

Prevalence of foot and mouth disease virus in apparently healthy buffaloes brought to Islamabad slaughterhouse in Pakistan

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Received 20 April 2017; received in revised form 14 November 2017; accepted 15 November 2017

Abstract. This study was conducted to determine the sero-prevalence of foot and mouth disease (FMD) in buffaloes (*Bubalus bubalis*) slaughtered at Islamabad slaughterhouse, Pakistan. Sera and oro-pharyngeal (OP) fluid were collected from 630 healthy buffaloes. These animals originated from various livestock markets from 14 districts of Punjab. The age of animals ranged from 1 to 15 years. Sera were analyzed using FMD virus (FMDV) non-structural proteins (NSP) and indirect ELISA. The sero-prevalence of FMD was 47.1% (n=297). There was a strong association between age of animal and its risk of being positive for FMD NSP ELISA (Odds ratio 1.27; p<0.001; CI 1.22–1.32). The OP fluids were tested for the presence of FMDV by real time PCR. FMDV specific signals were detected in 33 samples (11.1%). Virus recovery attempts were made after treating them with tri-chloro-tri-fluoro-ethane onto LFBK cell line and four FMD isolates were recovered. This study indicated high sero-prevalence of FMDV in buffaloes slaughtered at abattoir in Islamabad. Furthermore, the virus recovery from these animals is suggestive of their possible role in persistence and transmission of FMDV to other animals.

INTRODUCTION

Foot and mouth disease (FMD) is a vesicular disease of cloven-footed animals including domesticated ruminants and more than 70 wildlife species. Its aetiological agent, FMD virus (FMDV), is classified within the *Aphthovirus* genus of family *Picornaviridae* (Tekleghiorghis *et al.*, 2016; Barasa *et al.*, 2008). FMD is endemic in Pakistan and causes severe economic losses in livestock industry in terms of poor production performance, high morbidity and mortality in addition to ban on the export of livestock and their products in International market (Ferrari *et al.*, 2013; Tamilselvan *et al.*, 2009; Zahur *et al.*, 2006). In FMD endemic regions like India, the FMD associated economic losses had been recorded up to 2.7–3.6 billion US dollars

annually (Ganesh, 2012) whereas, in Pakistan these losses had been estimated around 1 million US dollars annually (Zulfiqar, 2003). Further, Ferrari *et al.* (2013) documented one third reduction in milk production even after sixty days of FMD infection showing the down-regulation of production performance in dairy animals due to FMD in Pakistan.

The recovered animals, with apparently healthy conditions, exhibit persistent FMD infection and play an important role in subsequent proliferation of FMDV in susceptible populations. The FMDV can be recovered from epithelial cells of pharyngeal region, lymph nodes in the dorsal soft palate, pharyngeal tonsil, palatine tonsil, lateral retropharyngeal lymph node and mandibular lymph nodes (Juleff *et al.*, 2008). The FMDV can persist for 3–5 years in various tissues

of cattle and African buffaloes after the infection (Jori and Etter, 2016) and such animals can act as silent carriers of FMDV (Bronsvooort *et al.*, 2016).

Most of such work has been focused on cattle and African buffaloes and there are hardly any studies demonstrating the persistence of FMDV in Asian buffaloes. Therefore, a cross sectional study was conducted to determine the sero-prevalence of FMDV in Pakistani buffaloes and to investigate the presence of FMDV in apparently healthy buffaloes slaughtered at Islamabad slaughterhouse in Pakistan.

MATERIALS AND METHODS

Selection of animals and collection of data

This study was conducted on apparently healthy buffaloes which were bought to the abattoir of Islamabad, the capital of Pakistan for slaughtering. All the animals were subjected to anti-mortem examination before sampling to ascertain the presence of clinical signs and/or healed lesions of FMD infection in buccal cavity, on muzzle, hooves and mammary gland. Out of 882 animals, a total of 630 animals having no previous evidence of FMD were selected for this study.

