ADAPTATION OF FOOT & MOUTH DISEASE VIRUS SUBTYPES PAN ASIA-II, TUR-06 AND SINDH-08 TOWARDS THE VACCINE DEVELOPMENT AND SEROLOGICAL FINDINGS BY SPC ELISA AND SERUM NEUTRALIZATION TEST

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ABSTRACT: FMD serotype "O" with subtype PAN Asia-II, serotype "A" with subtype Tur-06 and serotype "Asia-1" with subtype Sindh-08 were adapted on the LFBK cell line and subsequently on BHK-21. After inactivation these subtypes were introduced for the development of oil based trivalent vaccine against FMD. The study was conducted on the experimental animals (cow calves) at Livestock Production Research Institute (LPRI), Okara. Twenty one unvaccinated cattle calves of age group 4-6 month were used in the study, 16 were injected with experimentally prepared oil based FMD vaccine at the dose rate of 2cc per cow calf at day 0 and 28 post priming while five cattle calves were kept as control. The FMD oil based vaccine containing subtypes PAN ASIA-II, TUR-06 and SINDH-08 showed satisfactory immune response on ELISA and Serum Neutralization Test (SNT) at day 28 post priming and day 28 post booster. The study aimed to strengthen the national control strategy against FMD by the incorporation of prevalent field strains of FMD virus for the vaccine development.

Keywords: FMD Serotypes O, A and Asia-1, BHK-21 Cell Line, LFBK- Cell Line, Virus Adaptation, Vaccine Development, ELISA, SNT.

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INTRODUCTION

Foot and Mouth Disease (FMD) also recognized as Apthus Fever is a viral disease caused by FMD virus, a member of genus (Apthovirus) of the family (Picornaviridae) which is further divided into seven serotypes and more than 100 sub serotypes having a little or no cross protection among serotypes. FMDV serotypes (SAT 1, SAT 2 and SAT 3) are restricted to Africa, while FMDV serotypes O and Serotype A are distributed worldwide and Serotype Asia-1 only limited to Asia (Domingo *et al*., 1990). FMD is highly contagious and fatal disease of cloven footed animals that limiting the worldwide trade of animals and their by products from source countries. (Abubakar *et al*., 2012). It affects almost all the cloven footed animals. It causes decrease in the productivity of the animals. FMD is associated with high rate of morbidity almost 100% and depending upon the age of the animal variable rate of mortality 1-100%. The mortality rate may reach up to 100% in the young animals. (OIE, 2018)

Out of seven antigenically different serotypes, FMD virus serotypes A, O and Asia 1 are more prevalent in Pakistan while VP1 region of the viral genome is associated with sequence variability (Naveed *et al*., 2018). It is difficult to control Foot and Mouth Disease (FMD) as FMD virus had no evidence of cross protection. Furthermore, a periodic mutation in the virus genome and emergence of new subtypes of FMD viruses is also a significant issue for the field based disease control programs as application of vaccines in the field becomes inefficient (Mahapatra and Parida., 2018). Most of the developed countries have eradicated the FMD, but Pakistan has still key concerns in form of heavy economic losses every year due to poor disease reporting mechanism, lack of awareness and poor management practices etc (Sanaullah *et al*., 2019).

The FMD is one of the major constraints for the development of livestock, therefore considered as the most serious epizootic alarming disease in the world. (Park *et al*., 2013; Mohapatra *et al*., 2015). Different inactivated adjuvant FMD vaccines are currently used worldwide including Pakistan. With the emergence of new sub serotypes of virus in the fields with no or little cross immunity it is incumbent to develop vaccines with the changing environment (Chowdhury *et al*., 2016). Control programs for FMD depends upon the background history of the disease, the ability of the affected countries for proper funding, the availability of the technical expertise, the geography of the region/country and the application of the legislation for the animal health. There are many different techniques used for the control and eradication of FMD around the world. The initial step for the effective control is early detection of disease.

