 36 samples were collected in July 2009. These animals were reportedly healthy, and no clinical FMD was observed in the farms during the study period.

**RNA extraction**

RNA was extracted from all the oral swab samples using MagNA Pure LC Total Nucleic Acid Isolation kits (Roche, Mannheim, Germany) with a MagNA Pure LC robotic system (Roche) following the manufacturer’s instructions. Briefly, tubes containing the swabs in RLT buffer were thawed and thoroughly homogenized by vortexing. The tubes were centrifuged at 664 \( g \) for 10 min, and supernatant (200 \( \mu l \)) was added to lysis/binding buffer (300 \( \mu l \)). Extracted nucleic acid from each sample was eluted in water (50 \( \mu l \)). Positive (FMDV serotype A22/Iraq) and negative (water) extraction control samples were included in each group of RNA extractions.

**cDNA synthesis and real-time RT-PCR**

The cDNA was synthesized using a TaqMan cDNA synthesis kit from Applied Biosystems (Naerum, Denmark). Briefly, 1.5 \( \mu l \) of 10× TaqMan RT-buffer, 3.3 \( \mu l \) of 25 mm MgCl₂, 3.0 \( \mu l \) of 2.5 mm each of dNTPs, 0.5 \( \mu l \) of 50 \( \mu M \) random hexanucleotide primers, 0.3 \( \mu l \) of 20 U/\( \mu l \) RNase inhibitor and 0.375 \( \mu l \) of 100 U/\( \mu l \) MultiScribe reverse transcriptase with 6 \( \mu l \) of RNA sample were mixed and incubated at 48°C for 45 min followed by 95°C for 5 min.

The cDNA samples were tested initially in real-time RT-PCR (rRT-PCR) diagnostic assay targeting the 3D\textsuperscript{pol} coding region (Callahan et al., 2002). The reaction mixtures per sample consisted of 12.5 \( \mu l \) of 2× TaqMan Universal master mix (Applied Biosystems), 2.25 \( \mu l \) each of 10 pmol/\( \mu l \) of forward and reverse primers and 1.5 \( \mu l \) of 5 pmol/\( \mu l \) probe and 7 \( \mu l \) cDNA. Thermal cycling and concurrent fluorescence detection were performed in an MX4000 Multiplex quantitative PCR system (Stratagene, Amsterdam, The Netherlands) with the assay cycling conditions of 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 60 s and then maintained at 4°C. A cycle detection threshold (i.e. the cycle at which a target sequence is detected \([Ct]\)) was recorded for each test sample. Samples with Ct values <50 were tested in a second rRT-PCR assay using primers and probe that target the 5’ untranslated region (UTR) of the FMDV genome (Reid et al., 2002). Samples with a Ct value of <40 in either of the assays were considered as positive. For each set of reactions, positive controls (A22/Iraq virus RNA for the 3D assay and a 5’ UTR RNA transcript derived from cDNA from the O/UKG/2001 virus for 5’ UTR assay) and a negative synthesis control (water) were included.

Samples with Ct values ≤30 were selected for the determination of serotype and subtype of the virus by amplifying and sequencing the whole VP1 coding sequences; these data have been reported previously (Jamal et al., 2011a,b) and submitted to GenBank.

**Statistical analysis**

Multivariate logistic regression analysis (Doho et al., 2003) was applied to the data to determine the relationship between the FMD infection in apparently healthy animals (response variable) and explanatory variables including animal species (cattle or buffalo), country of origin (Pakistan or Afghanistan), locality of live animal markets, district of origin of the animals, and year and month of sample collection. Interactions between the response variables were also included in the model. Using a forward selection process, all of the explanatory variables, with and without interactions, were added to the
model. The model with the lowest residual deviance was selected if the likelihood ratio test showed that addition of the new variable resulted in a significantly ($\alpha = 0.05$) better model than the model without the additional variable. If the two levels of a variable (e.g. two districts) produced similar results, they were combined. All the analyses were performed using statistical software, R (http://www.r-project.org). Samples from dairy colonies were not included in the analyses as this production system is only present in Pakistan. Moreover, samples from Landhi dairy colony, Karachi, were not collected at random; rather they were collected from a selected cohort of animals.

