

DETECTION OF FOOT AND MOUTH DISEASE VIRUS SHEDDING IN MILK OF APPARENTLY HEALTHY BUFFALOES AND CATTLE OF PUNJAB, PAKISTAN

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ABSTRACT

Foot and mouth disease is among the top listed livestock diseases causing severe economic losses. The aim of the present study was to determine the seroprevalence of FMD and detection of FMD virus shedding in milk of apparently healthy buffaloes and cattle of Pakistan. A total of 30 dairy farms were selected and registered in rural areas of Punjab consisting of minimum 15 animals. A total of 180 serum samples were collected and subjected to Non structural proteins (NSP) ELISA. The milk of sero-positive animals were collected and detected for the presence of FMD virus using reverse transcriptase PCR (RT-PCR). The results of current study showed overall seroprevalence 71.66% (129/180) with 65.38% in buffaloes and 76.47% in cattle. The FMD virus was detected in 24.03% (31/129) of sero positive samples. Among the FMD virus positive samples 65.51% belongs to serotype "O" and 35.48% belongs to Asia I, while none of the other serotypes were detected. The detection of FMD virus from the milk of apparently healthy buffaloes and cattle is an alarming situation and it may be considered as a potential role in the transmission of FMD.

Keywords: *Bubalus bubalis*, buffaloes, sero prevalence, FMD, Milk, RT-PCR, Pakistan

INTRODUCTION

Foot and mouth disease is an acute and infectious disease of domestic animals including cattle, buffalo, sheep, goats and swine having communicable potential (Longjam *et al.*, 2011). The disease is caused by FMD virus belonging to Family *Picornaviridae* and genus *Aphthovirus* having single stranded genomic RNA with positive polarity. There are seven reported serotypes O, A, C, Asia I, SAT 1, SAT 2 and SAT 3 of FMD virus which are antigenically and immunologically different and each serotype has a vast range of distinct subtypes (Kitching *et al.*, 2005). The difficulty regarding the control of foot and mouth disease is due to its wide host range and geographical distribution along with poor cross immunity, antigenic diversity and establishment of a carrier state (Mdetele *et al.*, 2014).

Foot and mouth disease is endemic in Pakistan with the following prevalent serotypes A, O and Asia I (Abubaker *et al.*, 2012). FMD causes

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heavy economic losses to the livestock industry such as high morbidity in adult animals, treatment costs, reduced milk production, loss of working ability in draught animals of developing countries, reproductive disorders and high mortality in young ones. FMD is also responsible for export barriers in animals and animal products (Gleeson *et al.*, 2003). The cattle population of Pakistan is susceptible to FMD with variation in the course, severity and prevalence of disease due to the fact of cross breeding of indigenous with exotic animals and import of foreign breeds (Hussain *et al.*, 2008).

The shedding of the virus plays an important role in the transmission of FMD and a prolonged, asymptomatic infection may occur in ruminants as well as the virus may be present in all body secretions of the infected animals (Thomson, 1994). In carrier state of foot and mouth disease, virus can be isolated twenty eight days after infection from the oesophageal-pharyngeal fluid and this state can be found in both vaccinated and non vaccinated animals after infection (Sutmoller, 2003). The importance of carrier state to trigger an outbreak is still controversial (Alexandersen *et al.*, 2002). It is reported that the virus can persist for more than three years in cattle and it can survive up to 7 weeks post infection in the udders of non vaccinated cattle that were infected experimentally via intramammary routes (Burrows *et al.*, 1971). There are different routes of FMDV transmission such as direct contact, aerosols, fomites and animal products like meat, milk, semen, urine and embryos etc (Donaldson, 2000).

The objective of the present study was to determine the seroprevalence of foot and mouth disease in apparently healthy cattle of Pakistan and isolation of FMD virus from milk of seropositive cattle. The isolation of FMD virus from apparently healthy seropositive cattle could be a mechanism

for the transmission of this disease in Pakistan.