The antigenic variation in the viral genome is one of the major concerns for vaccine failure in the animals. For the successful and effective disease control strategies continuous research and development is needed

and modern trends should be adopted for safe, improved and efficacious vaccine production. Different studies are available regarding phylogenetic analysis of FMD virus in Pakistan in different areas but the real effort should be towards isolation and adaptation of prevalent strains and incorporate them as a better candidate in the preparation of vaccines. Keeping in view the above mentioned facts, this research work has been undertaken for the adaptation of isolated FMDV serotypes "O"(Pan-Asia-II), "A"(Tur-06), and "Asia-1"(Sind-08) on LFBK αVβ6 cell line and finally on BHK-21 cell line for vaccine seed development. Furthermore, the serological findings of experimentally prepared vaccine were also analyzed in cow calves by ELISA and SNT to evaluate the vaccine efficiency.

MATERIALS AND METHODS

Isolation and Adaptation of Virus: Foot and Mouth Disease Research Center is the premier institute in Pakistan for FMD vaccine production and research and development related activities. The institute received confirmed field isolates of serotype "O" (Pan Asia-II) and serotype "Asia-I (Sindh-08) by courtesy from FAO, Pakistan (Food and Agriculture Organization) while serotype "A" (Tur-06) was received form SAP Ankara, Turkey, prevalent in Pakistan. These isolates were wild type which had to be adapted on cell lines for further studies. LFBK αVβ6 is considered to be more effective for isolation and adaptation of FMD virus so this cell line was used in initial isolation and adaptation of virus. (LaRocco *et al*., 2013).

LFBK cells were propagated in Dulbeco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. On completion of the monolayer (24-48hrs), LFBK cells were seeded with sub-serotypes of FMD Virus "Pan Asia-II", "Tur-06" and "Sindh-08" in individual disposable tissue culture flasks in different labs due to avoid mixing of these subtypes.. The percentage of cytopathic effect (CPE) was observed under inverted microscope (18-24hrs of infection) in between (80-90 %.) The sub-serotypes "Pan Asia-II", "Tur-06" and "Sindh-08" were successfully adapted on LFBK cell line at passage level 5 with $TCID_{50}$ $10^{6.8}$, $TCID_{50}$ $10^{7.6}$ and $TCID_{50}$ $10^{7.2}$ respectively. $TCID_{50}$ was calculated according to Reed and Muench (1938). These LFBK adapted sub-serotypes were further processed for the adaptation on BHK-21 cell line for the vaccine development. The BHK-21 cells were propagated on Glassgow's Minimum Essential Medium (GMEM) supplemented with 10% fetal bovine serum, and antibiotics (Shahiduzzaman *et al*., 2016). After the observation of the complete monolayer of the BHK-21 cell line (24-48hrs) in disposable cell culture flasks, the monolayer was seeded with LFBK adapted sub-serotypes "Pan Asia-II", "Tur-06" and "Sindh-08". These sub-serotypes were successfully adapted on BHK-21 upto the passage level 10. The percentage CPE was observed in between the 80-90% and $TCID_{50}$ of BHK-21 adapted sub-serotypes "Pan Asia-II", "Tur-06" and "Sindh-08" was $10^{8.0}$, $10^{7.8}$ and $10^{7.0}$ respectively on passage level 10.

The harvested material of isolated sub-serotypes was stored at -80^0C. For the ready to use vaccine development the freeze thaw process was done with the harvested material. These sub-serotypes were inactivated individually by Binary Ethyl Imine (BEI) (1mM) and formalin (0.04%) in combination. The harmful effects of the BEI were neutralized by the addition of 20% sodium thiosulphate after 24hrs of inactivation. The formaldehyde effects were neutralized by adding 20% sodium bisulphite in final concentration (Soliman *et al*., 2013).

Inoculation of Inactivated Virus in Mice: The inactivated virus was inoculated into five baby suckling mice (2-4) days old intraperitoneally @100ul to confirm the complete inactivation. The mice found dead within 24 hours were considered to be as nonspecific deaths. The mice were observed for 7 days for paralysis or deaths. (OIE, 2018).

Preparation of Trivalent Inactivated FMD Vaccine: The preparation of FMD vaccine was followed by the formulations as described by Gamil (2010) and El-Sayed *et al*. (2012). The inactivated harvested material of sub-serotypes "Pan Asia-II", "Tur-06" and "Sindh-08" was pooled with montanide oil ISA-50 with ratio 1:1. The mixture was homogenized at 10000rpm in the sterile environment to ensure even out the montanide oil and inactivated virus. The safety and sterility tests of this vaccine were ensured and followed as per SOPs of F&MDRC.

