Reverse transcription-polymerase chain reaction (RT-PCR) based detection and economic impact of foot-and-mouth disease in District Faisalabad, Pakistan during the year 2015

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Abstract

The aim of this study was to evaluate the economic impact of the disease by using milk production records and to determine the serotypes circulating in the region during 2015. Sampling was done from different outbreaks initially on the basis of clinical signs and later reverse transcriptase-polymerase chain reaction (RT-PCR) was employed for the conformation of FMDV genome. Out of total 88 samples, 73 were found positive which were then serotyped into type O (n=44), Asia1 (n=18) and A (n=06). The economic impact was analyzed by recording milk loss at four affected farms. Their average milk yield was observed 9.2 liters before the onset of disease that decreased dramatically after the disease. Milk loss of 225 and 195 liters was recorded for buffalo and cattle respectively, during 70 days of the study period.

الكشف عن مرض حمى القلاعية بطريقة تفاعل البلمرة المتسلسل المعاكس والتاثير الاقتصادي للمرض في منطقة فيصل آباد، باكستان خلال العام ٢٠١٥

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هدفت الدراسة لتقييم الأثر الاقتصادي لمرض الحمى القلاعية باستخدام سجلات إنتاج الحليب وتحديد الأنماط المصلية المنتشرة في المنطقة خلال عام ٢٠١٥. تم أخذ عينات من مناطق مختلفة في البداية على أساس العلامات السريرية ومن ثم تم استخدام تفاعل البلمرة المعنكس لتحديد جينات FMDV من مجموع ٨٨ عينة، تم العثور على ٧٣ حالة إيجابية ومن ثم تم تصنيفها وعلى النحو التالي نوع O (٤٤ عينة)، نوع ASIA 1 (١٨ عينة)، و نوع A (المجموع ٦ عينات). تم تحليل الخسائر الاقتصادية عن طريق خسائر انتاج الحليب في ٤ مزارع مصابة، لقد سجل معدل انتاج الحليب قبل الاصابة ٩٫٢ لتر وكان انخفاض الحليب من ٢٢٥ لتر الى ١٩٥ لتر للجاموس والابقار على التوالي خلال ٧٠ يوم فترة الدراسة.

Introduction

Foot and mouth disease (FMD) is a disease of cloven-hoofed animals and causes severe socioeconomic losses by infecting domestic animals, i.e. buffalo, cattle, goats, sheep and pigs. The causative agent of this notorious disease belongs to genus *Aphthovirus,* family *Picornaviridae* and known as FMD virus (FMDV). The virus lack envelope,

has 8.3 kilobases, +ve sense RNA genome (1). Seven serotypes (O, C, A, Asia1 and SAT1, SAT2, SAT 3, are known for this virus (2). Within each serotype, mµltiple subtypes are found which sometimes do not protect against the same serotype. The distribution of the FMDV serotype is not uniform, generally, serotype O and A are widely distributed throughout the globe as compared to other serotypes. Serotype Asia 1 is limited to the Asia, while SAT 1, SAT 2, SAT 3 are found normally in Sub-Saharan Africa and serotype C has not been reported since 2005 anywhere (3). In Pakistan, three serotypes A, O and Asia 1 are prevalent (4) and occurrence of O is more as compared to other serotypes (5-9). Signs and symptoms include lesions on foot, mouth, udder etc. as shown in the fig. 1 Pakistan is one of the top (42 million tonnes) milk producing countries in the world and 95% of milk comes from small peri-urban and rµlers livestock farmers (10,11). Main milk producing animals are buffalo and cattle contributing 62% and 34 % respectively. Production of milk from cows increased (from 7.42 to 9.4) million tonnes. 35 million peoples are involved in livestock and obtain 30-40% of their income (9,12-14). The current study focuses on effective diagnosis and calculation of economic loss due to a decrease in milk production. Very few studies on economics importance of this disease are present, although the disease is very old in its origin (15). Studies revealed 20% to 24% loss in milk production in cattle and 19% in sheep (15). Local cattle breed showed 35%, cross breed 17% and Holstein Friesian 37% decrease in milk production (16). FMD caused 19% milk loss in turkey (17) and 25% in the UK (18). Post-FMDV 216 to 370 US dollars loss was recorded in a study of 62 Cambodian farmers (19). FMD caused 7.6% to 10% burden on the income of poor livestock farmers. This interview-based study revealed that 84% of the farmers agreed to pay 1.3 US dollars/animal to protect their animals for six months (20). In Pakistan, if we invest one US dollar on vaccination we can save milk loss of 5.7 US dollars (21).