MATERIALS AND METHODS

Study design and sample collection

A cross sectional analytical study was designed with the aim to determine the seroprevalence of Foot and mouth disease in buffaloes and cattle of Punjab, Pakistan and to detect the FMD virus shedding in milk of seropositive but apparently healthy animals. Thirty rural dairy farms having minimum 15 animals were selected and registered. The selected farms also had a previous history of FMD outbreak within 6 months. To make the total of 180 samples, 6 actively lactating animals were selected randomly from each registered (n=30) dairy farm. All the animals were examined properly for any signs and symptoms of current FMD infection. Blood samples were collected in gel and clot activator tubes (Improvacuter, China) from jugular vein and transported immediately to laboratory. The blood samples were allowed to clot for an hour at 4°C and centrifuged to separate the serum. The serum was stored in sterile eppendorf tubes at -20°C.

Detection of non structural proteins (NSP) antibodies of FMD

The NSP antibodies against foot and mouth disease virus were detected in serum samples (n=180) with the help of 3ABC-ELISA SVANOVIR kit (Svanova Diagnostic, Sweden) as the procedure described by (Nawaz *et al.*, 2014). Precisely, the test sera along with positive and negative controls were added to 96 well plates coated with FMD 3ABC antigen and incubated at 37°C. After washing, HRP conjugated antibodies were added and again incubated. After washing

TMB substrate was added and incubated in dark. The reaction was terminated by adding stop solution. The optical density (OD) of the samples was measured at 405 nm in (Biotek, USA) ELISA reader.

Collection of milk samples from FMD sero positive animals

The milk samples were collected only from those animals that were declared positive by NSP ELISA test. A total of 10 ml milk was collected from each animal in a sterile falcon tube and shifted to the laboratory using ice box. The milk samples were processed as soon as possible for the extraction of viral RNA.

Detection of FMD viral genomic RNA from milk samples

Milk samples were subjected to genomic extraction with QIAamp Viral RNA Mini kit (Qiagen, Dusseldorf, Germany) as per the manufacturer's guidelines. The extracted RNA was eluted in 50 µl elution buffer. Rapid reverse transcription and amplification of viral RNA was performed by one step RT-PCR kit (Affymetrix Inc, USA) according to the standard procedure. The primer sequences used for amplification were (IF: 5'GCCTGGCTTCCAGGTCT3'/ IR: 5'CAGTCCCCTTCTCAGATC3') and the final volume of reaction mixture was adjusted to 50 µl as described by (Reid *et al.*, 2000). The amplicon was identified on 1.5% ethidium bromide stained agarose gel by electrophoresis.

Confirmation of FMD virus serotypes

An indirect sandwich ELISA developed in United Kingdom by Institute for Animal Health was employed for detection of various FMD virus serotypes from RT-PCR positive samples as

described by (Nawaz *et al.*, 2018).

RESULTS AND DISCUSSION

Pakistan is a truly agricultural state and livestock have a great importance in the economy of this country. It is responsible for nearly 11.4% of total GDP and contributes 58.3% of agricultural value added. The total population of buffaloes and cattle in Pakistan is 37.7 million and 44.4 million heads respectively and majority of them are residing in Punjab, Pakistan. A total of 56080 thousand tons milk production was recorded during the year 2016 to 2017 consisting of 34122 thousand tons from buffaloes and 20143 thousand tons from cattle (Economic survey of Pakistan, 2016).

Foot and mouth disease (FMD) is endemic in Pakistan prominently with serotype "O", "Asia I" and "A". The role of milk in the transmission and spread of FMD virus is still not clearly understood in developing countries of Asia particularly in animals which were naturally exposed to FMD or became a carrier. The animals from which we can recover live virus 28 days post infection are known as carriers and approximately half of the recovered animals become carrier irrespective of their vaccination status (Parida, 2009). In the current study the seroprevalence of FMD in apparently healthy buffaloes and cattle was determined with the help of NSP ELISA and isolation of FMD virus from the milk of NSP sero-positive animals from Punjab, Pakistan.

