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Evidence of foot-and-mouth disease virus excretion in the milk of apparently healthy vaccinated buffaloes in Islamabad, Pakistan

Hafiz Iftikhar AHMED, Umer FAROOQ*, Aamer Bin ZAHUR, Khalid NAEEM, Asma LATIF, Hamid IRSHAD
Animal Health Laboratories, Animal Sciences Institute, National Agricultural Research Centre, Islamabad, Pakistan

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Abstract: The aim of the study was to determine the seroprevalence of foot-and-mouth disease (FMD) and isolation of the FMD virus (FMDV) in the milk of apparently healthy Asian buffaloes. A cross-sectional study was conducted in periurban dairy farms (n = 20) on ≥20 animals per farm in the suburbs of Islamabad, Pakistan. Sera samples (n = 200) were collected to monitor FMDV seroconversions. The analysis of serum samples using ELISA indicated a high seroprevalence of FMD (78%; 156/200) in Asian buffaloes. Milk samples were collected from FMDV seropositive animals only, and virus-specific signals were detected in 12.2% (19/156) of the animals by reverse transcriptase real-time PCR (rRT-PCR). Milk samples positive for FMDV using rRT-PCR were inoculated into LF-BK cells for virus isolation. Two FMDV isolates (serotypes O and Asia-1) were recovered from the milk samples. A high seroprevalence of FMD and isolation of FMDV from the milk of healthy Asian buffaloes may represent a possible role for healthy Asian buffaloes as an FMD reservoir.

Key words: FMD, seroprevalence, ELISA, milk, buffalo, LF-BK, real time PCR

1. Introduction

Foot-and-mouth disease (FMD) is an extremely contagious vesicular viral disease of cloven-footed animals, including domestic ruminants, pigs, and more than 70 wildlife species (1). The disease has a serious impact on the socioeconomic conditions of farmers and subsequently suppresses agricultural development in poor countries, altering trade patterns besides compromises on food security (2). FMD is endemic to Pakistan with the prevalent serotypes being O, A, and Asia-1 (3). Disease outbreaks are reported throughout the year (4). Disease-related losses are estimated at 6.0 billion Pakistani rupees annually (5).

The shedding of the virus plays an important role in disease transmission and its persistence in endemic settings. Subsequent to acute FMDV infection, a prolonged, asymptomatic infection may occur in ruminants, and the virus may be present in all body secretions of the infected animal (6). In disease-recovered cattle, the virus continues to be isolated in esophageal-pharyngeal fluid (7), epithelial cells, and lymph nodes in the pharyngeal region (8). The virus is reported to persist for 3.5 years in cattle (9), 5 to 12 months in sheep and goats (10), 14 days in camels and llamas (11), and up to 5 years in the African buffalo (12). The role of infected cattle milk has previously been explored by various experts before the onset of clinical signs during outbreaks and during acute phases of the disease (10). A study reported on the survival of FMDV for up to 7 weeks postinfection in the udders of nonvaccinated cattle that were infected experimentally via intramammary routes (13). Although FMDV has been observed in the milk of cattle by real-time PCR and virus isolation (14), there is no information regarding the presence of the virus in the milk of buffaloes.

The present study aimed to determine the seroprevalence of FMD in healthy vaccinated Asian buffaloes and isolation of FMDV from the milk of seropositive animals for FMD. The isolation of FMDV from normal, healthy vaccinated buffaloes that experienced FMD during the previous 6 months could have a possible role in the persistence and transmission of the virus in endemic settings in Pakistan.

2. Materials and methods

2.1. Selection of animals and blood collection

Twenty periurban dairy farms possessing more than 20 buffaloes (*Bos bubalis bubalis*), vaccinated against FMDV serotypes O, A, and Asia-1, and having a previous history of FMD during the previous 6 months in Islamabad, Pakistan, were registered. Ten lactating animals from each farm were selected randomly for the study. Each animal

* Correspondence: umerfarooq@usa.com

was examined before sampling to ascertain the presence of clinical signs and/or healing lesions of FMD infection. These clinical signs include FMD blisters in the buccal cavity, muzzle, hooves, and mammary glands. In total, 200 blood samples were collected from buffaloes by jugular vein puncture using vacutainer tubes (BD Vacutainer Serum, Franklin Lakes, NJ, USA). Blood was transferred to the Animal Health Laboratories, Animal Sciences Institute, National Agricultural Research Centre (NARC) in Islamabad in a cold box within 1–2 h. These blood samples were allowed to clot overnight and sera were harvested in labeled 2-mL cryovials after centrifugation at 600 × *g* for 10 min and stored at –20 °C until further use. Ethical approval for this study was given by NARC's Institutional Animal Ethics Committee.