**Relationship between viruses from clinical and subclinical cases of FMDV infection**

Based on the phylogenetic trees reported previously (Jamal et al., 2011a,b), closely related viruses were selected for further analysis using TCS trees that were generated using statistical parsimony analysis implemented in the programme TCS v1.21:6 (Clement et al., 2000) to establish the relationships between FMDVs from subclinical and clinical cases.

**Results**

**Samples from live animal markets**

A total of 797 oral swab samples were collected from apparently healthy animals in live animal markets, and they comprised 497 samples from four locations in Pakistan (Lahore, TM Khan, Gondal and Chichawatni), 180 samples from three sites in Afghanistan (Kabul, Nangarhar and Badakhshan) and 120 samples from Rudaki and Gissar in Tajikistan. Of these, 35 samples were found positive in the rRT-PCR diagnostic assays, comprising 13 from Pakistan and 22 from Afghanistan. All the samples originating from Tajikistan were found negative for FMDV RNA. The details of the test results for the samples collected in the different countries are summarized in Table 2.

The samples from Lahore and TM Khan (Pakistan) plus Nangarhar (Afghanistan) all tested negative for FMDV RNA. Two of 129 swabs (~2%) and 11 of 123 (9%) samples from Gondal and Chichawatni, respectively, tested positive in the rRT-PCR assays (Table 2) but the level of viral RNA was low and the samples were not analysed further. In Afghanistan, 12 of 81 (15%) samples from Kabul and 10 of 20 (50%) samples from Badakhshan tested positive for FMDV RNA (Table 2). Among the positive samples from Kabul, three were collected in July 2008 (one was characterized as A-Iran05$^{\text{BAR-08}}$), four were collected in September 2008 (two were characterized as a recent variant of the serotype O-PanAsia lineage, which has been designated as O-PanAsia-III, Jamal et al., 2011a), one was collected in October 2008 and four were collected in April 2009 (one was characterized as A-Iran05$^{\text{AFG-07}}$). Only three of the ten positive oral swab samples from Badakhshan were further characterized; one was identified as A-Iran05$^{\text{AFG-07}}$, while two belonged to the recent A-Iran05$^{\text{BAD-09}}$ sublineage. Details about the dates of collection of oral swabs in live animal markets that tested positive for FMDV RNA are given in Table 3.

The relationship between the presence of FMDV RNA in apparently healthy animals and various other parameters was ascertained using multivariate logistic regression analyses. No significant difference ($P > 0.05$) was found between cattle and buffaloes, country, locality of animal markets, and year and month of sample collection on the prevalence of FMDV in these apparently healthy animals. No significant interaction ($P > 0.05$) was found between the different variables included in the model. However, a
significant difference \((P \leq 0.05)\) in the prevalence of FMDV in these animals was noted between districts from where the animals were brought to the live animal markets. The prevalence was highest in the Shewa district (in Badakhshan province of Afghanistan), Chichawatni (in Pakistan) and Kabul (Afghanistan). No significance difference \((P > 0.05)\) was found between the districts of Attock, Lahore, Sahiwal and Sanghar in (Pakistan) and Nangarhar (in Afghanistan).

Oral swab samples from dairy colonies

A total of 704 oral swab samples were collected from dairy colonies in Pakistan. These included samples from Harbanspura dairy colony, Lahore \((n = 232)\), and the dairy colony in Nagori, Karachi \((n = 136)\), which all tested negative for FMDV RNA. However, 41 of 336 \((12\%)\) oral swab samples from the Landhi dairy colony, Karachi, tested positive for FMDV RNA (Table 4). These samples were collected periodically from a cohort of 179 animals (see Materials & Methods). Locations of the source markets and the number of animals purchased are shown in Table 5.

Oral swab and serum samples were collected from these animals on the first day of their entry into the colony (Feb/March, 2009). Thirty-nine \((22\%)\) of 179 animals were found to be positive for FMDV RNA, while 130 \((73\%)\) had anti-NSP antibodies (see Table 6). All the FMDV RNA–positive animals originated from Punjab province (Table 5). Among the sera from FMDV RNA–positive animals, 29 were positive and 10 negative for anti-NSP antibodies (Table 6). The highest proportion of animals testing positive for FMDV RNA were purchased from DG Khan \((18 \text{ of } 21)\) and Pakpattan \((9 \text{ of } 14)\) in Punjab. Animals purchased from Sindh province were all negative for FMDV (Table 5). Seven of the samples that had high levels of FMDV RNA (low Ct values) were further characterized by sequencing amplicons corresponding to the coding region for the VP1 capsid protein. Five FMD viruses belonged to the A-Iran05BAR-08 sublineage, whereas two viruses grouped into the A-Iran-05 AFG-07 sublineage (Jamal et al., 2011b). Animals positive for the A-Iran-05 BAR-08 sublineage originated from two different live animal markets (four from DG Khan and one from Pakpattan), whereas the animals positive for the A-Iran05 AFG-07 viruses were purchased from the Chichawatni live animal market.

