



RESEARCH PAPER

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Complement fixation analysis for seroepidemiology of foot-and-mouth disease in District Muzaffargarh

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Key words: Foot and mouth disease, Complement fixation test, Epidemiology, Seroprevalence.

<http://dx.doi.org/10.12692/ijb/13.2.154-159>

Article published on August 30, 2018

Abstract

Foot-and-Mouth Disease (FMD) is considered as most colossal due to huge economic losses caused by the disease. It affects majority of cloven-footed animals including buffaloes, cattle, sheep, goats, camels, wild animals. District Muzaffar-Garh is known as one of the most important districts in Punjab, Pakistan, with significant livestock population. The data regarding its epidemiological occurrence is insufficient at the field level. The study was designed to investigate a cross-sectional survey to determine the seroprevalence of FMDV in selected areas of district Muzaffargarh. The sampling was carried out and completed through multistage random sampling for the seroprevalence. Blood samples and epithelial samples were collected from clinically affected animals. For sera preparation, the blood samples were collected in vacutainer tubes. The sera were examined for the complement fixation test (CFT). Epithelial tissues and oropharyngeal fluids samples of clinically positive cases were collected and processed. A total of 200 samples (cow and buffalo 100 from each) were collected and processed for CFT. The current data clearly revealed that the percentage of FMD positive sample in cow (76%) was comparatively higher as compared to a percentage (68%) in buffalo (but statistically the ratio of positive samples in cow and buffalo was non-significant ($P>0.05$)). The data showed that 68 samples out of total 100 samples were positive for buffalo and 76 samples out of total 100 samples were positive for the cow.

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Introduction

Livestock sector in Pakistan has an imperative importance in agriculture economy. The livestock sector is considered as the backbone for sustaining the livelihood of 35-40 million people. This sector is playing an important role in the daily life of people and serves as a source of highly nutritive food in the form of milk and meat. In nation GDP of Pakistan, the share of livestock sector is 11.7%, while being a major contributor in the agriculture sector of Pakistan, its contribution is 58.6% in agriculture economy (Economic Survey of Pakistan, 2016-2017).

Nature has blessed Pakistan with unique geography, environment, and natural resources. The most populous province of Pakistan is Punjab (both in terms of human as well as livestock populations). In this province, there are 17.75 million buffalo and 14.41 million cattle (Livestock Census, 2006). Most of the buffalo population (>71%) is located in irrigated areas of Punjab and this contributes around 70% of total milk production in the country (Bilal *et al.*, 2006). The milk from these animals is also supplied in markets located in urban areas to earn income (Bilal *et al.*, 2006).

Numerous factors including management, feeding, reproduction and disease control are the key factors which determine the economy of the farmers. The benefits achieved from these animals are adversely affected by poor husbandry/ management practices, inadequate supply of feed and fodder and incidence of infectious diseases. Foot-and-Mouth Disease (FMD) is the most common viral disease of dairy animals (cattle and buffalo) which includes many regions. These regions include Asia, Australia, Middle East, Africa, New Zealand, America and Chile (Brito *et al.*, 2017).

Overall case fatality and morbidity rates caused by Foot-and-Mouth Disease (FMD) are 2.12% and 11.89% in cattle and buffalo respectively (Pattnaik *et al.*, 2012). FMD is known as a highly contagious disease in cloven-footed animals (Kitching, 2005) including domesticated buffalo, cattle, goats, sheep, and deer. Bison, African buffalo, Wild boar, Yak,

Asian and African elephants and Fallow deer also suffer from the disease (Schaftenaar, 2002). In all Pakistan FMDV isolated between 2005 and 2008 revealed homology to Pan Asia serotypes (Saeed *et al.*, 2011). However, FMD serotype O PanAsia 2 lineage was predominantly found in southern Punjab, Pakistan (Khan A., *et al.*, 2011).