Data regarding different determinants including age, sex, breed of animal and purchase market were recorded on a specially designed questionnaire. The study was carried out from September 2012 to December 2012. All the experimental procedures were approved by the Institutional Animal Ethics Committee of National Agricultural Research Center (NARC), Islamabad, Pakistan.

Sample collection

The selected animals (n=630) were bled for sera collection by jugular vein puncture using sterile vacutainer tubes (BD Vacutainer®, USA). The oro-pharyngeal (OP) fluid was collected through probang cup by inserting into the oro-pharynx and mixed with an equal amount of Glasgow Minimum Essential Medium (GMEM) without fetal bovine serum (FBS) and transferred to 25 ml falcon tubes

in duplicate. The collected samples were labeled and placed on icebox immediately and shipped to Animal Health Laboratories, Animal Sciences Institute, NARC, Islamabad, Pakistan. Both the sera samples and OP fluids were stored at -80°C till further testing.

Detection of FMDV specific antibodies in sera

Serum samples (n=630) were examined for the presence of non-structural proteins (NSP) antibodies against FMDV using indirect enzyme linked immuno-sorbent assay (I-ELISA; CHEKIT FMD-3ABC bo-ov kit, IDEXX Laboratories, USA) following Barend *et al.*, 2006. Briefly, the test and control sera were diluted 1:100 with diluent buffer. The diluted sera (100 µl) was dispensed in FMDV antigen pre-coated micro-titration plate wells and incubated for 60 minutes at 37°C. Anti-ruminant IgG per-oxidase conjugate (100 µl) was dispensed after rinsing into the micro-titration plate wells and re-incubated for 60 minutes at 37°C. After washing, 100 µl of TMB (3,3',5,5'-tetramethylbenzidine) substrate was added into each well and incubated at room temperature for 15 minutes in darkness followed by the addition of 100 µl of stop solution. The micro-titration plates were read using ELISA plate reader (Immunoskan MS, BDSL, Finland) at 450 nm wavelength.

Detection of FMDV specific genome in OP fluid

FMDV specific RNA was extracted from OP samples of seropositive animals using QIAamp® Viral RNA Minikit (Qiagen, GmBh, Germany) by following the manufacturer's instructions. The controls were included in each run. The extracted RNA was subjected to real time PCR (RT-PCR) using universal primers 1F *vis* 5'-GCCTGGTCTTTCCAGGTCT 3' and 1R *vis* 5'-CCAGTCCCCTTCTCAGATC-3' located in the 5' un-translated region (UTR) (Reid *et al.*, 2000, 2002). The real time PCR was performed using the core reagents kit (Taq Man®, EZ-RT-PCR core reagent). The final volume of the reaction mixture was adjusted to 25µl by adding 2.5µl of template RNA. The reaction plate was loaded and run on ABI 7500 real time PCR system (Applied Biosystems®, USA) using ABI Prism

SDS 7500 software and Ct values were recorded.

Processing of OP fluid for virus recovery

Each of the OP fluid was treated with trichloro-tri-flouro-ethane (TTE) following Kitching (2002). Briefly, TTE and OP were mixed equally and homogenized using vortex mixer followed by centrifugation at 1000×g for 10 minutes at 4°C. Then a 500µl of supernatant was placed on a 0.45 µm cellulose acetate spin X filter tube for centrifugation at 4200×g for 10 minutes at 4°C. The filtrate was used as a source of virus to infect LFBK cell line.

Isolation, propagation and confirmation of FMDV

Processed OP fluid filtrate (500 µl) was inoculated onto LFBK cell line followed by incubation for 12 hours. Thereafter, cell lines were examined for the presence of cytopathic effect (CPE) twice daily. If no CPE's were observed, three blind passages were conducted by freeze/thaw technique (Sobrinho *et al.*, 2001) and re-inoculated onto fresh monolayer of LFBK and each passage was examined for the appearance of CPE for 48-72 hours. The samples were declared negative if no CPE's appeared after three blind passages (Jae *et al.*, 2009). In most of the cases, CPE's developed after 12 to 24 hours and these included increased refractivity, rounding of the cells, detachment of the cells from the surface and cellular clumping. The isolates recovered on LFBK cell line were confirmed using RT-qPCR (Reid *et al.*, 2000, 2002).

