



Detection of Foot and Mouth Disease Viruses in Cattle Using Indirect Elisa and Real Time PCR

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Abstract

The purpose of this study was to find out how common Foot and Mouth Disease (FMD) was in cattle slaughtered at the Rawalpindi slaughterhouse in Pakistan. n=606 healthy cattle's sera and Oro-Pharyngeal (OP) fluid were collected. These animals came from ten different livestock markets throughout Punjab. The animals ranged in age from one to fourteen years. FMD virus (FMDV) nonstructural proteins (NSP) and indirect ELISA were used to examine sera. FMD was found to be present in 44.55 % of the population (n=270). The age of the animal and its chance of being positive for FMD NSP ELISA (Odds ratio 1.27; P<0.001; CI 1.22–1.32) were shown to be strongly linked. Real-time PCR was used to check for the presence of FMDV in the OP fluids. In 31 samples, FMDV-specific signals were found (11.48%). After treating the viruses with tri-chloro-tri-flouroethane and transferring them to the LFBK cell line, four FMD isolates were obtained. The prevalence of FMDV was found to be high in buffaloes slaughtered at a Rawalpindi abattoir in this investigation. In addition, the viral recovery from these animals suggests that they may play a role in FMDV persistence and transmission to other animals.

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Abbreviations: FMDV: Foot and Mouth Disease Viruses; NSP: Non-structural proteins; GMEM: Glasgow Minimum Essential Medium; IELISA: Indirect Enzyme-Linked Immunosorbent Assay; PCR: Polymerase Chain Reaction.

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Introduction

Foot and Mouth Disease (FMD) is a vesicular disease that affects cloven-footed animals such as domesticated ruminants and over 70 species of wildlife [1]. FMD virus (FMDV), the etiological agent, belongs to the Aphthovirus genus of the Picornaviridae family [2,3]. FMD is endemic in Pakistan, resulting in significant economic losses in the livestock industry due to poor production, high morbidity, and mortality, as well as a ban on the export of livestock and their products on the international market [4,5]. FMD-endemic regions such as India have reported annual economic losses of up to 2.7–3.6 billion US dollars [6], whereas these losses in Pakistan have been estimated at around 1 million US dollars [7,8]. It was documented that one-third reduction in milk production even after sixty days of FMD infection in dairy animals in Pakistan [1], demonstrating the down regulation of production performance in dairy animals caused by FMD. The recovered animals, despite appearing to be in good health, have persistent FMD infection and play a key role in the spread of FMDV in susceptible populations [9]. The FMDV can be retrieved 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 [10]. After infection, FMDV can be found in various tissues of cattle and African buffaloes for up to three years [11], and these animals can act as silent carriers of the virus [12]. The majority of this research has focused on cattle and African buffaloes, with few studies demonstrating FMDV persistence in Asian buffaloes. Therefore, a cross sectional investigation was done to establish the prevalence of FMDV in Pakistani cattle's and to investigate the presence of FMDV in seemingly healthy cattle is slaughtered at Rawalpindi slaughterhouse in Pakistan.

Materials and methods

Selection of animals and collection of data

This investigation was conducted on apparently healthy cattle's who were bought to the abattoir of Rawalpindi, Pakistan for slaughtering. All the animals were submitted to anti-mortem examination before sampling to ascertain the existence of clinical signs and/or healed lesions of FMD infection in buccal cavity, on snout, hooves and mammary gland. Out of n=902 animals, a total of n=606 animals exhibiting no previous indications of FMD were selected for this investigation. A custom prepared questionnaire was used to collect data on many determinants such as age, sex, animal breed, and buying market. The research was conducted from May to August 2021. The University of Veterinary and Animal Sciences (UVAS) in Lahore, Pakistan, approved all of the experimental protocols.

Collection of samples

The chosen animals (n=606) were led by jugular vein puncture with sterile vacutainer tubes (BD Vacutainer®, USA) for sera collection. The oro-pharyngeal (OP) fluid was collected using a probang cup inserted into the oropharynx, mixed with an equal volume of Glasgow Minimum Essential Medium (GMEM) without fetal bovine serum (FBS), and transferred in duplicate to 25 ml falcon tubes. The samples were immediately tagged and kept in an icebox before being delivered to the SB Diagnostic Lab in Rawalpindi, Pakistan. Both the sera samples and the OP fluids were kept at -80°C until they were tested further.