Trade of infected/ contaminated animals and their products & by-products play an important role in the spread of trans-boundary diseases including Foot-and-Mouth Disease (Sutmoller *et al.*, 2003). In Pakistan, Foot-and-Mouth Disease (FMD) is considered as an endemic in cattle and buffalo and its outbreaks are reported every year across the country (Jamal *et al.*, 2010). Outbreaks may occur even after multivalent vaccines (using inactivated gel based) (Knowles *et al.*, 2005). Uncontrolled/ unchecked livestock movement from one area/province to other, increasing the contact of healthy animals with infected animals and unavailability of an effective vaccine is also the key factor for Foot-and-Mouth Disease outbreaks in thickly livestock populated areas (Brito *et al.*, 2017). By practicing effective vaccination programs along with controlled/checked livestock movement and proper animal's hosing disinfection are the key factors to control Foot-and-Mouth Disease (Singh *et al.*, 2007).

The currently available data/ research studies in Pakistan is insufficient to reflect the true status of disease in the country because most of the research has been conducted at farm level (Abubakar *et al.*, 2012). There is a dire need to collect the data from the field level to investigate the true picture of Foot-and-Mouth Disease in field level. Different serotypes involved in disease outbreak also need to be investigated since there is no cross-protection among serotypes. The diagnosis of FMD outbreaks are generally based on clinical signs. In Pakistan, the currently available research data is not sufficient to depict the real magnitude of Foot-and-Mouth Disease. Therefore, the present study has been designed to investigate the epidemiological aspects and the risk factors associated with Foot-and-Mouth Disease prevalence.

Materials and methods

Study area and sample collection

The study was conducted in different areas of District Muzaffargarh during the period of Jan-March 2018 and the epithelial and blood samples were collected from cow and buffalo ($n=200$; 100 from each species). These samples were transferred and processed in the BSL-3 laboratory and the Institute of Microbiology, University of Agriculture Faisalabad.

Preparation of antigen

Epithelium samples from infectious bovine tongue were injected intra-dermally in rats. After 24 hours, the fluid was aspirated from vesicles. This was further processed for centrifugation @1500 rpm for 15 min. The vesicular fluid was stored at -20°C .

Serum preparation

Serum was separated from the blood samples by centrifugation at 600 rpm for 3 minutes. Serum samples were inactivated through heating at 56°C for 30 minutes & for further use, it was stored at -20°C for further use.

Compliment

The compliment was taken from guinea pig as described by (Sedeh *et al.*, 2008).

Washing of sheep red blood cells (rbc's)

A 10 mL of blood was taken from sheep with a sterilized syringe (10mL) in vacutainer. The blood was processed by centrifugation. The washing was done through the following steps;

- I. Centrifugation of blood was performed at 1500 rpm for 15 minutes in centrifuge tubes.
- II. Plasma and serum was discarded after centrifugation
- III. Normal saline was added to the settled RBC's and volume was made to 13mL. Mixing was done gently and slowly to avoid lysis of RBC's.
- IV. Centrifugation was performed 2nd time for 3-5 minutes.
- V. The supernatant was discarded and steps III and IV was repeated for 3 times.

- VI. Finally, the supernatant was discarded and 40% suspension of washed RBC's was performed for further use.

Dilution Of Rbc's

To make a total volume of 100mL, 98ml normal saline and 2mL sheep RBC's was mixed gently.

Developing Amboceptors In Rabbits

Antibodies produced against sheep RBC's (known as amboceptors) were used in CFT. Washed RBC'S were injected to rabbit according to the maintained schedule.

Blood Collection

After 2 weeks (14 days) of the last injection in rabbits, blood was collected (10 mL) from these rabbits. Centrifugation of blood sample was performed at @600 rpm for 5 mins and the serum was separated. The serum sample was inactivated through heating at 56°C for 30 mins and was stored at -20°C .

Titration Of Amboceptor

The amboceptors were titrated according to the following protocol,

- I. Amboceptors were diluted 2 folds.
- II. In each well, 2% RBC's were added
- III. Agglutination was checked for 4HA.
- IV. The dilution of this 4HA was performed.
- V. 50 μl of amboceptor from this dilution was mixed with 50 μl of 2% RBC's.

