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Preparation of Inactivated Trivalent FMD Vaccine and Determination of Antibody Titre in Vaccinated Cattle

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Authors' contributions

This work was carried out in collaboration between all authors. Author MMRC did the study design and wrote the protocol. Authors MLH, SA and MR helped in designing and doing experiments. Authors MFRK and KBA did the statistical analysis and literature searches while analyses of study was by author KHMNHN. Authors MTR and MBR critically checked the manuscript for finalization. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: This research work was conducted for isolation and molecular detection of Foot and Mouth Disease (FMD) virus from the field samples, development of inactivated trivalent FMD vaccine, and determination of antibody titer in vaccinated cattle.
Methodology: A total of 10 samples (tongue epithelium) were collected from FMD affected cattle from Gazipur, Mymensingh, and Pabna districts of Bangladesh during May 2014. Inoculum was prepared from the sample and the associated FMDV was propagated in BHK-21 cell lines. Besides, viral RNA was extracted for molecular detection (RT-PCR) of the serotypes involved. The isolated serotypes were used as seed virus in preparation of binary ethyleneimine (BEI) inactivated trivalent FMD vaccine using saponin and oil adjuvants. For the determination of antibody production in

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response to our trivalent FMD vaccine, a total of 25 cattle aging 11-22 months were used**.** Sera of vaccinated cattle were collected on day 0, 21, 35, 49, 63. The sera were subjected for the determination of antibody level using enzyme-linked immunosorbent assay (ELISA). The titer values were statistically analyzed using one-way ANOVA to see immune response against the serotypes.

Results: Three serotypes of FMDV were detected; these were FMD serotype-O, A and Asia-1, of which serotype-O was mostly prevalent, followed by serotype-A and Asia-1. The highest mean antibody titer was found on day 63 in all serotypes. Sixteen cattle (80%) out of 20 vaccinated cattle obtained protective antibody titer. The titer values of the vaccinated cattle were statistically significant against O, A and Asia-1 serotypes.

Conclusion: FMD serotype-O, A, and Aisa-1 are prevailing in Bangladesh. A trivalent inactivated FMD vaccine has been prepared successfully using circulating virus of Bangladesh. The vaccine can be used to combat FMD in Bangladesh.

Keywords: FMDV; RT-PCR; vaccine; ELISA; vaccinated cattle.

1. INTRODUCTION

Foot and mouth disease (FMD) is a highly infectious febrile disease of cloven hooved animals like cattle, sheep, pigs, goats, deer, elephant, and water buffalo. The disease causes huge economic loss through reducing meat and milk production, trade restriction, and mortality of young animals [1]. The typical clinical sign of FMD is the appearance of blisters (or vesicles) on tongue, nose, lips, teats, in the oral cavity, above the hooves, and between the toes. Lameness and reluctance to move or eat may occur due to rupture of blisters. Besides, hypersalivation, fever, loss of weight, depression may also present [2].

The causal agent of FMD, Foot and mouth disease virus (FMDV), is a positive sense, non-envelop, single stranded RNA virus. The genome size of this virus is approximately 8,500 bases which are surrounded by 4 structural proteins (VP 1-4) [3]. There are 7 immunologically distinct serotypes (A, O, C, SAT1, SAT2, SAT3, and Asia1) of FMDV; the serotypes do not cross protect each other. Thus, each serotype requires individual vaccine strain to induce immune response in susceptible animal [4].

In Bangladesh, huge economic loss (about 125 million US$/year) is incurred due to the outbreaks of FMD [5]. Previous studies on sero-epidemiological and molecular epidemiological investigations of FMDV indicated that 3 FMDV serotypes (O, A, and Asia-1) are prevailing in Bangladesh [6-12]. For the control of FMD using inactivated FMD vaccines has been used for the immunization of cattle, buffalo, sheep and goat in Bangladesh since last four decades. However, the disease appears as endemic throughout the year and as epidemic during rainy and winter seasons. Preparation of vaccine using circulating FMDV would be the best choice to control the disease in Bangladesh. In our previous study, we assessed the effects of age, sex, and breed on immune response in cattle after vaccination with FMD trivalent vaccine [13]. Few studies have been conducted on molecular detection of prevalent FMDV serotypes in Bangladesh and production of inactivated FMD vaccine by using binary ethyleneimine (BEI). The present study was undertaken for identification, adaptation of FMDV in BHK-21 cell line and detection of FMDV by RT-PCR and development an effective, economic and highly protective vaccine for the FMD prevalent in Bangladesh.