The results of the present study indicated the overall seroprevalence of FMD in buffaloes and cattle of selected farms of Punjab was 71.66% (129/180). The prevalence of antibodies was found highest in exotic cows (79.16%) as compared to cross breed (76.66%) and local indigenous

breeds (72.91%) making the cumulative cattle seroprevalence 76.47% while in buffaloes it was 65.38% as shown in Table 1.

NSP ELISA is a valuable tool to affirm current or previous replication of virus in the host, irrespective of vaccination condition. The inactivated FMD vaccine have moderately purified virus without NSP in them. Therefore, the response against these NSP's in unvaccinated animals is indication of exposure to live virus instead of a response to vaccine (Diego *et al.*, 1997). The results of the presented study are very close to the previous findings 72.62% of (Lazarus *et al.*, 2012) and 75.1% of (Olabode *et al.*, 2013). In contrast a lower level of antibodies was found against FMD in the results of (Mwiine *et al.*, 2010); (Kibore *et al.*, 2013) with 61% and 52.5% respectively. On the other hand (Nawaz *et al.*, 2014) reported FMD seroprevalence nearly 20% in Punjab, Pakistan. This huge variation in the prevalence percentage of FMD was might be due to spatiotemporal difference and difference in the animal selection in these studies, as in present study, the animals were selected who are infected with FMD in the past six months.

The seroprevalence of FMD was found 65.38% and 76.47% in buffaloes and cattle respectively. These results are in accordance with the conclusions of (Nawaz *et al.*, 2014), (Abubakar *et al.*, 2009); (Abubakar *et al.*, 2012). One of the reasons behind these results is as a permanent resident of Indo-Pak region, buffaloes are more resistant to the circulating field virus and secondly the induction of massive exotic blood and cross breeding increases the trend towards high vulnerability of cattle towards FMD (Zulfiqar, 2003).

Among the NSP positive (n=129) samples, the FMD viral RNA was detected in the milk of 24.03% samples (31/129) with the help of reverse transcriptase polymerase chain reaction (RT-PCR)

as shown in Figure 1. The presence of FMDV in the milk of healthy animals is an alarming situation. The animals are continuously secreting FMD virus in milk without any clinical signs posing a risk to lives, young calves and transmission of FMD to other animals. These results coincide with the previous findings of (Reid *et al.*, 2006) that FMD virus can be detected in milk samples up to 23 day post infection. A recent finding in Islamabad, Pakistan is also in agreement to our results (Ahmed *et al.*, 2017). The virus shedding in milk can possibly infect other animals by three different ways: firstly, if they drink the milk, secondly by inhaling droplets or aerosols and thirdly the milk may contaminate people who handle animals (Sellers, 1969).

The RNA positive samples were subjected to indirect sandwich ELISA and found that 64.51% of the samples (20/31) were belonged to serotype "O" and remaining 35.48% were serotype "Asia I" (11/31) of FMD virus. These results are very similar to the findings of (Jamal *et al.*, 2010; Abubakar *et al.*, 2012; Nawaz *et al.*, 2018) in Pakistan. While in the neighboring countries (Bhattacharya *et al.*, 2005) found the same pattern with least involvement of serotype "A" in India.

The present study concludes that higher sero conversion against FMD virus and secretion of FMD virus in milk indicates its possible potential role in the spread and transmission of FMD in buffaloes and cattle of Pakistan. There is a need of comprehensive comparative study between healthy vaccinated animals, outbreak recovered animals and outbreak recovered vaccinated animals of the same area to establish the role of virus shedding in milk with the transmission of FMD.