Materials and methods

Sample collection

Total 41 outbreaks were recorded during this study from district Faisalabad and 88 samples were collected in autoclaved plastic vials having a 15 ml capacity filled with 8-10 ml glycerolized buffer saline. Detail of the samples collected is given in the table 2. Samples were brought to the lab by keeping in the icebox and stored at -70C for further processing.

RNA extraction

RNA extraction was carried out with the help of PavorPrep Viral Nucleic Acid Extraction Kit (Favorgen, China) according to Manufacturer's protocol. Briefly, 570µl of lysis buffer is added to 150 µl of sample (serum/epithelial tissue supernatant) and incubated for 10 minutes at room temperature. Then ethanol (750 µl) precipitation is carried out after which RNA is transferred to silica based column and centrifuged at 8000g for 1 min. One washing with 500 µl of wash buffer1 and two with wash buffer 2 was carried out on 8000g for 1min after every washing. After washing RNA was eluted using 35µl elution buffer in a centrifuge tube for 2 min at 8000g and stored at -70º C until further use.

Figure 1: Showing lesions on the hoof and mouth epithelium during a FMD outbreak in District Faisalabad.

Complementary DNA Synthesis (cDNA)

Fermentas Revert Aid First Strand cDNA Kit was used for cDNA preparation. Briefly, 5µl RNA template was incubated with 1µl of random hexamer primer (0.2 ug/µl) and 6µl nuclease-free water at 65º C for 5 minutes than ice chilled and short spin. After that, 5X reaction buffer 4µl, Ribolock 1µl, dNTPs (10mM) 2 µl, revert Aid (reverse transcriptase) 1 µl were added into it and incubated in PCR machine with 25º C for 5 minutes, 42º C for 60 minutes and 70º C for minutes to terminate the reaction. After synthesis, cDNA was stored at -20 C for future use.

PCR

cDNA was subjected to the general FMD detection primers (1F, 1R) as well as typing primers using following procedure: 1 µl cDNA, 1 µl Forward primer (1F) (10 pM/µl), 1 µl Reverse primer (1R) (10 pM/µl),5µl 10X Taq polymerase buffer, 5µl MgCl2 (25mM),1µl 4X dNTP mix (10 mM each), 0.2 µl Taq plus DNA polymerase (5U/µl) and 35 µl Double Deionized water (ddH2O) in a micro PCR tube. Thermal cycling conditions were 94º C for 10 min, followed by 35 cycles at 95º C for 45s, 55º C for 45 s and 72º C for 45s. A final extension was carried out at 72º C for 10 min.