2. MATERIALS AND METHODS

2.1 Sample Collection and Processing

Tongue epithelium (n=10) samples were collected from fields during FMD outbreaks in Gazipur, Mymensingh, and Pabna districts of Bangladesh during May 2014, and the samples were transported to the Virology Laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh-2202, Bangladesh. The samples were processed and 10% suspensions were prepared using phosphate buffered saline (PBS). After centrifugation of the suspension at 5,000 rpm for 10 min, the supernatant was collected which was treated with antibiotics. After antibiotic treatment, the fluid was filtered using 0.22 µm filter. After sterility test on blood agar media, the virus suspension was propagated into BHK-21 cell culture.

2.2 Adaptation and Propagation of FMDV in Bhk-21 Cell Line

The confluent monolayer of BHK-21 cell line was infected with 1 ml of inoculum, and monitored the cytopathic effects as per the procedure described by Chowdhury et al. [13]. The infectious fluid was harvested after 48-72 h of post infection.

2.3 Viral RNA Extraction and RT-PCR

For molecular detection and serotyping of the FMDV by RT-PCR, viral RNA was extracted by SV Total RNA isolation System® (Promega, USA). The FMDV type specific oligonucleotide primers are mentioned in Table 1. The RT-PCR was carried out using Access RT-PCR system® (Promega, USA) as per the protocol of the manufacturer [14].

2.4 Virus Purification and Inactivation

Aseptically, the harvested culture medium from FMDV infected BHK-21 cell culture was centrifuged at 7,000 rpm for 20 min. The BHK-21 monolayer ($TCID_{50}$) was inactivated by BEI. Appropriate amount of BEI was added and incubated at 37°C for 24 h for inactivation of the FMDV properly. The sodium thiosulfate NaO(OH) was used to neutralize the media. The inactivation was assessed by infecting fresh BHK-21 cell culture.

2.5 Preparation of Vaccine

BHK-21 cell culture fluid containing FMDV was taken in a sterile test tube. After inactivation of virus, the inactivated fluid was emulsified with appropriate amount of saponin, mineral oil according to the prospectus guide. For sterility test, little amount of vaccine was inoculated onto blood agar and incubated at 37°C overnight to check the presence of contaminated bacteria or fungi in the prepared vaccine. The prepared vaccine was then stored at 4°C until use.

2.6 Vaccination of Cattle

Prepared FMD vaccines were administered subcutaneously in neck region at the dose rate of 6ml at group of A, B, C, D respectively except control cattle. A total of 25 cattle consisting of 13 females (local and hybrid) and 12 males (local and hybrid) were randomly selected for investigation. Cattle were classified into five groups according to the sex and breed. Group A, B, C, D were local male, local female, cross male, cross female respectively while group E was non-vaccinated group. In vaccinated cattle 11 animals were aged <12 months, and 9 were of >12 months.

2.7 Serum Sample Collection

Sera were collected from all groups of cattle at day 0, 21, 35, 49 and 60 after post vaccination using sterile syringe and needle in sterile eppendorf tube. The tubes were then was centrifuged for 15 minutes at 1000 rpm to have more clear serum from the blood. The serum was then collected in sterile eppendorf tube and was preserved at -20°C refrigerator until used for the further study.

2.8 ELISA

The sera samples were tested by ELISA**.** The kit is a liquid phase blocking ELISA technique for the detection of FMDV antibodies in serum as described by Hamblin et al. [15,16]. For titration assay in Five-fold dilution range was followed, as indicated in Table 2.

The titre of the test serum demonstrating replicate PI values above 50 PI can be assessed by reference to Table 2. An antibody titre of ≥112 indicates that the animal, at the time of bleeding, was protected against infection from the homologous antigen of the particular FMDV serotype. The higher the antibody titre, the greater is the confidence of protection.