Data Analysis

The data obtained were analyzed using Chi-square (χ^2) test and multiple logistic regression methods to calculate odds ratios.

RESULTS

The overall prevalence of anti-FMDV antibodies in buffaloes was 47.1% (n=297/630) examined at Islamabad slaughterhouse, Islamabad, Pakistan. These buffaloes were brought for slaughtering from 14 districts of

Punjab. The prevalence of FMDV in buffaloes ranged from 35% to 57.7%. The highest prevalence was observed in Kharian district and lowest in Rahim Yar Khan. All the animals of the study were brought to the market from different home territories from where the butchers have purchased them for slaughtering and there was no association of sero-prevalence of FMD with the purchase market ($\chi^2=8.52$; p=0.81). The district wise FMDV NSP sero-conversions in buffaloes are presented in Table 1. The average age of the sampled animals was 5.41±4.1 years ranging from 1 to 15 years. The prevalence of anti-FMDV antibodies in different age groups ranged from 16.8% to 76.8%. The highest prevalence (76.8%) was observed in animals over 9 years of age and lowest (16.8%) in up to 1-year-old calves (Table 1). There was a strong association between age of animal and its risk of being positive for FMD (OR 1.27; p<0.001; CI; 1.22- 1.32). The microscopic analysis of LFBK cells revealed various cytopathogenic effects of FMDV including rounding of LFBK cells, detachment, vacuolization and clumping.

The amount of the target sequence that was present in given samples of OP was analyzed and out of 297 OP fluid samples, 33 (11.1%) were positive for FMDV using RT-qPCR. Four FMDV isolates were recovered from positive OP fluid samples on LFBK cell line. The presence of FMDV in OP fluid samples was shown in Fig. 1. The graphical presentation of delta Rn plotted against PCR cycle number in the figure expressed the Ct values in exponential phase.

DISCUSSION

FMD is considered endemic in Pakistan and outbreaks are reported throughout the year in livestock and dairy production units in peri-urban areas (Jamal *et al.*, 2010). It can be speculated that virus is surviving in the susceptible population even without of apparent outbreak(s) in the herds. But such animals can be speculated as potential risk factor for recurrence of outbreaks of FMD in Pakistan. Keeping in view, this study was conducted to determine the sero-prevalence

Table 1. Prevalence of Foot and mouth disease (FMD) in buffaloes slaughtered at Islamabad slaughterhouse using indirect ELISA and real time PCR