Sera from a total of 49 animals from the cohort were found negative for anti-NSP antibodies on their entry into the colony. However, FMDV RNA was detected in

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**Table 3.** Details of the FMDV RNA–positive oral swabs collected from apparently healthy animals in live animal markets

<table>
<thead>
<tr>
<th>Date of collection</th>
<th>Country</th>
<th>District of origin</th>
<th>Location of market</th>
<th>Total samples</th>
<th>Total Positive</th>
<th>Cattle positive</th>
<th>Buff. positive</th>
<th>No. typed</th>
<th>Serotype</th>
<th>Sublineage identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>July 2008</td>
<td>Afghanistan</td>
<td>Kabul</td>
<td>Kabul</td>
<td>20</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>A</td>
<td>Iran05BAR-08</td>
</tr>
<tr>
<td>Sept. 2008</td>
<td>Afghanistan</td>
<td>Kabul</td>
<td>Kabul</td>
<td>21</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>O</td>
<td>PanAsia-III</td>
</tr>
<tr>
<td>Oct. 2008</td>
<td>Afghanistan</td>
<td>Kabul</td>
<td>Kabul</td>
<td>20</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Jan. 2009</td>
<td>Pakistan</td>
<td>Sahiwal</td>
<td>Chichawatni</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Feb. 2009</td>
<td>Pakistan</td>
<td>Attock</td>
<td>Gondal</td>
<td>29</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>April 2009</td>
<td>Afghanistan</td>
<td>Kabul</td>
<td>Kabul</td>
<td>20</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>A</td>
<td>Iran05AFG-07</td>
</tr>
<tr>
<td>May 2009</td>
<td>Pakistan</td>
<td>Chichawatni</td>
<td>Chichawatni</td>
<td>23</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>July 2009</td>
<td>Afghanistan</td>
<td>Shewa</td>
<td>Badakhshan</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>3</td>
<td>A</td>
<td>Iran05AFG-07 ((n = 1))</td>
</tr>
<tr>
<td>Aug. 2009</td>
<td>Pakistan</td>
<td>Chichawatni</td>
<td>Chichawatni</td>
<td>20</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

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**Table 4.** Details of FMDV RNA–positive oral swab samples collected from animals in the Landhi Dairy Colony, Karachi

<table>
<thead>
<tr>
<th>Month of collection</th>
<th>Total samples</th>
<th>Total positive</th>
<th>Cattle positive</th>
<th>Buff. positive</th>
<th>No. typed</th>
<th>Serotype</th>
<th>Sublineage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feb/March 2009</td>
<td>179(^a)</td>
<td>39(^a)</td>
<td>12</td>
<td>27</td>
<td>7</td>
<td>A</td>
<td>A-Iran05BAR-08 ((n = 5)) (^b)</td>
</tr>
<tr>
<td>July 2009</td>
<td>36</td>
<td>2(^d)</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>A</td>
<td>A-Iran05BAR-08d</td>
</tr>
</tbody>
</table>

\(^a\)Market of origin shown in Table 5.  
\(^b\)From live animal market in DG Khan \((n = 4)\) or P. Pattan \((n = 1)\).  
\(^c\)From live animal market, Chichawatni.  
\(^d\)Tested positive after a period of 4 months in the Landhi dairy colony.
the oral swab samples from 10 of these animals. Thus, just thirty-nine of the 179 animals were found negative for both FMDV RNA (in oral swabs) and anti-NSP antibodies (in serum samples) on their arrival into the colony (Table 6).