Table 1. Foot and mouth disease virus type specific primers used for RT-PCR

Serotype	Primers	Sequence (5'-3')	Size (bp)	Reference
O	FMDOF	ACC AAC CTC CTT GAT GTG GCT	1301	[17]
	FMDOR	GAC ATG TCC TCC TGC ATC TG		
Asia-1	FMDAsia1F	TAC ACT GCT TCT GAC GTG GC	914	[18]
	FMDAsia1R	GAA GGG CCC AGG GTT GGA CTC		
A	FMDAF	TAC CAA ATT ACA CAC GGG AA	866	[17]
	FMDAR	GAC ATG TCC TCC TGC ATC TG		

Table 2. Test serum antibody Titre - Five-fold dilutions

Serum dilution	Sera with different replicate PI values >50 (+)							
	1	2	3	4	5	6	7	8
50	+-	++	++	++	++	++	++	++
250	--	--	+-	++	++	++	++	++
1250	--	--	--	--	+-	++	++	++
6250	--	--	--	--	--	--	+-	++
Antibody Titre	50	11	250	560	1250	2800	6250	>6250

2.9 Statistical Analysis

One way ANOVA test were performed to analysis the data of antibody titre for comparison of antibody titres from different experimental groups. All statistical analyses were performed with SPSS 20 version.

3. RESULTS AND DISCUSSION

In Bangladesh, FMD is a great threat for livestock population. The FMDV is circulating in Bangladesh almost around the year; however, the outbreak reaches to peak during winter [10]. In this study, 80% (n=8/10) field samples were found to be positive by RT-PCR (Fig. 1). The FMDV from the positive samples were successfully adapted in BHK-21 cell line; the cell showed characteristics cytopathic effects like swelling, clumping, rounding of cells and breaking down of intercellular bridge (Fig. 2). Several cell lines were used for FMDV adaptation by different researchers, of which Vero cell and BHK-21 cell lines are prominently used. However, BHK-21 is one of most sensitive cell line for FMDV [2].

The FMDV adapted in BHK-21 cell line were reconfirmed by RT-PCR using specific primers (Table 1). RT-PCR is a highly sensitive, specific, rapid and reliable tool for specific identification of FMDV [12,14,17,19]. Out of 10 tongue samples 5 (50%) were positive for FMDV serotype-O, 2 (20%) were positive for FMDV serotype-A, and 1 (10%) was positive for FMDV serotype-Asia-1 (Table 3). The findings of cell culture following infection with FMDV were correlated with the findings of Hossen et al. [12], Alam et al. [14], and Olabode et al. [20].

In this study, after detection FMDV O, A, Asia-1 serotypes, a trivalent FMD vaccine had been developed. To determine the potency of O, A and Asia-1 serotypes of FMDV in a tissue culture fluid the $TCID_{50}$ assay was done using tenfold serial dilution of O, A and Asia-1 serotypes separately. The lowest dilution factor that showed >50% CPE were 10-7, 10-5, 10-6 for O, A, Asia-1 serotype, respectively. The result of $TCID_{50}$ for O serotype was 7.5 for 0.1 ml. So, for 1 ml $TCID_{50}$=7.5+1=108.5 $TCID_{50}$/ml. The result of $TCID_{50}$ for A serotype was 107 $TCID_{50}$/ml and 107.5 $TCID_{50}$/ml for Asia-1. The vaccine was