District	Serum Samples					Probang Samples
	Total	Calves (Up to 1 year old)	Young Stock (1-3 year old)	Adults (3-9 years old)	Old Stock (Over 9 years old)	*Real time-PCR
Rawalpindi	26/53 (49.1)	3/10 (30.0)	4/15 (26.7)	14/22 (63.6)	5/6(83.3)	5/26(19.2)
Gujranwala	50/119 (42.0)	7/44 (15.9)	6/25 (24.0)	12/16 (75.0)	25/34 (73.5)	7/50(14)
Lahore	23/42 (54.8)	2/10 (20.0)	3/6 (50.0)	10/16 (62.5)	8/10(80.0)	3/23(13)
Sialkot	16/42 (38.1)	1/9(11.1)	1/5 (20.0)	9/19 (47.3)	5/9(55.6)	2/16(12.5)
Jhelum	20/39 (51.3)	1/9(11.1)	3/10 (30.0)	6/8(75.0)	10/12 (83.3)	2/20(10)
Kharian	56/97 (57.7)	1/4(25.0)	2/17 (11.8)	27/44 (61.4)	26/32 (81.3)	4/56(7.1)
Sheikhupura	25/61 (40.9)	3/21 (14.3)	5/15 (33.3)	5/18 (66.7)	5/7(71.4)	1/25(4)
Narowal	6/16 (37.5)	0/3(0.0)	1/4 (25.0)	3/6(50.0)	2/3(66.7)	1/6(16.7)
Bahawalpur	8/18 (44.4)	1/7(14.3)	1/4 (25.0)	2/3(66.7)	4/4(100.0)	1/8(12.5)
Okara	27/47 (57.5)	3/12 (25.0)	3/8 (37.5)	13/18 (72.2)	8/9(88.9)	1/27(3.7)
Multan	6/17 (35.3)	0/2(0.0)	1/7 (14.3)	3/5(60.0)	2/3(66.7)	1/6(16.7)
Gujrat	11/25 (44.0)	2/11 (18.2)	2/6 (33.3)	2/3(66.7)	5/5(100.0)	2/11(18.2)
Bahawalnagar	16/34 (47.1)	1/7(14.3)	2/6 (33.3)	4/7(57.1)	9/14(64.3)	2/16(12.5)
Rahim Yar Khan	7/20 (35.0)	1/6(16.7)	1/5 (20.0)	3/6(50.0)	2/3(66.7)	1/7(14.3)
Total	297/630 (47.1)	26/155 (16.8)	35/133 (26.3)	120/191 (62.8)	116/151 (76.8)	33/297 (11.1)

The figures in parenthesis are presenting per cent prevalence values; * = Real Time-PCR was performed on ELISA positive samples only.

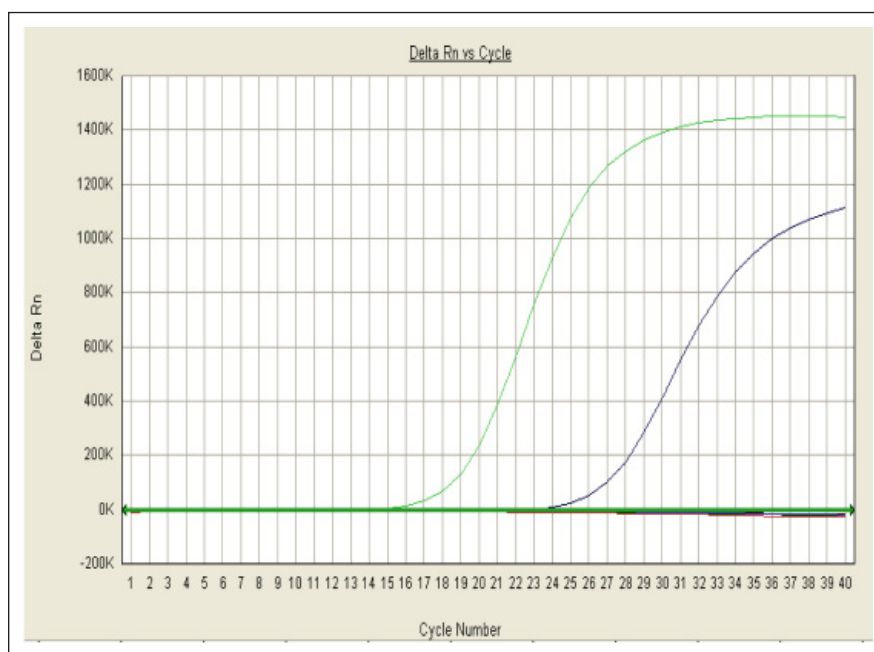


Figure 1. Graphical presentation of amplified FMD viral genome extracted from LFBK cell line during geometric phase (exponential phase).

of FMDV in apparently healthy buffaloes to rule out their role in subsequent spreading of FMDV to draw the attention of veterinary health professionals towards this important issue of animal health and production concern.

A non-specific proteins (NSP) ELISA was used as a diagnostic tool to determine the sero-prevalence of FMD. The NSPs are removed from the virus during vaccine preparation; therefore, no NSP antibodies are produced in vaccinated animals. Thus NSP

ELISA for FMD can discriminate vaccinated animals from infected animals and is an effective tool for detection of previous FMDV infection in the animals. So this test was used to detect the past exposure of FMD in apparently healthy buffaloes (Bronsvooort *et al.*, 2006).