Further oral swab and blood samples were collected in April, May, June and July from animals that had been found negative for anti-FMDV NSP antibodies in the first sampling round. The swab samples obtained in the second \((n = 45)\), third \((n = 38)\) and fourth \((n = 38)\) rounds of collection were all found to be negative for FMDV RNA. However, two of 36 samples collected in July 2009, during a fifth round of sampling, tested positive, and these came from two different farms within the colony. Only one virus could be characterized, and this was A-Iran05BAR-08. Thus, only two animals were detected as becoming positive for FMDV RNA while within the colony and these were subclinical infections. However, of the 10 animals that were positive for FMDV RNA but negative for NSP antibodies on the first day of their arrival into the colony, eight became anti-NSP antibody positive after one month. This may indicate that the animals that were positive for FMDV RNA but were negative for NSP antibodies on the first day of their arrival into the colony were either at a preclinical stage of infection or at a subclinical stage of infection. These eight animals that were anti-NSP antibody positive also included the two animals that were found to be FMDV RNA positive at the 5th round of oral swab sampling. This suggests that these two animals had become infected with a different strain of virus (probably of a different serotype) than their initial infection. Of the remaining two anti-NSP antibody–negative animals, one seroconverted after 2 months within the colony, while the other remained negative throughout the study. Furthermore, of 39 animals that were negative for both the viral RNA and anti-NSP antibodies on their first day, 27 became anti-NSP antibody positive, while nine remained negative throughout the study period and three animals lost to follow after one month in the colony (see Table 6). As sampling only occurred at intervals of one month, it is possible that some animals became infected and then recovered between the visits and thus appeared clinically healthy (and FMDV RNA negative) on each occasion.

### Table 5. Analysis of the oral swab samples taken from animals on their first day of entry into the Landhi dairy colony, Karachi

<table>
<thead>
<tr>
<th>Province of origin</th>
<th>Location of market from where the animals were purchased</th>
<th>Proportion of samples positive for FMDV RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cattle</td>
</tr>
<tr>
<td>Punjab</td>
<td>Arifwala</td>
<td>0/1</td>
</tr>
<tr>
<td>Punjab</td>
<td>Burewala</td>
<td>1/7</td>
</tr>
<tr>
<td>Punjab</td>
<td>Chichawatni</td>
<td>5/28</td>
</tr>
<tr>
<td>Punjab</td>
<td>DG Khan</td>
<td>5/5</td>
</tr>
<tr>
<td>Punjab</td>
<td>Gago</td>
<td>0/1</td>
</tr>
<tr>
<td>Punjab</td>
<td>Okara</td>
<td>0/4</td>
</tr>
<tr>
<td>Punjab</td>
<td>Pakpattan</td>
<td>1/1</td>
</tr>
<tr>
<td>Punjab</td>
<td>Khanpur</td>
<td>0/0</td>
</tr>
<tr>
<td>Sindh</td>
<td>Sanghar</td>
<td>0/0</td>
</tr>
<tr>
<td>Sindh</td>
<td>Talhar</td>
<td>0/4</td>
</tr>
<tr>
<td>Sindh</td>
<td>Tando Saidan</td>
<td>0/0</td>
</tr>
<tr>
<td>Sindh</td>
<td>TM Khan</td>
<td>0/0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>12/51</td>
</tr>
</tbody>
</table>

### Table 6. Detection of FMDV RNA (in oral swabs) and antibodies to FMDV NSPs (in serum samples) in animals on their first day of arrival into the Landhi dairy colony, Karachi

<table>
<thead>
<tr>
<th>Result</th>
<th>Anti-NSP antibodies positive</th>
<th>Anti-NSP antibodies negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMDV RNA positive</td>
<td>29</td>
<td>(10^4)</td>
<td>39</td>
</tr>
<tr>
<td>FMDV RNA negative</td>
<td>101</td>
<td>39 (10^4)</td>
<td>140</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>49</td>
<td>179</td>
</tr>
</tbody>
</table>

\(8\) Eight animals became positive for anti-NSP antibodies within the first month of their arrival within the colony. Of the remaining two negative animals, one became positive on day 60, whereas the other remained negative throughout the study period.

\(9\) Twenty-seven animals tested positive for anti-NSP antibodies within the first month of their arrival in the colony, three lost to follow, while nine remained negative throughout the study period.