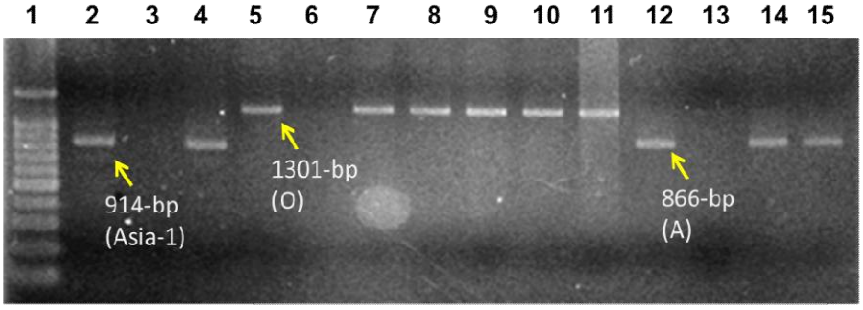

Fig. 1. Agarose gel electrophoresis of PCR products: Image showing that Lane-1: 100 bp DNA marker, Lane 2, 5, 12: positive control of Asia-1, O, A respectively. Lane 4, 7-11, 14-15: samples of Asia-1, O, A. Lane-3, 6, 13: negative control of Asia-1, O, A respectively

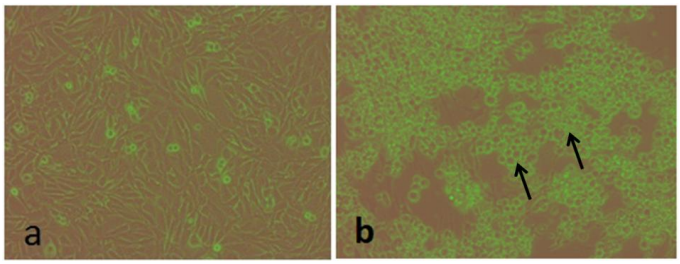

Fig. 2. FMDV propagation in BHK-21 cell line. a) Normal (uninfected) BHK-21 cell line, b) FMDV infected BHK-21 cell line (observed under 40x). The infected cells become round and flat. The intercellular bridge was broken down, and finally the cells were died (arrows).

Table 3. Detection of FMDV serotypes by one step RT-PCR

Source of samples	No. of samples	Types of samples	Serotypes of FMDV positive by RT-PCR		
			A	O	Asia1
Gazipur	5	Tongue epithelia	-	3	1
Mymensingh	3	Tongue epithelia	-	2	-
Pabna	2	Tongue epithelia	2	-	-
Total No.	10		2	5	1

formulated with antigenic preparation exhibiting the $TCID_{50}$ value.

No cattle showed titre against FMD before vaccination. One way ANOVA test has done to analyze the antibody titre of vaccinated cattle. Figs. 3-5 presented that the results of antibody production were gradually increased from 21 to 63 days in all groups against O, A, Asia-1 serotype ($P<0.01$). The highest mean antibody found at 63 days of all groups where highest value of antibody titre was 498.00±138.64 in Group-B and lowest value in Group C that was 294.00±256.18 at 63 days.

In group A, 4 cattle were produce protective antibody titre (≥112.00) out of 5. In group B, all cattle produced protective antibody where it was 3 out of 5 in group C and 4 out of 5 in group D. Different factor can be responsible for unprotected animal according to Doel [21] who reviewed that stimulus variables of host that species, breed, individuality, age, health, physiological state, other stress factors (*e.g.*, husbandry, climate), FMD immune status and dose, route, volume, purity of virus, virus strain (e.g., physical and antigenic characteristics), adjuvant(s) which influence the immune response to FMDV and vaccine. In total 20 cattle

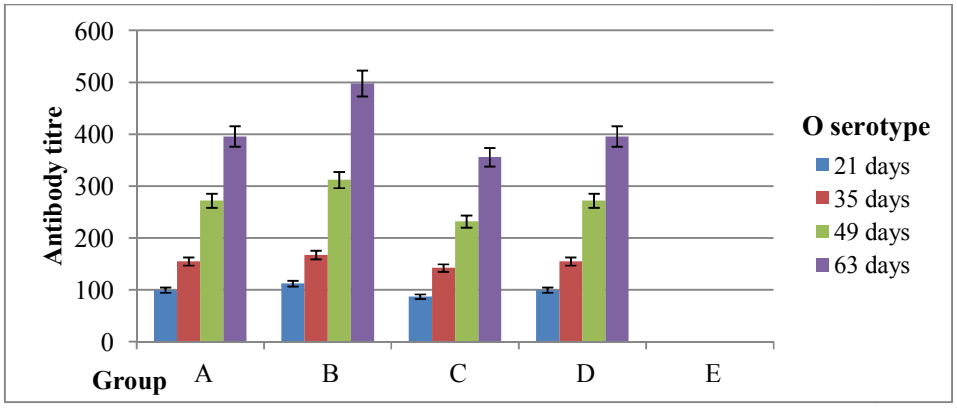

Fig. 3. Graphical presentation of mean antibody titre of different groups against FMD 'O' serotype