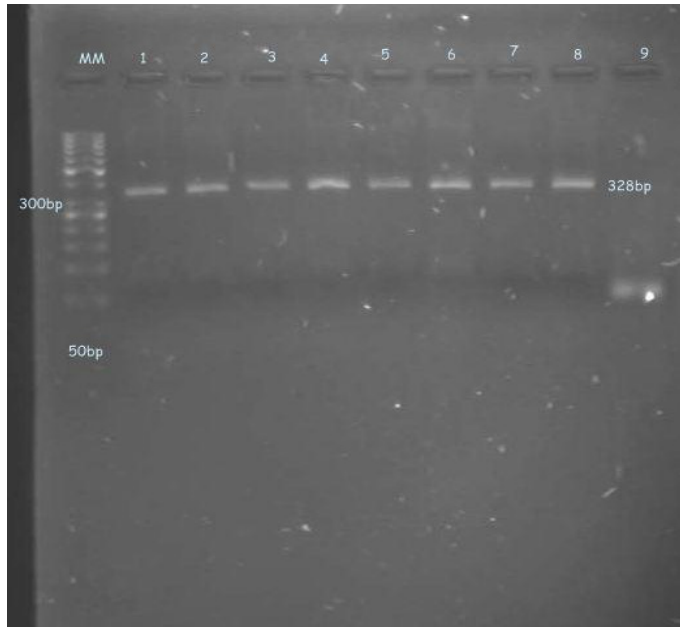


Figure 1. DNA amplified product (328 bp) of FMD virus using universal primers.
 Lane 1-7: Bands showing positive (328 bp) product of FMD virus,
 Lane 8: Band showing positive control,
 Lane 9: Band showing negative control,
 Lane M: 50 bp known ladder as marker

Table 1. Seroprevalence of FMD in buffaloes and cattle of Punjab, Pakistan.

Specie/Breed	Total Sampled	Total Positive	Percentage
Buffalo	78	51	65.38%
Indigenous/Local cattle	48	35	72.91%
Cross breed cattle	30	23	76.66%
Exotic breed cattle	24	19	79.16%
Total	180	129	71.66%

REFERENCES

- Abubakar, M., G. Ferrari, M. Hussain, E. Haq, M.J. Arshed and Q. Ali. 2009. Prevalence of foot-and-mouth disease virus serotypes in Pakistan. *Pak. J. Zool.*, **9**: 351-355.
- Abubakar, M., M.J. Arshed, Q. Ali and M. Hussain. 2012. Spatial trend of foot and mouth disease virus (FMDV) serotypes in cattle and buffaloes, Pakistan. *Viol. Sin.*, **27**: 320-323.
- Ahmed, H.I., U. Farooq, A.B. Zahur, K. Naeem, A. Latif and H. Irhsad. 2017. Evidence of foot-and-mouth disease virus excretion in the milk of apparently healthy vaccinated buffaloes in Islamabad, Pakistan. *Turk. J. Vet. Anim. Sci.*, **41**: 431-434.
- Alexandersen, S, Z. Zhang and A.I. Donaldson. 2002. Aspects of the persistence of foot-and-mouth disease virus in animals- the carrier problem. *Microbes Infect.*, **4**: 1099-1110.
- Burrows, R., J.A. Mann, A. Greig, W.G. Chapman and D. Goodridge. 1971. The growth and persistence of foot-and-mouth disease virus in the bovine mammary gland. *J. Hyg.-Camb.*, **69**: 307-321.
- De Diego, M., E. Brocchi, D. Mackay and F. De Simone. 1997. The nonstructural polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. *Arch. Virol.*, **142**: 2021-2033.
- Donaldson, A.I. and R.F. Sellers. 2000. *Foot-and-Mouth Disease. Chapter in Diseases of Sheep*, 3rd ed. In Martin, W.B. and I.D. Aitken (ed.) Blackwell Science, Oxford, UK.
- Gleeson, L.J., K. Bauer and H.A. Aidaros. 2003. A review of the status of FMD in South East Asia and approach to control and eradication. *Scientific and Technical Review*, **21**: 465-475.
- Hussain, M., H. Irshad and M. Khan. 2008. Laboratory diagnosis of transboundary animal diseases in Pakistan. *Transbound. Emerg. Dis.*, **55**: 190-195.
- Jamal, S.M., S. Ahmed, M. Hussain and Q. Ali. 2010. Status of foot and mouth disease in Pakistan. *Arch. Virol.*, **155**: 1487-1491.
- Kibore, B., C.G. Gitao, A. Sangula and P. Kitala. 2013. Foot and mouth disease seroprevalence in cattle in Kenya. *J. Vet. Med. Anim. Health*, **5**: 262-268.
- Kitching, R.P., A.M. Hutber and M.V. Thrusfield. 2005. A review of foot and mouth disease with special consideration for the clinical and epidemiological factors relevant to predictive modelling of the disease. *Vet. J.*, **169**: 197-209.
- Lazarus, D.D., W.J.G. Schielen, Y. Wungak, D. Kwange and F.O. Fasina. 2012. Sero-epidemiology of foot and mouth disease in some border states of Nigeria. *Afr. J. Microbiol. Res.*, **6**: 1756-1761.
- Longjam, N., R. Deb, A.K. Sarmah, T. Tayo, V.B. Awachat and V.K. Saxena. 2011. A brief review on diagnosis of foot-and-mouth disease of livestock: Conventional to molecular tools. *Veterinary Medicine International*, 17p.
- Mdetele, D., C. Kassanga, M. Seth and K. Kayunze. 2011. Seroprevalence of foot and mouth disease in the wildlife-livestock interface and non interface areas in Tanzania. *Research Opinions in Animal and Veterinary Sciences*, **4**: 208-211.