Titration Of Complement

- I. The complement was diluted (2 fold).
- II. This diluted complement was added with 50 μl of 2% RBC's
- III. The complement was 2 fold diluted and it was added with 50 μl of amboceptor and 50ul of 2% RBC'S and 50ul of Amboceptor.
- IV. Highest dilution at which lysis occurred was observed.
- V. 8HA was selected and diluted for further use.

Procedure For Complement Fixation Test

- I. A 50 μl of normal saline in all wells of titer plat
- II. A 50 μl serum in all wells except 12th well
- III. A 50 μl of antigen was add in all wells

- IV. Plate was incubated at 37°C for 15 min
 V. A 50 µl of sheep RBC's were added in all wells
 VI. A 50 µl of amboceptor in all wells
 VII. The plate was incubated at 37°C for 1hour
 VIII. A 50 µl of complement was added in all wells except for 12th well

Results

The objective of current study was to determine the seroprevalence of Foot-and-Mouth Disease in district Muzaffargarh.

Evaluation parameters

The samples were collected from clinically positive cow and buffalo. No. of positive samples was examined through the complement fixation test. A total of 200 samples (100 from each species) were collected and processed for CFT. The no. of positive samples (through complement fixation test) are given in table 1. The formation of button in the multiwell plate indicated positivity samples while lysis indicated status of the negative samples as presented in plate 1. The current data clearly revealed that the percentage of FMD positive sample in cow (76%) is comparatively higher in cow as compared to 68% in buffalo (but statistically the ratio of positive samples in cow and buffalo was non-significant ($P>0.05$)). The data showed that 68 samples out of total 100 samples were positive for buffalo and 76 samples out of total 100 samples were positive for the cow. The graphical presentation of positive and negative samples is given in Fig. 1.

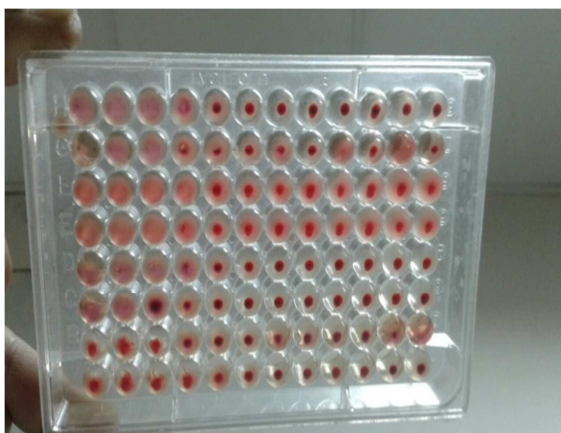


Plate 1. The button formation and lysis is indicating the status of positive and negative samples on the multiple well plate after incubation at 37°C.

Table 1. No. of the positive sample (%) of FMD with complement fixation test.

Species	Total Number of samples	Negative	Positive	Positive (%)	P- Value
Buffalo	100	32	68	68 %	0.21 ($P>0.05$)
Cow	100	24	76	76 %	
Total	200	56	144	72 %	

The data is statistically not significant at $P>0.05$.

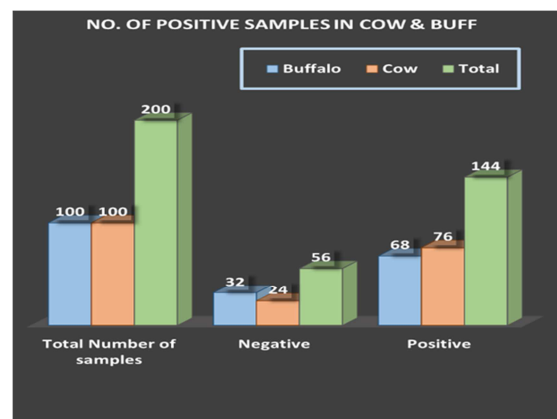


Fig. 1. Graphical presentation of positive samples for FMD through complement fixation test in cow & buffalo.