Table 1: The oligonucleotide primers used for RT-PCR diagnosis and serotyping

Primer *	Sequence 5' → 3'	Location	Sense	Specificity
1F	GCCTGGTCTTTCCAGGTCT	5'UTR	Forward	All types
1R	CCAGTCCCCTTCTCAGATC	5'UTR	Reverse	All types
P33	AGCTTGTACCAGGGTTTGGC	2B	Reverse	All types
P38	GCTGCCTACCTCCTTCAA	1D	Forward	Type O
P74	GACACCACTCAGGACCGCCG	1D	Forward	Type O
P75	GACACCACCCAGGACCGCCG	1D	Forward	Type O
P90	GTCATTGACCTCATGCAGACTCAC	1D	Forward	Type A
P91	GTCATTGACCTCATGCAAACCCAC	1D	Forward	Type A
P92	GTCATTGACCTTATGCAGACTCAC	1D	Forward	Type A
P76	GACACCACACAAGACCGCCG	1D	Forward	Type Asia1
P77	GACACGACTCAGAACCGCCG	1D	Forward	Type Asia1
P87	GTCATTGACCTCATGCAGACCCAC	1D	Forward	Type Asia1
P88	GTTATTGACCTCATGCAGACCCAC	1D	Forward	Type Asia1
P89	GTCATTGACCTCATGCACACCCAC	1D	Forward	Type Asia1

* Primers were adapted from previous studies (22,23).

Economic study

Milk production data were collected from 4 farms (Total no. of the animals were 75) at different FMD outbreak locations. Data of 22 clinically affected animals (10 buffalos, 12 cattle) was noted for 70 days by interview and follow up. The diagnosis was done primarily based on clinical signs that were later confirmed by RT-PCR. Data was collected in the form of a questionnaire and visit of the location. The questionnaire consists of two parts; first deal with the name, address and no. of animals at the farm while the second one deals with the milk production data of the animals. The focus was affected animals and their data collection by follow-up. The measurement of milk was done in litres usually used by the farmers to sell their milk. Milk price varies with season and during this study period; it was kept according to the Faisalabad market, i.e. 0.4 USD for cattle and 0.5 USD for buffalo per litre milk for economic loss calculations.

Results

Consensus primer pair 1F and 1R was used for the detection of the FMDV while remaining primers mentioned in the table No. 1 were used for the detection of serotypes prevalent in Pakistan viz. Type A, O, Asia 1. 88 samples were subjected to these primers collected from different outbreak regions of District Faisalabad. Six samples were found positive for serotype A, 18 for serotype Asia 1 and 44 for serotype O. Bands of 328 bp with consensus primer pair and of 292, 432 and 702 bp (fig. 2) for serotype O, Asia1 and A respectively with typing primers was found when electrophoresed by 1.5% agarose gel (Invitrogen, Carlsbad, USA), prepared in 1×Tris–borate–EDTA buffer and stained with 0.5 g/ml ethidium bromide The

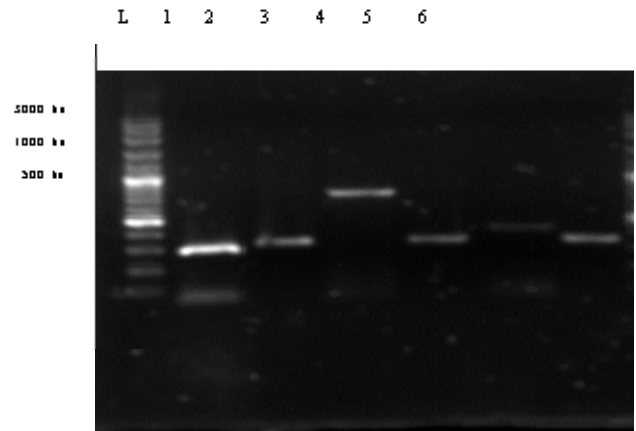amplification products were visualized using a transilluminator at 302 nm.

Figure 2: Lane 2, 4 and 6 shows the PCR product (328 bp) with consensus primer while lane 1, 3, and 5 shows the PCR products of type Asia1 (292 bp), type A (732 bp) and type O (402 bp) respectively with type specific primers**.**

Economic study

Not all questionnaire data is presented there only milk production loss is shown in Table 3 before and after the outbreak of FMD. Vaccination was absent in the majority of outbreaks and was irregular in some. Out of total 75 animals, 22 were found positive for FMD by laboratory diagnosis.

One of the main signs of FMD is a sudden drop in milk production. In this study, data of 22 lactating animals were recorded for 70 days by follow-up. Buffaloes show more decline and slow recovery in average milk production (Fig.