Our study reported high sero-prevalence of FMD in buffaloes slaughtered at Islamabad slaughterhouse. However, such a high sero-prevalence has also been observed in Ethiopian (48.1%) and Kenyan (52.5%) cattle (Megersa *et al.*, 2009; Kibore *et al.*, 2013). However, other Ethiopian studies reported lower sero-prevalence in cattle (Abunna *et al.*, 2013; Duguma *et al.*, 2013). For example, 21.59% sero-prevalence was observed in cattle during 2013 at Ethiopia (Duguma *et al.*, 2013). A likely explanation of such a high prevalence of FMD in buffaloes may be fact that most of the animals slaughtered in Pakistan are either culled due to old age, poor health, disease or some other factor. Thus, many of these animals may have been exposed to FMD during their lifetime. Thus this study might have overestimated the prevalence of FMD in buffaloes in Pakistan, which may not be representative of the general population where the true prevalence may be on the lower side.

Moreover, similar to our findings, some previous studies had also reported high FMD sero-prevalence of NSP antibodies in adult animals compared to young animals (Kibore *et al.*, 2013; Olabode *et al.*, 2013). For example Kibore *et al.* (2013) reported high sero-prevalence of FMD in adult cattle (>2 years) compared to calves <1 year to 2 years. Another study conducted in Ethiopian cattle also reported high FMD sero-prevalence in adult cattle compared to young animals (Megersa *et al.*, 2009). Age is an important risk factor for many infectious diseases including FMD. This is due to the fact that older animals have a longer history of exposure to the disease agent than young animals and are thus more likely to develop a disease (Thrusfield, 2007).

During the present study, LFBK cells were used for the recovery of FMDV from the OP fluids. LFBK cell line can be easily maintained in the laboratory and is highly

susceptible to all serotypes and subtypes of FMDV due to presence of principal receptor for FMDV (LaRocco *et al.*, 2013). Many other primary and continuous cell lines are being used for isolation of FMDV such as bovine thyroid (BTY). The BTY is reported to be highly susceptible to a wide range of FMDV serotypes but this cell line loses its susceptibility for FMDV after multiple passages. Though BHK-21 cell line is easier to maintain but it has less susceptibility for FMDV serotypes (Ferris *et al.*, 2002, 2006).

The animals were brought to the abattoirs from 14 districts of the country. Most of these animals travelled long distance to reach abattoir for slaughtering. There is a possibility that these slaughterhouses may act as source of transmission of FMDV to other areas. The virus passages in susceptible population may give rise to new variants of the virus. For example Kitching *et al.* (2007) reported that during FMDV persistence in animals generated new variants, which were having differences in their VP1 sequence in persistently infected cattle. Therefore, a study is required to analyze the sequential differences of viruses recovered from OP fluid collected from buffaloes. Moreover, their relationship with the circulating FMDV is required.

Conclusively, high sero-conversions against FMDV in slaughterhouses, detection of causative agent in OP fluid and recovery of viruses from the buffaloes at the slaughterhouse indicates a potential role of persistent FMD infection in FMDV transmission and persistence in local large ruminant's population.

Acknowledgements. The work described here was partially supported by Agricultural Research Service (ARS), United States Department of Agriculture (USDA) implemented project No. 1940-32000-057-58, Characterization of the Local Isolates of FMD Viruses and Development of Vector-Based Vaccines. The authors are also thankful to ARS-USDA scientists Luis L Rodriguez, Dr. Zaheer Ahmed and their team for their technical support and cooperation. The authors are also thankful to Dr. Mansoor Ahmed (Director), Dr. Anjum Shahbaz (In-

charge Islamabad slaughterhouse) and Dr. Abdul Basit (Assistant Director), Livestock and Dairy Development Department, Punjab for their cooperation during this study.

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