Sera for the detection of FMDV-specific antibodies

Following [13], serum samples (n=606) were tested for non-

structural proteins (NSP) antibodies against FMDV using an indirect enzyme linked immuno-sorbent assay (IELISA; CHEKIT FMD-3ABC bo-ov kit, IDEXX Laboratories, USA). Using diluent buffer, the test and control sera were diluted 1:100. The diluted sera (100 l) was poured into pre-coated FMDV antigen micro-titration plate wells and incubated at 37°C for 60 minutes. After cleaning, 100 l of antiruminant IgG peroxidase conjugate was poured into the microtitration plate wells and incubated for 60 minutes at 37°C. After cleaning, each well was filled with 100 l of TMB (3,3',5,5'-tetramethylbenzidine) substrate, which was incubated at room temperature for 15 minutes in the dark before being filled with 100 l of stop solution. At 450 nm, the micro-titration plates were read using an ELISA plate reader (Immunoskan MS, BDSL, and Finland).

FMDV-specific genome detection in OP fluid

Following the manufacturer's recommendations, FMDV specific RNA was isolated from OP samples of positive animals using the QIAamp® Viral RNA Minikit (Qiagen, GmbH, Germany). Each run had its own set of controls. In the 5' untranslated region (UTR), the isolated RNA was subjected to real-time PCR (RT-PCR) using universal primers 1F vis 5'GCCTGGTCTTTCCAGGTCT 3' and 1R vis 5'-CCAGTCCCCTTCTCTCAGATC3' [14,15]. The core reagents kit (Taq Man®, EZ-RT-PCR core reagent) was used to perform the real-time PCR. By adding 2.5 l of template RNA to the reaction mixture, the final volume was increased to 25 l. Ct values were recorded when the reaction plate was loaded and performed on an ABI 7500 real-time PCR machine (Applied Biosystems®, USA) using ABI Prism SDS 7500 software.

OP fluid processing for virus recovery

Following Kitching's instructions, each OP fluid was treated with trichloro-tri-fluoro-ethane (TTE). TTE and OP were mixed evenly and homogenized using a vortex mixer, then centrifuged for 10 minutes at 4°C at 1000g. The supernatant was then centrifuged at 4200g for 10 minutes at 4°C using a 0.45 m cellulose acetate spin X filter tube. The filtrate was utilised to infect the LFBK cell line with virus.

FMDV isolation, spread, and confirmation

The LFBK cell line was injected with processed OP fluid filtrate (500µl) and incubated for 12 hours. After that, cell lines were tested twice a day for the presence of cytopathic effect (CPE). If no CPEs were found, three blind passages were performed using the freeze/thaw approach [16] and re-inoculated onto new monolayers of LFBK, with each passage being evaluated for 48-72 hours for the appearance of CPE. If no CPEs developed after three blind passages, the samples were deemed negative. CPEs, which included increased refractivity, rounding of the cells, separation of the cells from the surface, and cellular clumping, appeared in the majority of patients within 12 to 24 hours. RT-qPCR was used to confirm the isolates isolated on the LFBK cell line [14,15].

Analyze the data

To generate odds ratios, the data was examined using the Chi-square (χ^2) test and multiple logistic regression methods.

Results

Anti-FMDV antibodies were found in 44.55% (n=270/ 606) of buffaloes investigated at the Rawalpindi slaughterhouse in Pakistan. These cattle were brought from ten different areas in Punjab to be slaughtered. FMDV prevalence in cattle was found

to be between 34.55% and 54.10%. Murree had the highest occurrence, while Lahore had the lowest. All of the animals in the study were transported to the market from various home areas where butchers bought them for slaughtering, and there was no link between the prevalence of FMD and the buying market ($\chi^2=8.62$, $P=0.81$). Table 1 shows the FMDV NSP conversions in cattle for each district.

The average age of the animals sampled was 5.5 ± 3.2 years, with a range of 1 to 14 years. Anti-FMDV antibodies were found in 16.22% to 61.54 % of people in various age groups. The highest prevalence (88.0%) was found in animals over 9 years old, while the lowest (8.33%) was seen in calves under a year old (Table 1).

Table 1: Prevalence of Foot and mouth disease (FMD) in cattle's slaughtered at Rawalpindi slaughterhouse using indirect ELISA and real time PCR.