4) as compared to cattle which shows a rapid decline, but quick recovery (Fig. 3). In general, both graphs depict abrupt decrease in milk production at day 5-7 after the FMD outbreak and gradual recovery takes place in 65-70 days (Fig. 5). Predicted value was calculated as the mean value of production at pre-FMD (day 0) and day 70 which showed a linear trend. Total loss of 225 litres (51% of the predicted value) per lactating buffalo and 195 litres (31% of the predicted value) per lactating cattle was recorded during 70 days of this study period. Total loss of 2061 USD (per

litre price of ilk was kept 0.4 and 0.5 USD per litre for cattle and buffalo milk respectively) was recorded in 22 affected animals. We assumed expense of good quality vaccination is equal to 450 USD (3 USD/Dose) for all 4 farms (75 animals) annually (the vaccination rate was keptr). Milk production twice a year).

The ratio between predicted milk loss and vaccination was 4.58 that means that 1 USD spent on vaccination could save 4.58 USD of milk loss.

Table 2: FMDV outbreak locations and results of Faisalabad district

Administrative regions	No. of outbreaks	No. of samples	FMDV detected	FMDV typed		
				A	Asia1	O
Chak Jhumra	06	19	17	-	-	07
Jaranwala	05	07	05	02	-	05
Sadhar	18	40	38	03	13	22
Sumandri	03	09	08	-	03	08
Tandlianwala	09	13	05	1	02	02
Total	41	88	73	06 (12%)	18 (25%)	44 (60%)

Table 3: Milk production of buffalos and cows during FMD outbreak

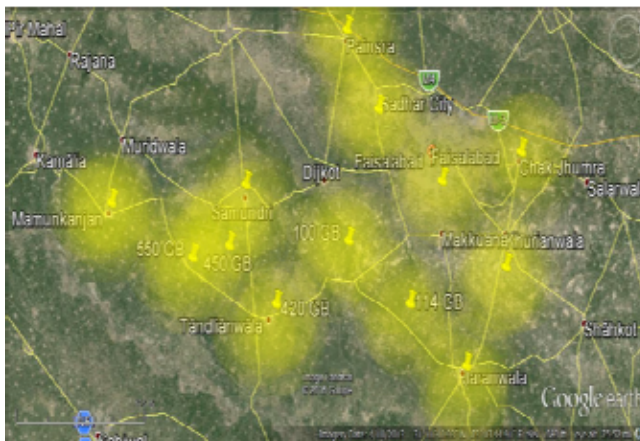|	Pre-FMDV	Day 1	Day 3	Day 5	Day 7	Day 10	Day 15	Day 22	Day 30	Day 40	Day 55	Day 70
Average	9.2	6.6	4.16	2.83	2.6	3.0	3.48	3.94	4.07	4.69	5.46	6.25
Standard deviation	3.37	2.34	1.89	2.84	2.76	3.01	2.83	3.29	3.82	3.44	4.36	4.77

Figure 4: Map depicts outbreak zones of 10 Km radius that were generated using Google earth software.
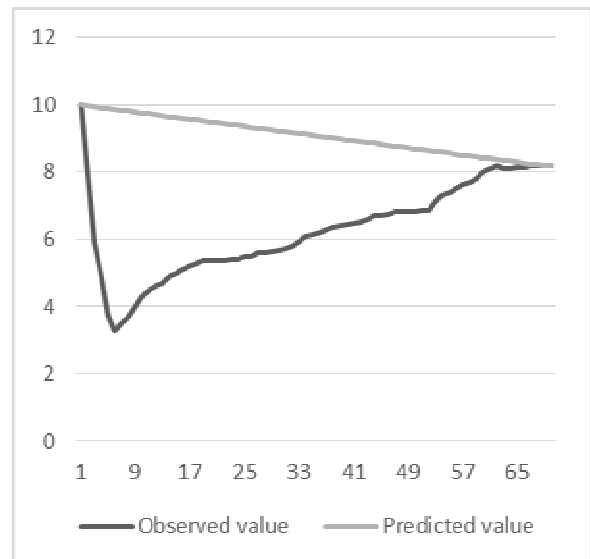