Discussion

Foot-and-Mouth Disease is enzootic in all most all parts of Pakistan. Pakistan having a large livestock population faces lots of uncontrolled infectious and noninfectious health problems. FMD is one of the most important infectious diseases, hindering the production of livestock and causing large economic losses in this sector. FMD remains mostly uncontrolled in Pakistan, as in general prophylactic vaccination is not practiced, being practiced by a small portion of farmers. In a country where FMD is enzootic like Pakistan, and where a large population of susceptible livestock exists, with imperfect vaccination, disease reporting and investigation, such serological surveys are very important in understanding the epidemiology of FMD.

To build up a prompt seroprevalence profile of the enzootic FMD occurrence in Muzaffargarh, 200 sera samples of cattle and buffalo from various area of the district were investigated, using CFT, and overall seroprevalence of 72% was obtained. This Fig. (72%) is higher from the previous prevalence reported (Abubakar *et al.*, 2012).

The difference in the results may be due to the difference in the study area within the same country. Where differences in management practices, herd size and movement of animals exists, as also attributed by (Abubakar *et al.*, 2012). The high prevalence than the previous studies may also be due to the geography of the study area, and selection of samples from clinically positive samples.

The single most important cause of increased incidence of FMD appeared to be the animal movement. Livestock markets especially the market held before the Eid ul Azha festival are playing important role in the spread of FMD in Pakistan due to large-scale movement of livestock and sheep/goat for religious consumption i.e. sacrificing animals. In many reported outbreaks, there was a history of the introduction of unsold animals from these markets into the flock when they were brought back. Prior to seventies, the FMD used to be seasonal, particularly during wheat harvesting season in Pakistan (March-April) and also in September-October, related to animal movement. It used to be a mild or sub-clinical disease and affected animals recovered in a few days.

A significant ($p < 0.05$) difference in species susceptibility was found in Buffalo (16.04%) and cattle (23.09%) which was supported by the observation of (Abubakar *et al.* 2009) on the prevalence of FMD which was 34.00% in buffaloes and 64.00% in cattle of Pakistan. It may be due to the introduction of extensive exotic cattle blood and cross-breeding which leads to high susceptibility of cattle towards FMD (Zulfiqar, 2003). In the study (Zeeshan.*al.* 2014) revealed a significant variation on the seropositivity of Foot-and-Mouth Disease among the three age groups. The significantly higher seroprevalence of FMD in young and adult animals than in calves observed in the current study is in agreement with the previous reports of Mohamoud *et al.* (2011). The low seroprevalence of FMD recorded in young calves could be associated with low frequency of exposure to disease. In addition, farmers keep their young calves near the homestead and away from the grazing adult animals (Thrusfield, 2007).

The seroprevalence of FMD was 11.50% in herds having only large ruminants and 21.57% in herds having large as well as small ruminants (mixed). In previous studies, it has been shown that small ruminants are important reservoirs for FMD infection in cattle and buffaloes (Gelaye *et al.*, 2009). The results showed significant ($p < 0.05$) difference between both groups. The seroprevalence of FMD in cattle population (76%) was higher as compared to Buffalo (68%) but no significant difference was found statistically. It may be because of the higher proportion of cattle and the free movements of cattle being brought all the year from other districts of Punjab and inter-provincial to this part of the country.

For the first time in southern Punjab, a comprehensive study was conducted for the investigation status of FMD to understand the complex situation of FMD. Results of this study shed light on the epidemiological situation of FMDV in this region and will help to develop a better understanding of the evolutionary situation of circulating virus. The genetic analysis of FMDV has emphasized the need for continuous molecular epidemiological investigations to monitor the virus serotypes and serotype-variants involved in disease outbreaks especially in comparison with serotype used in vaccination. Similar investigations are continuously required in the disease-endemic areas of Pakistan for the improvement of vaccination and disease control strategies to avoid the emergence of new outbreaks with diverse-variants of the virus.

In conclusions, the results of the present study will be useful in future for launching control programs in the country, knowing the high FMDV prevalent zones and trends and pattern of FMD in time and space.

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