District	Serum Samples					Probang Samples
	Total	Calves (up to 1 year old)	Young (1-3 years old)	Adults (3-9 years old)	Older (over 9 years old)	Real Time PCR
Rawalpindi	24/60 (40)	3/13 (23.08)	4/15 (26.67)	8/15 (53.33)	9/17 (52.94)	5/24 (20.83)
Attock	19/45 (42.22)	2/8 (25)	4/10 (40)	7/13 (53.85)	6/14 (42.86)	3/19 (15.79)
Murree	33/61 (54.10)	1/12 (8.33)	5/14 (35.71)	14/16 (87.50)	13/19 (68.42)	1/33 (3.03)
Jhelum	24/62 (38.71)	2/10 (20)	3/13 (23.08)	11/17 (64.71)	8/22 (36.36)	2/24 (8.33)
Gujrat	30/56 (53.57)	1/7 (14.29)	3/11 (27.27)	8/16 (50)	18/22 (81.82)	2/30 (6.67)
Kharian	31/68 (45.59)	3/13 (23.08)	2/15 (13.33)	11/18 (61.11)	15/22 (68.18)	3/31 (9.68)
Faisalabad	41/78 (52.56)	1/14 (14.29)	5/18 (27.78)	12/21 (57.14)	22/25 (88.00)	5/41 (12.20)
Sheikhupura	22/56 (39.29)	1/11 (9.09)	4/13 (30.77)	6/14 (42.86)	11/18 (61.11)	3/22 (13.64)
Lahore	19/55 (34.55)	1/9 (11.11)	2/13 (15.38)	7/15 (46.67)	9/18 (50.00)	3/19 (15.79)
Gujranwala	27/65 (41.54)	2/14 (14.29)	5/16 (31.25)	11/17 (64.71)	9/18 (50.00)	4/27 (14.81)
Total	270/606 (44.55)	18/111 (16.22)	37/138 (26.81)	95/162 (58.64)	120/195 (61.54)	31/270 (11.48)

There was a clear link between an animal's age and its probability of contracting FMD (OR 1.27; $P<0.001$; 1.22-1.32). The microscopic examination of LFBK cells demonstrated that FMDV causes rounding, detaching, vacuolization, and clumping, among other cytopathogenic consequences. The amount of the target sequence contained in specified OP fluid samples was evaluated, and 31 (11.48 %) of the 270 OP fluid samples tested positive for FMDV using RTqPCR.

Discussion

FMD is considered endemic in Pakistan, with outbreaks occurring throughout the year in peri-urban cattle and dairy production units [17]. It's possible that the virus is surviving in the vulnerable population despite no visible outbreaks in the herds. However, such animals could be a risk factor for the return of FMD epidemics in Pakistan. With this in mind, this study was carried out to evaluate the prevalence of FMDV in apparently healthy cattle in order to rule out their role in later FMDV dissemination and to attract veterinary health specialists' attention to this critical issue of animal health and production concern. The prevalence of FMD was determined using a non-specific proteins (NSP) ELISA as a diagnostic technique. Because the NSPs are eliminated from the virus during vaccine manufacturing, inoculated animals do not create NSP antibodies. As a result, the NSP ELISA for FMD can distinguish between vaccinated and infected animals and is a useful method for detecting prior FMDV infection in the animals. As a result, this test was utilized to detect past FMD exposure in otherwise healthy cattle [18]. The frequency of FMD in cattle slaughtered at the Rawalpindi slaughterhouse was found to be high in our study. However, Ethiopian (48.1%) and Kenyan (52.5%) cattle have also been shown to have such a high incidence [19, 20]. Other Ethiopian

research, on the other hand, found a lower frequency in cattle [21, 22]. In Ethiopia, for example, the prevalence of the disease was found to be 21.59 % in cattle in 2013 [22]. The fact that most of the animals slaughtered in Pakistan are either culled owing to old age, poor health, sickness, or some other condition may be a possible explanation for such a high frequency of FMD in cattle. As a result, many of these animals may have been exposed to FMD at some point in their lives [9]. As a result, the prevalence of FMD in cattle in Pakistan may have been overstated in this study, which may not be typical of the broader population, where the true frequency may be lower. Furthermore, prior research have revealed significant FMD prevalence of NSP antibodies in mature animals compared to young animals, which is consistent with our findings [20,23].

For example, [20] found that mature cattle (>2 years) have a higher prevalence of FMD than calves, who are susceptible to all serotypes and subtypes of FMDV due to the existence of the FMDV main receptor [24]. Many different main and continuous cell lines, such as bovine thyroid, are employed to isolate FMDV (BTY). The BTY has been reported to be extremely susceptible to a wide range of FMDV serotypes, but after repeated passes, this cell line loses its FMDV susceptibility. Though the BHK-21 cell line is less susceptible to FMDV serotypes, it is easier to maintain [25-27]. The animals were transported to the abattoirs from ten different districts throughout the country. The majority of these animals had to travel a significant distance to reach the abattoir where they would be slaughtered. There's a chance that these slaughterhouses could be a source of FMDV transmission to other places. As the virus spreads among a susceptible population, new virus variations may emerge. For example, [28] found that FMDV persistence in animals resulted

in the emergence of novel variants with variations in their VP1 sequence in persistently infected cattle [29]. As a result, research on the sequential differences of viruses isolated from OP fluid collected from calves is required. Furthermore, they must have a relationship with the FMDV that is now circulating. In conclusion, high conversions against FMDV in slaughterhouses, discovery of the causal agent in OP fluid, and viral recovery from cattle at the slaughterhouse suggest that chronic FMD infection may play a role in FMDV transmission and persistence in the local large ruminant population.

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