Figure 5: Observed and predicted data of 12 cattle after and before FMD onset.
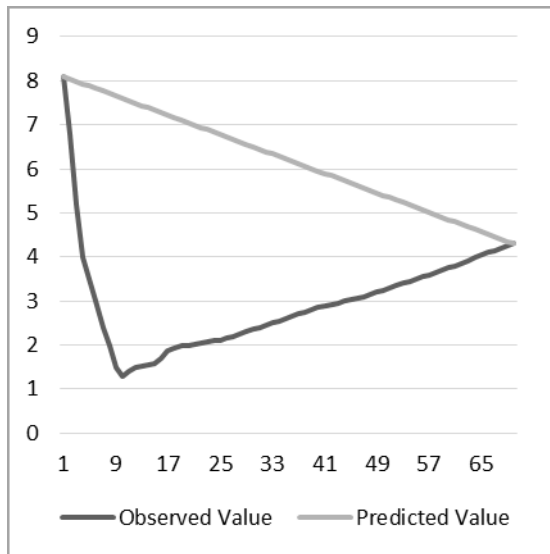
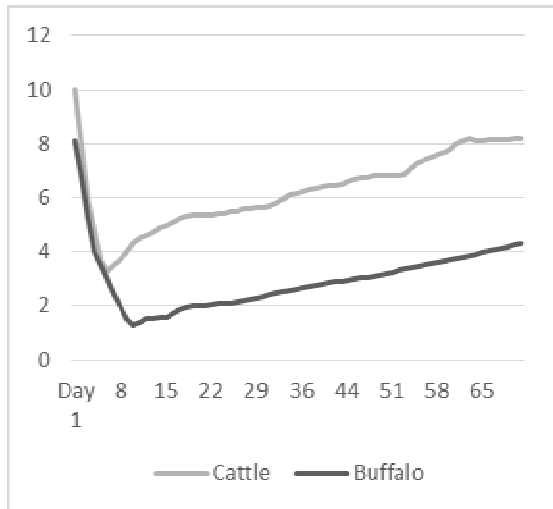Figure 6: Observed and predicted data of 10 buffaloes after and before FMD onset.

Figure 7: Trend of Average Milk production of studied animals.

Discussion

Out of total 88 cases detected positive for FMD based on clinical signs in the field, 73 was found positive in the laboratory. This indicates an accurate clinical diagnosis by field veterinarians in 83% cases, which shows sufficient skills of field staff of Livestock Department of District, Faisalabad. This also reveals that signs are so prominent/severe in most of the cases to facilitate diagnosis. Serotype O was found most frequently (60%) for the outbreaks studied, followed by serotype Asia 1 (25%)

and then serotype A (12%). Five samples that were found positive with consensus primers were not typed by the typing primers. This shows that there is a need for the development of regional typing primers as the virus differ from region to region and conserved portion of the virus genome in one geographical setting may be not so for the other one. Different parts of the world have developed their own consensus as well as typing primers (22). Buffer zones for FMD ring vaccination are kept of 5-10 km radius (23) because FMDV spread is predicted to cover a distance of 10 km (24). This finding is used to predict the zones of 10 km radius in Faisalabad district that illustrate that zones are intermingling with one another and it is very difficult to distinguish their boundaries indicating that almost whole study area is in the reach of FMDV because of these reported outbreaks. In this economic loss study, only the milk loss was considered. Other losses due to FMD, such as weight loss, mortality in young calves, abortions, etc. were not included in this study. The study of economic losses due to these can emphasise more on the need of need of regular vaccination in the country. However, only milk production losses indicate that if one USD is spent on vaccination, it can save 4.58 USD of milk loss.

Acknowledgment

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