- Mwiine, F.N., C. Ayebazibwe, W. Olaho-Mukani, S. Alexandersen and K. Tjornehoj. 2010. Prevalence of Antibodies against foot-and-mouth disease virus in cattle in Kasese and Bushenyi districts in Uganda. *International Journal of Animal and Veterinary Advances*, **2**: 89-96.
- Nawaz, Z., M. Arshad, S.U. Rahman and Z. Iqbal. 2014. Epidemiology of foot and mouth disease in buffaloes and cattle of Punjab using non structural proteins ELISA. *Pak. J. Agric. Sci.*, **51**: 507-511.
- Nawaz, Z., S. UrRehman, A.B. Siddique, M.A. Zahoor and S. Ali. 2018. Investigations of foot and mouth disease outbreaks in different districts of Punjab, Pakistan. *Buffalo Bull.*, **37**: 81-87.
- Olabode, O.H., H.M. Kazeem, M.A. Raji and N.D. Ibrahim. 2013. Seroprevalence of foot and mouth disease virus antibodies in trade cattle (*Bos indicus*) in Kwara state of Nigeria. *Vet. World*, **6**: 828-832.
- Pakistan Economic Survey. Government of Pakistan. 2016. http://www.finance.gov.pk/survey_1617.html.
- Parida, S. 2009. Vaccination against foot-and-mouth disease virus: Strategies and effectiveness. *Expert Review of Vaccines*, **8**: 347-365.
- Reid, S.M., N.P. Ferris, G.H. Hutchings, A.R. Samuel and N.J. Knowles. 2000. Primary diagnosis of foot-and-mouth disease by reverse transcription polymerase chain reaction. *J. Virol. Methods*, **89**: 167-176.
- Reid, S.M., S. Parida, D.P. King, G.H. Hutchings, A.E. Shaw, N.P. Ferris, Z. Zhang, J.E. Hillerton and D.J. Paton. 2006. Utility of automated realtime RT-PCR for the detection of foot-and-mouth disease virus excreted in milk. *Vet. Res.*, **37**: 121-132.
- Sellers, R.F. 1969. The nature and control of foot and mouth disease. *Int. J. Dairy Technol.*, **22**(2): 90.
- Sutmoller, P., S.S. Barteling, R.C. Olascoaga, K.J. Sumption. 2003. Control and eradication of foot and mouth disease. *Virus Res.*, **91**: 101-144.
- Thomson, G.R. 1994. Foot-and-mouth disease, p. 825-952. In Coetzer, J.A.W., G.R. Thomson and R.C. Tustin (ed.) *Infectious Diseases of Livestock*. Oxford University Press, Oxford, UK.
- Zulfiqar, M. 2003. Draft report for development of national disease control policy for foot and mouth disease in Pakistan. p. 90. *Support for Emergency Prevention and Control of Main Transboundary Animal Diseases in Pakistan Rinderpest, FMD, PPR*. Pakistan.