The TCS trees (Figs 2, 3 and 4) were generated (using sequences determined previously, Jamal et al., 2011a,b) to ascertain the relationships of FMDV viruses of specific sublineages detected in oral swab samples from apparently
healthy animals with those detected in clinically suspect cases of FMD from Pakistan, Afghanistan, Iran and Bahrain. Two different sublineages (A-Iran05AFG-07 and A-Iran05BAD-09) within the A-Iran05 lineage were detected in the live animal market at Badakhshan. The A-Iran05AFG-07 virus (A/BAD/AFG/L3024/2009, accession number HQ439305) detected in an oral swab sample from a clinically healthy animal in the Shewa (Badakhshan) market showed 100% identity in the VP1 coding sequence with another virus (A/BAD/AFG/L2822/2009, accession number HQ439305) detected from a clinically diseased animal. The two animals were from the same district in Badakhshan, and the samples were collected one day apart. Similarly, viruses belonging to the A-Iran05BAD-09 sublineage detected in clinically healthy animals, collected in July 2009 (e.g. A/BAD/AFG/L3025/2009, accession number HQ439306), showed a single synonymous nucleotide substitution (within the VP1 coding sequence) with the viruses from the clinical cases of disease (e.g. A/BAD/AFG/L2813/2009, accession number HQ439295) collected in May 2009 from Badakhshan province (Jamal et al., 2011b). A TCS tree (Fig. 2) was generated to identify the relationships between the viruses belonging to the A-Iran05AFG-07 sublineage that originated from Pakistan and Afghanistan. The origin of A/SIN/PAK/L574/2009 (accession number HQ439244) and A/SIN/PAK/L578/2009 (accession number HQ439245) viruses, both detected from apparently healthy animals at Landhi dairy colony, Karachi, and two viruses from clinically diseased animals each from Afghanistan (A/JAW/AFG/L1439, accession number HQ439278) and Pakistan (PAK/76/2009, accession number GU384686) appears to be the same.

An A-Iran05BAR-08 sublineage virus detected from a clinically healthy animal from a live animal market in Kabul (Afghanistan) in 2008 (A/KAB/AFG/L1163/2008, accession number HQ439262) is very closely related both to the viruses responsible for clinical disease outbreaks in Bahrain in 2008 and to the viruses detected in oral swab samples collected from clinically healthy animals in Landhi dairy colony, Karachi (Pakistan), on their introduction to the colony in 2009 (see Jamal et al., 2011b). A TCS tree generated to ascertain the relationship between these viruses shows the accumulation of 6–7 nucleotide changes between them within the VP1 coding region (Fig. 3). A single non-synonymous substitution was found between the A-Iran05BAR-08 viruses detected in a sample taken on arrival within the colony (e.g. A/SIN/PAK/L693/2009, accession number HQ439246) and a virus (A/SIN/PAK/L3213/2009, accession number HQ439260) detected in a clinically healthy animal after 4 months in the Landhi dairy colony.

The O-PanAsia-III viruses (Jamal et al., 2011a) obtained from clinically healthy animals in the live animal market in Kabul in 2008 (O/KAB/AFG/L1163/2008, accession number HQ439262) are very closely related to the viruses detected from clinically diseased animals in Afghanistan (e.g. O/KAB/AFG/L643/2009, accession number HQ439233), Pakistan (e.g. O/ISL/PK/L1412/09, accession number HQ439214) and Iran (O/IRN/7/2009) in 2009 (see Fig. 4). The viruses detected in oral swab samples from Kabul (e.g. O/KAB/AFG/L1231/2008, accession number HQ439230) showed two non-synonymous
nucleotide substitutions in the VP1 coding region compared with another virus responsible for clinical disease in Kabul (O/KAB/AFG/L643/2009, accession number HQ439233). Similarly, three or four synonymous nucleotide substitutions were noted between this virus (i.e. O/KAB/AFG/L1231/2008) and certain other O-PanAsia-III viruses circulating in Pakistan (O/ISL/PAK/L1412/2009, accession number HQ439214) and Iran (IRN/7/2009) in 2009 (Fig. 4).