2.2. Detection of FMDV specific antibodies in sera

Serum samples (n = 200) were analyzed for the presence of NSP antibodies against FMDV by indirect enzyme-linked immunosorbent assay (I-ELISA bo-ov kit, Idexx Laboratories, Westbrook, ME, USA). The assay was performed as described by Brocchi et al. (15). In brief, the test sera and control sera (positive and negative controls) were diluted with diluent buffer at a concentration of 1:100. The diluted sera (100 µL) were dispensed in FMDV pre-antigen coated microtitration plate wells and incubated for 60 min at 37 °C. After rinsing the microtitration plate, 100 µL of antiruminant IgG peroxidase conjugate (Westbrook, ME, USA) was dispensed into the microtitration plate wells and again incubated for 60 min at 37 °C. After washing, 100 µL of TMB (3,3′, 5,5′-tetramethylbenzidine, Westbrook, ME, USA) substrate was added to each well of the microtitration plate. The plate was incubated at room temperature for 15 min in the dark and then 100 µL of stop solution was added to each well. The microtitration plate was read with an ELISA plate reader (Immunoskan MS, BDSL, Brussels, Belgium) using a 450 nm filter.

2.3. Collection of milk sample from NSP-positive animals

The milk samples were only collected from buffaloes that tested positive with I-ELISA. Milk from each quarter (10 mL) was collected in a sterilized 15-mL falcon tube and transported to the Animal Health Laboratories, Animal Sciences Institute, NARC in Islamabad in a cold box within 1–2 h. These milk samples were stored at –70 °C until further use.

2.4. Detection of FMDV-specific genome in the milk samples

FMDV-specific RNA was extracted from the milk samples using a QIAamp Viral RNA Mini kit (Qiagen, Düsseldorf, Germany) following the manufacturer's instructions. The controls (positive and negative) were included in each run. The extracted RNA was subjected to reverse transcriptase real time PCR (rRT-PCR) using a primer set described by Reid et al. (16). Core reagents kits (Taq Man, EZ-RT-PCR core reagent and N808-0236, Applied Biosystems, MA, USA) were used during rRT-PCR. The final volume of the reaction mix was adjusted to 25 µL by adding 2.5 µL of template RNA. The reaction plate was loaded and run on an ABI 7500 real-time PCR system (Applied Biosystems, MA, USA) using ABI Prism SDS 7500 software, and Ct values then were recorded.

2.5. Processing of milk samples for recovery of FMDV on cell culture

Milk samples positive using rRT-PCR were thawed in a biosafety cabinet. The milk samples were treated with trichlorotrifluoroethane (TTE, 133266.1612; 1, 1,2-triclorotrifluoro etano, Panreac Quimica SA, Barcelona, Spain) following Kitching et al. (17). In brief, an equal amount of TTE and milk sample (1 mL each) were thoroughly mixed, followed by centrifugation at 1000 × *g* for 10 min at 4 °C. Next approximately 500 µL of clear supernatant was placed on a 0.45-µm cellulose acetate spin X-filter tube for centrifugation at 16,000 × *g* for 10 min at 4 °C. The filtrate obtained was used to infect the cell line.

2.6. Isolation and propagation of FMDV

The LF-BK cell line derived from bovine calf kidney cells grown in 25-cm^2 cell culture flasks was used for FMDV recovery. An amount of 500 µL of filtrate was inoculated in duplicate to 25-cm^2 cell culture flasks for virus isolation. This cell line was maintained on Dulbecco's modified Eagle's medium-high glucose (DMEM, D7777-50L, Sigma-Aldrich, Steinheim, Germany) supplemented with 2% bovine serum albumin. The infected cell culture was examined for the appearance of cytopathogenic effect (CPE) for 48 h. In the case of no CPEs, these cells were frozen/thawed and inoculated onto fresh cultures and examined for CPEs for another 48 h (18). The CPEs were shriveling, swelling/rounding of the cells, increased refractivity, detachment of cells from the flask surface, and clumping of cells. If no CPEs appeared after 3 blind passages, the sample was declared negative.

2.7. Confirmation of FMDV isolates

FMDV isolates were confirmed by rRT-PCR (16) after extracting viral RNA. These FMDVs were recorded using indirect sandwich ELISA (19).

3. Results

The overall seroprevalence of FMD in buffaloes in these farms (n = 20) was 78% (156/200), ranging from 40% to 100%.