Vaccine Efficacy Trial: The research trial was conducted in LPRI, Okara, Pakistan. 21 young cow calves of age group 4-6 month were divided in two groups to evaluate the level of antibody titers against FMDV sub-serotypes used in vaccine. The sample size was determined by using power and sample size method using Minitab 17. 16 cow calves were placed in Group A and experimentally prepared vaccine was injected deep intramuscularly at the dose rate of 2cc at 0 day, 28^{th} days and 56 days while 5 cow calves were kept in Group B and considered as control (no vaccination). The serum samples were collected from both groups at 0 day, 28^{th} day post vaccination and 28^{th} day post booster vaccination. The serum samples were further analyzed by Solid Phase Competitive ELISA (SPC ELISA) using commercially available kit (IZLER, Italy) and Virus Neutralization test for development of antibody titer (OIE, 2018).

RESULTS

SPC ELISA Titers: SPC ELISA (IZLER Italy) kit interprets the standard positive as for the presence of specific antibodies if the PI % is more than 70% (Reference Kit).

At day 0 all the animals of Group A and Group B were considered as negative for the anti FMDV serotype "O, A and Asia-1"ELISA titers because no animal showed PI >70%. The ELISA results showed that at day 28 post priming all the 16 cow calves that were kept in group A showed the percentage inhibition (PI) more than 80 % against serotype "O". Post booster vaccination at 56 days, all the animals were positive with PI >80%. Almost similar results were found against Asia-1 and A serotypes where 14 and 13 animals were positive for FMD antibody titer at 28 days. Considerable increase were there when we found 16 animals positive for Asia-1 and 15 animals showed high titers against A serotype at 56 days post booster (Table-1 and Graph-1). All these results were recorded at different dilution factors i.e. 1:10, 1:30, 1:90 and 1:270. According to reference kit, the animals showed titer at dilution factor 1:10 and 1:30 are highly positive but our results even showed titers >70% at 1:90 and 1:270 for some animals strengthened the results (Table 2-4 and Graph 2-4).

It can be concluded from the results of this research trial that on day 28 post-priming more than 90 % of the experimental animals were positive for antibodies against serotypes "O", "A" and "Asia-1" at dilution factor 1:10. A remarkable response was observed in case of 28 day post-booster vaccination that 100% animals were positive for the antibodies on dilution factors 1:10 and 1:30 and a remarkable response of vaccine was recorded at DF 1:90 and 1:270.

Serum Neutralization Test (SNT): The results from SNT expressed that on day 28 post-priming, 14 cow calves showed the satisfactory antibody titers > 1.5 log 10 SN titers for the FMDV sub-serotypes "Pan Asia-II", "Tur-06" and "Sindh-08". Only two cow calves were below the range of protective SN titers. On day 56 post priming all the 16 cow calves from the group A demonstrated the sufficient sero-specific SN titers i.e. >1.8. (Table-5 and Graph-5)

Statistical Analysis: The results obtained from ELISA and Serum Neutralization Test were subjected to Analysis of Variance at 95% CI using Minitab 17. The antibody titers obtained through these tests showed highly significant results with P- Value less than 0.05%.

Table 1. Antibody Titers (PI) Against Different Serotypes of FMD by SPC ELISA.

FMD Serotype	Day of Serum Collection	No. of Positive Animals	Antibody Titer (PI)
O	0	0	0
Asia-1	0	0	0
A	0	0	0
O	28	16	>80%
Asia-1	28	15	>70%
A	28	13	>70%
O	56	16	>80%
Asia-1	56	16	>70%
A	56	15	>70%

Table 2. No. of Positive Animals Against Serotype O through SPC ELISA at Different Dilution Factors.

Dilution factor	No. of Positive Animals at Day 0	No. of Positive Animals at Day 28	No. of Positive Animals at Day 56
10	0	16	16
30	0	10	12
90	0	8	10
270	0	2	2

Table 3. No. of Positive Animals Against Serotype Asia-I through SPC ELISA at Different Dilution Factors.