Discussion

FMD is endemic and widespread in both Pakistan and Afghanistan, and viruses of serotype O, A and Asia-1 are responsible for outbreaks in these countries (Schumann et al., 2008; Jamal et al., 2010). In the present study, oral swab samples were collected from clinically healthy animals and tested for the presence of FMDV RNA using sensitive and virus-specific rRT-PCR assays. This system has been used previously for the detection of FMDV in oral swab samples collected from apparently healthy animals (Klein et al., 2007, 2008; Paixão et al., 2008; Reid et al., 2009) and milk samples (Reid et al., 2006) and for the determination of within- and between-pen transmission of FMDV in pigs (Eble et al., 2006). In the present study, two production systems were examined for the purpose of detecting circulating FMDVs in the absence of clinical signs. These production systems included live animal markets in Pakistan, Afghanistan and Tajikistan plus dairy colonies, a particular production system that is present only in Pakistan. Both sectors, even if to a different extent, are characterized by the gathering together of a large number of animals from different areas and also by a high level of turnover of the animals, which may create suitable conditions for FMD to be maintained.
The present study revealed a higher prevalence of FMDV in healthy animals within the live animal markets compared with dairy colonies. This may be due to the higher frequency of mixing of animals in animal markets as compared to dairy colonies. Both serotype A (A-Iran05^AFG-07^ plus A-Iran05^BAR-08^ and a recent sublineage designated as A-Iran05^BAD-09^, Jamal et al., 2011b) and serotype O (O-PanAsia-III, Jamal et al., 2011a) FMDVs were characterized from these samples. It is noteworthy that serotype Asia-1 FMDVs were not detected in these samples from apparently healthy animals, although such viruses are in current circulation in Pakistan and Afghanistan (Jamal et al., 2011c). Ten of 20 samples collected in Badakhshan were found positive for FMDV, of which three were further characterized, one virus is within the A-Iran05^AFG-07^ sublineage, while the other two are within the A-Iran05^BAD-09^ sublineage. The high prevalence of FMDV within this live animal market either may be due to ongoing FMD in the area or to the mixing of the sampled animals, from which the samples were collected, just after their arrival from the summer pasture. The market is located in an area (province) that shares a common summer pasture for animals from Afghanistan, Pakistan and Tajikistan.

The Landhi dairy colony is the largest dairy colony in Pakistan and perhaps the biggest buffalo colony in the world (Klein et al., 2008). It has about 0.3 million large ruminants, of which 95% are water buffaloes. Most of the animals are kept for a single lactation period, and when they become dry, they are sent for slaughter or for breeding purposes. These are replaced with fresh lactating animals, which are purchased from different live animal markets in Sind and Punjab provinces. Thus, about 10–12% of the animals are replaced every month. It is thought that the Landhi dairy colony may be responsible for the maintenance and spread of FMD because of the high turnover of animals. An earlier study (Klein et al., 2008) showed that 98% of animals leaving the colony for being slaughtered were positive for antibodies against FMDV NSPs. These antibodies are generated within infected animals and also within animals vaccinated against FMD using non-purified vaccines.

In the present study, a cohort of animals, entering for the first time into the colony as replacement animals, were observed for 4 months to ascertain whether they became infected. Only two animals were found to become positive for FMDV RNA while being kept for 4 months in the colony, the virus responsible for one of these infections was characterized as being A-Iran05^BAR-08^. However, 39 of 179 animals were found to harbour FMDV RNA on the first day of their entry into the colony. Among these 39 animals, 29 were positive and 10 negative for anti-NSP antibodies (Table 6). These anti-NSP antibodies can persist for a long period (Paton et al., 2009) and do not necessarily indicate recent FMDV infection. It was also found that 130 of this cohort (73%) had antibodies to FMDV NSP proteins on their arrival at the colony (Table 6) and a further 36 animals developed these antibodies during their time in the colony (three animals lost to follow so these 36 animals constituted 78% of the animals that were initially identified as anti-NSP negative). Thus, after their time in the colony, 94% of this cohort was anti-FMDV NSP antibodies positive, which is consistent with the previous data from Klein et al. (2008). These earlier studies on oral swab samples collected from the Landhi dairy colony attributed the high incidence of the disease to the rainy season and a religious festival. The results of the samples collected in the current study from animals on the first day of their entry into the colony indicated that the newly introduced animals (purchased from live animal markets) may be an important source of FMDV, which can then be transmitted and maintained in the colony. The high incidence of the disease in the rainy season may be due to increased humidity, which may not only enhance the stability of the virus (Donaldson, 1972; Sorensen et al., 2000; Alexandersen and Donaldson, 2002; Alexandersen et al., 2002) but also impose environmental stress on the animals. Peak disease outbreaks reported between the months of January and March during 2002–2007 have also been attributed to livestock movements in Pakistan because of a religious festival in which animals are killed (Jamal et al., 2010).