FMDV-specific signals were detected in 12.2% (19/156) of the milk samples by rRT-PCR.

A total of 10.5% (2/19) FMDV was recovered from the milk on the LF-BK cell line. The FMDV isolates obtained were confirmed by rRT-PCR and characterized as serotypes O and Asia-1 using indirect sandwich ELISA.

4. Discussion

Pakistan is one of the developing countries where FMD is endemic in nature. The gross milk production of the country during 2013 and 2014 (July to March) was recorded as 50,990,000 tons, 31,252,000 tons of which was buffalo milk (20). The role of milk in the spread and maintenance of FMDV has not yet been explored in developing countries and especially in normal, healthy vaccinated animals, which may be the carriers of the disease. The carrier animals are defined as those that actively secrete virus in their excretions even after 28 days postinfection. In the present study, the seroprevalence of FMD in healthy buffaloes was determined along with isolation of FMDV from milk of seropositive healthy Asian buffaloes kept in periurban dairy colonies located in Islamabad, Pakistan.

In this study, NSP ELISA was used to detect the presence of FMD antibodies in the serum. NSP ELISA is simple to perform and suitable for large-scale serological surveillance in countries such as Pakistan (21). NSP antibodies against FMDV are produced in animals either by the use of impure vaccination or in the case of infection (22,23). Therefore, NSP fractions are removed from FMDV vaccines during preparation. Therefore, the presence of NSP antibodies in serum samples indicates ongoing viral activity in these ruminants. This means that detection of antibody response to the nonstructural polyprotein 3ABC using ELISA seems to be the most reliable indicator of a previous infection. The indirect ELISA used in this study can detect 3ABC antibodies, which are higher than other NSPs (24). Furthermore, 3ABC ELISA has already been used in ruminants to differentiate infected and vaccinated animals (25).

The seroconversion in buffaloes during this study is suggestive of an ongoing viral activity monitored by NSP ELISA. The overall seroconversion against FMDV NSP in buffaloes during this study was recorded as 78%. Previous studies have also reported high FMDV NSP seroconversions in cattle and buffaloes (26–28). The FMD NSP seroconversion in the present study is similar to a Nigerian study, in which a 75.1% seroprevalence was recorded in cattle (26). Another study conducted in Kenyan cattle reported a slightly lower seroprevalence (52.5%) of FMDV compared to our study (28). However, in contrast to our research, a very low seroprevalence (16%) of FMD was reported in buffaloes in 3 districts (Chakwal, Faisalabad, and Khanewal) of Pakistan (29). The difference in the prevalence rate may be number of animals and spatiotemporal differences in both studies. Moreover, there might be variation in the circulating pattern of viruses.

The animals included in this study were from periurban dairy farms, and the high percentage of FMDV NSP-positive animals may be due to the high turnover rate of animals in this type of production system. These animals are mainly kept for milk production and, after lactation, they are either slaughtered or sent back to farms for breeding, where they can get infections from disease-affected animals. These animals may sometimes travel hundreds of kilometers in various regions of the country without proper quarantine measures. This significant movement of animals in endemic areas of the country increases the chances of exposure to disease and vice versa if they are disease-affected.

The presence of FMDV in the milk of healthy vaccinated animals is quite alarming. Despite vaccination, animals may continue to secrete the virus in milk without any clinical signs. The milk from the disease-affected animals was able to cause mortalities among young calves even after pasteurization (30). Previously, the secretion of FMDV in milk was only reported in cattle (14). In the present study, the milk was treated with TTE. The purpose of this treatment was to dissociate the FMDV from the secretory antibodies present in the milk. Furthermore, TTE treatment eliminates bacterial and fungal contaminants and other inhibitors (31). The LF-BK cell line was used for the recovery of virus from the milk as it was reported that the cytopathogenic effects produced by the FMDV are more prominent in this cell line (18), and this cell line has already been used for the recovery of FMDV from diseased animals (16). Real-time PCR has also been used previously to detect FMDV signals from milk samples collected from FMD-affected animals (32).

During this study, seroconversions against FMDV and the evidence of FMDV excretion in the milk indicate a possible role of FMDV transmission and persistence in healthy Asian buffaloes. To our knowledge, this is the first study to report on the isolation of FMDV from the milk of healthy Asian buffaloes. However, it is important to compare the sequential differences of viruses recovered from the milk of healthy vaccinated animals with those recovered during outbreaks from the same region to pinpoint genome changes to further elucidate the role of these animals in the transmission of FMDV to other animals. Further studies are also required to estimate the duration of persistence of FMDV in healthy Asian buffaloes.

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