Dilution factor	No. of Positive Animals at Day 0	No. of Positive Animals at Day 28	No. of Positive Animals at Day 56
10	0	15	16
30	0	9	11
90	0	8	10
270	0	1	2

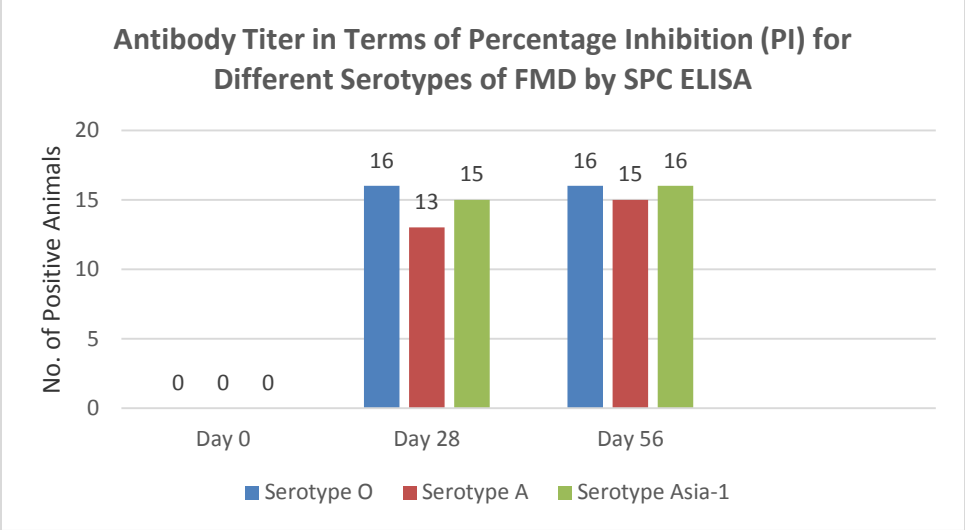
Table 4. No. of Positive Animals Against Serotype A at Different Dilution Factors through SPC ELISA.

Dilution factor	No. of Positive Animals at Day 0	No. of Positive Animals at Day28	No. of Positive Animals at Day 56
10	0	13	15
30	0	7	9
90	0	3	5
0	0	0	2

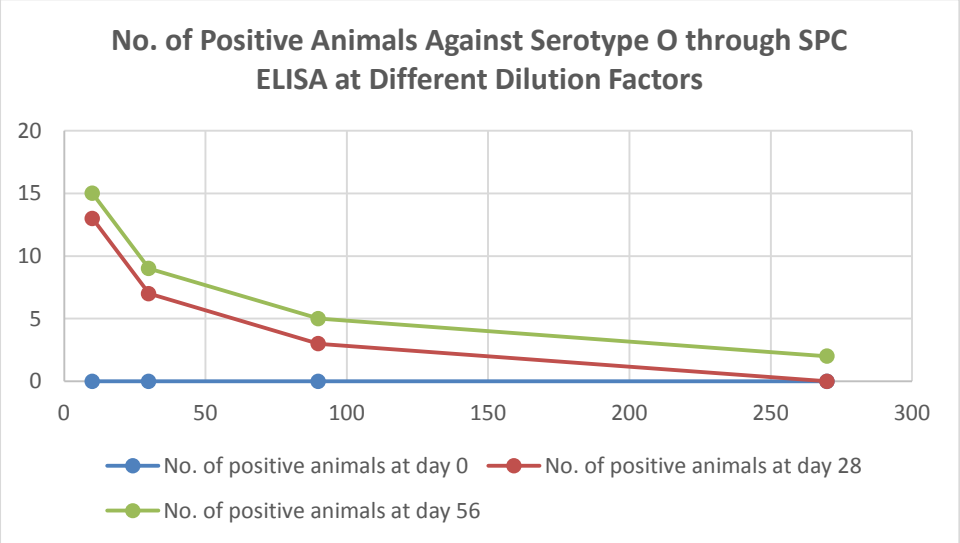
Table 5. Ab Titer of FMD Virus Serotypes (O, A, Asia-1) at Different Days Intervals.