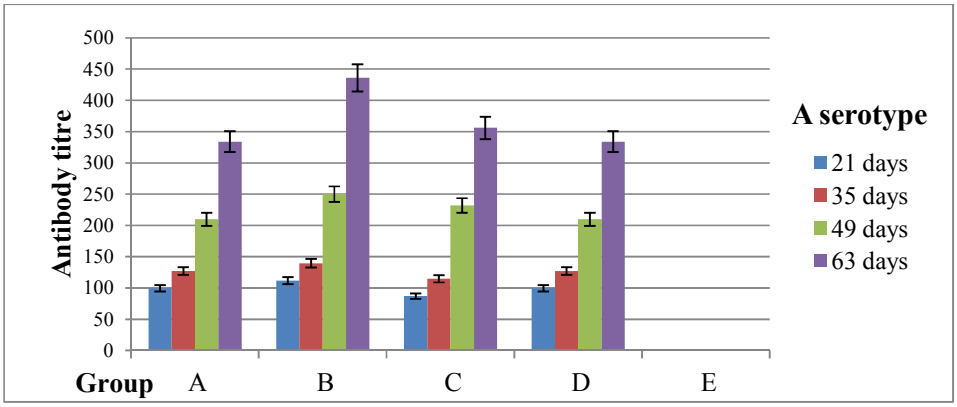

Fig. 4. Graphical presentation of mean antibody titre of different groups against FMD 'A' serotype

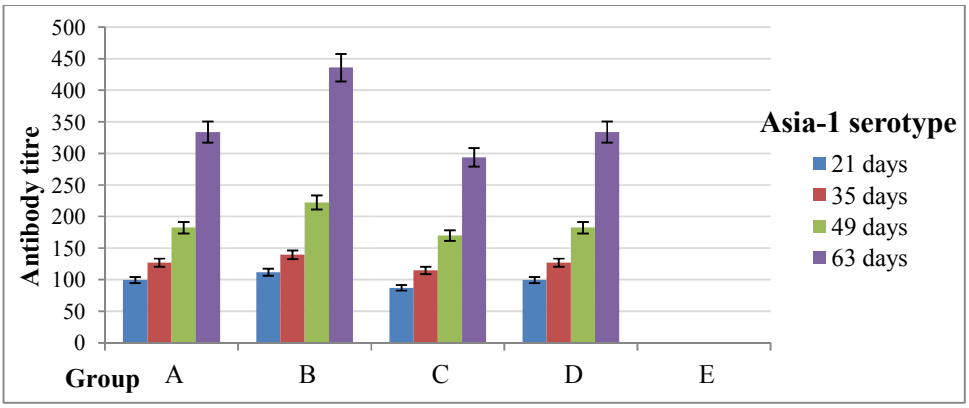

Fig. 5. Graphical presentation of mean antibody titre of different groups against FMD 'Asia-1' serotype

were vaccinated where four cattle were not obtained productivity against FMD. It indicates that the prepared trivalent FMD vaccine produced 80% protectivity in vaccinated cattle against FMD O, A, Asia-1 serotypes.

4. CONCLUSION

It is concluded that BEI inactivated trivalent FMD vaccine is capable to produce good immune response against FMD virus serotype-O, A and Asia-1. Although a few cattle are vaccinated to check the protective antibody, the results reflect the potency of the newly developed FMD vaccine, and the vaccine can be used to combat the FMD in Bangladesh.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Vaccination of the cattle was done, and the blood samples were collected from the cattle as per standard procedure without harming or giving stress to any animal.

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COMPETING INTERESTS

Authors have declared that they have no competing interests.

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