High levels of FMDV RNA were obtained within some of the swab samples; these are generally expected within samples from clinically infected animals, either with acute FMD or with healing FMD lesions. However, all the oral swab samples analysed here were collected from apparently healthy animals. This may suggest that some animals were in the early stages of infection before clinical disease appeared. All the 179 animals from the Landhi colony studied here were reportedly vaccinated with an imported trivalent purified vaccine on the day of their arrival into the colony. However, as the A22/Iraq vaccine strain, incorporated in most of the commercially available vaccines, is not expected to protect efficiently against the sublineage, A-Iran05^BAR-08^ (WRL-FMD, 2009, 2010a,b,c), this vaccination may not prevent infection completely and hence subclinical infections may occur.

Phylogenetic trees (e.g. neighbour-joining, Bayesian or maximum likelihood, as reported previously for FMD viruses from this region, Jamal et al., 2011a,b,c) cluster together closely related viruses but the putative origin of each strain cannot be ascertained. The TCS trees (as in Figs 2, 3 and 4), however, indicate the sequential relationships between the different viruses. These analyses help to identify the putative transmission pathways for the
viruses. TCS trees based on complete genome sequences have been used to determine the transmission pathways of serotype O FMDVs responsible for the 2001 and 2007 outbreaks in the United Kingdom (Cottam et al., 2008a,b). A TCS tree generated using the VP1 coding sequences has also been used to ascertain the origin of outbreaks owing to serotype Asia-1 FMDVs circulating in Pakistan, Afghanistan, Tajikistan, Kyrgyzstan and Hong Kong over a period of 8 years (Jamal et al., 2011c). The TCS trees generated in the present study (Fig. 3) using the VP1 coding sequences indicate that the origin of the A-Iran05BAR-08 viruses that caused outbreaks in Pakistan and Bahrain is the same. The viruses in Pakistan (A/SIN/PAK/758/2009 and A/SIN/PAK/3213/2009) were detected in clinically healthy animals, whereas those from Bahrain (BAR/6/2008 and BAR/7/2008) were isolated from clinical cases of FMD. Similarly, the origin of outbreaks because of O-PanAsia-III virus in Pakistan (e.g. O/ISL/PAK/L1412/2009) and Iran (O/IRN/7/2009) in 2009 seems to be the same (Fig. 4). From the sequence information available, there is not any obvious difference between the viruses obtained from apparently healthy and diseased animals but complete genome analysis is required to be confident about this.

Conclusions
A higher proportion of oral swab samples collected from apparently healthy animals in live animal markets were positive for FMDV RNA compared with those collected from dairy colonies. From the oral swab samples collected from clinically healthy animals, higher numbers of the samples analysed were positive for serotype A (A-Iran05AFG-07, A-Iran05BAR-08 and A-Iran05BAD-09 sublineages) compared with serotype O. No Asia-1 viruses were identified from healthy animals. From diseased animals, sampled during the same period, the serotype A viruses were also more prevalent than serotype O and Asia-1 (Jamal et al., 2011a); however, over a longer period of time, the serotype O viruses have been the most common in this region (Jamal et al., 2010). Detection of FMDV RNA in 22% of the animals on their arrival into the Landhi dairy colony shows that the newly introduced animals are a significant source of the disease in the colony. Detection of a new A-Iran05BAR-08 virus infection in an apparently healthy animal previously vaccinated with a trivalent vaccine containing A22/Iraq is consistent with the poor antigenic match between the vaccine strain and the A-Iran05BAR-08 sublineage (WRL-FMD, 2009, 2010a,b,c), which may have resulted in a subclinical infection.

Marketing and farming systems characterized by high turnover of the animals appear to create suitable conditions for FMDV to be maintained, and it is likely that they play an important role in the chain of transmission of the disease. Collection and testing of swab samples may be an important component of a surveillance programme in endemic settings. In this regard, animal live markets have proved to be a good collection point from which FMDV RNA can be detected from healthy animals. The procedure for collecting oral swabs (in contrast to the use of probang cups) is not invasive and is usually accepted by the traders in the market.

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Epidemiology of FMD in Pakistan and Afghanistan