FMD Virus Type	Day o	No. of Positive Animals	Day 28	No. of Positive Animals	Day 56	No. of Positive Animals
O	0	0	>1.8	16	>1.8	16
A	0	0	>1.5	15	>1.8	16
Asia -1	0	0	>1.5	13	>1.8	15

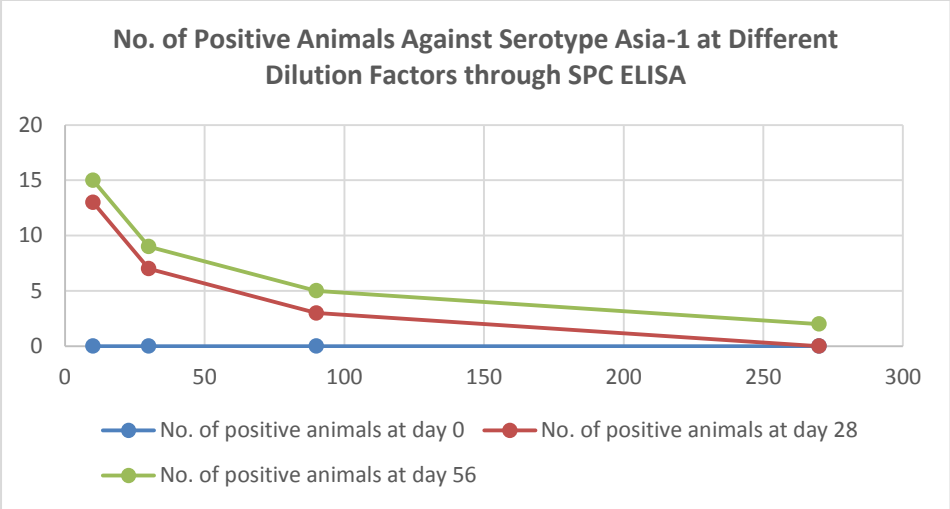
(The results are prepared @log10)



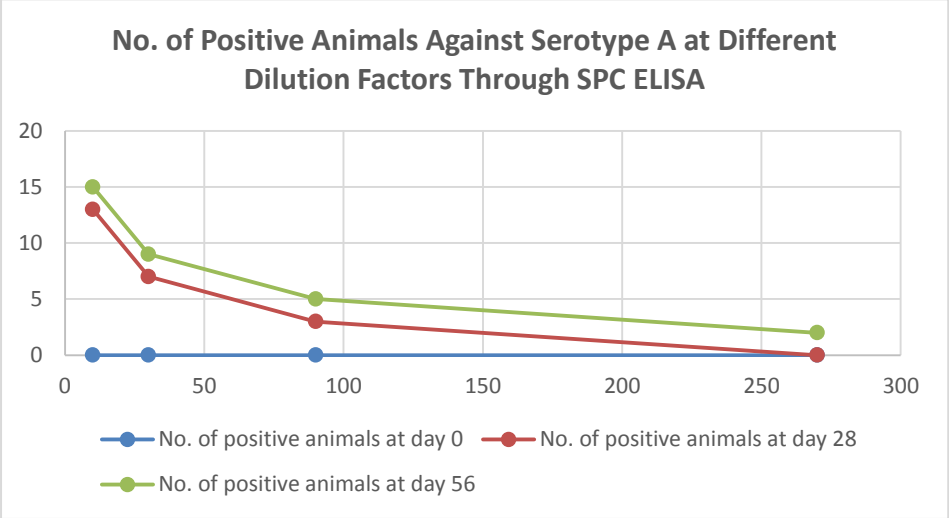
Graph. 1



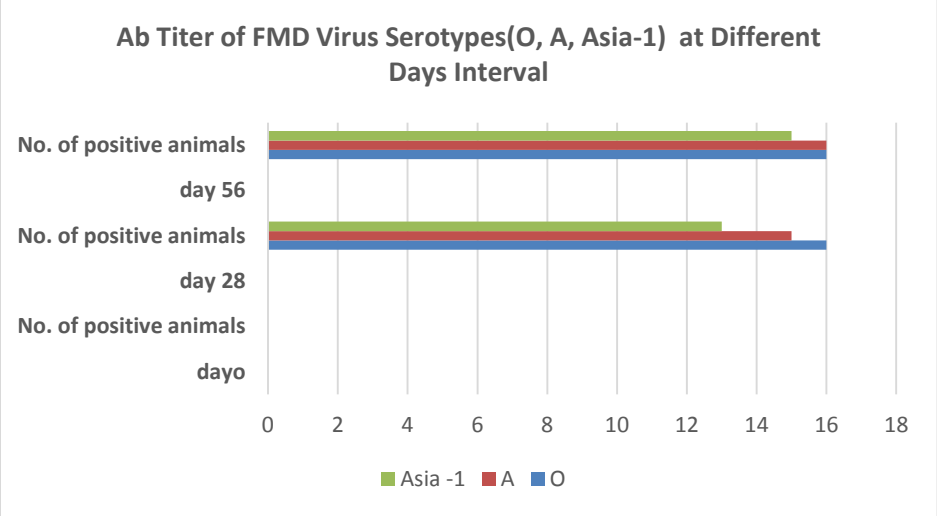
Graph. 2



Graph. 3



Graph. 4



Graph. 5

DISCUSSION

In endemic areas, vaccination is one of the key that is believed worldwide for the control strategy of foot and mouth disease. It seems relatively very simple to control foot and mouth disease by vaccination programs but rationally it is difficult to choose and decide either the vaccine to be used for the campaign is equally effective or not in field environment for the control strategy as reviewed by Park *et al*. (2013). In the present study the different sub-serotypes of FMD virus were used which are currently prevailing in Pakistan. (Abubkar *et al*., 2012). Due to variation in the serotypes and sub-serotypes of foot and mouth disease virus effective vaccine is the question for the successes of foot and mouth disease control programs. Main objective of the current study was to adapt the field virus on BHK-21 cell line and prepare an effective vaccine for the farmers.

The isolation and adaptation of virus on LFBK and BHK-21 are in agreement with that of LoRacco *et al*. (2015); Huang *et al*. (2011). The TCID 50 results are in correlation with Chowdhury *et al*. (2016) but more than as described by Mohammad *et al*. (2018).The vaccine trial results were in line with the results of Soliman *et al*. (2013) showing high ELISA and SN titers in guinea pigs. The results of ELISA titer are also in agreement with Trotta *et al*. (2015) who used Liquid Phase Blocking ELISA for estimation of FMD vaccine titer in cows. The results are also in correlation with the vaccine trial results of Ismail *et al.* (2013) showing SNT of >1.8 log 10 at day 28 and 56 post vaccination.

As the FMD virus strains do not provide cross immunity so it is the need of time for the South Asian countries like Pakistan to strengthen the continuous research and development related activities to identify prevalent strains in the field for effective vaccination. With the success of this experiment, the freshly adapted reference strains “Pan Asia-II”, “Tur-06” and “Sindh-08” will be incorporated in the vaccine production process of Foot and Mouth Disease Research Center, Lahore, Pakistan. In future, the research findings may be used as model for the isolation, adaptation of FMD virus, its molecular characterization and development of vaccines that will have further application in the national FMD control program.

Conclusion: Hence it can be accomplished, that the booster vaccination to achieve the highest protective titers for the foot and mouth disease should be considered as the model for the successful FMD vaccination programs to control the FMD effectively. On the basis of the findings from this research trial it is recommended that the after priming of animals with FMD oil based vaccine, booster dose on day 28 post-priming must be considered for better efficacy of the vaccine and protection against the disease.

Author Contribution Statement: Dr. Sajjad Hussain, Dr. Rashad Munir, Dr. Muhmmad Shoaib Noor, Dr. Abeera Mubarak, Dr. Rehan Rafique and Dr. Shaukat Ali conceived and designed the research project as a team. Dr. Sajjad Hussain provided institutional support as the head of F&MDRC and supervised the whole project while Dr. Rashad Munir and Dr. Shaukat Ali provided support in technical aspects of project. Dr. Abeera Mubarak conducted all practical aspects related to FMD virus adaptation, vaccine production and SNT. Moreover, Dr. Muhammad Shoaib Noor and Dr. Rehan Rafique practically performed this research based field trial and all aspects of SPC ELISA. Dr. Rehan Rafique wrote the manuscript while Dr. Abeera Mubarak and Dr. Shaukat Ali reviewed the study.

Conflict of Interest The authors declare that they have no conflict of interest.

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