Ali et al., J Virol Antivir Res 2019, 8:1
DOI: 10.4172/2324-8955.1000188

Journal of Virology & Antiviral Research

Research Article A SCITECHNOL JOURNAL

Comparative Sensitivity of *LFBK* and *LFBK αVβ6* Cell Lines for Isolation of Foot and Mouth Disease Virus from Riverine Buffaloes by Using Oro-Pharyngeal Fluids

Ali A[1,2*], Zahur AB[1], Farooq U[2], Latif A[2], Naeem K[2], Afzal M[3], Ullah RW[4], Shabana[5] and Muhammad ZY[6]

[1]*Wuhan Institute of Virology, University of Chinese Academy of Sciences, Huairou, China*

[2]*Animal Health Research Laboratories, Animal Sciences Institute, National Agricultural Research Centre (NARC), Park Road Islamabad, Islamabad, Pakistan*

[3]*Progressive Control of FMD in Pakistan (UN-FAO), GCP/PAK/123/USA, Islamabad, Pakistan*

[4]*Veterinary Research Institute, Zarar Shaheed Road Lahore Cantt, Pakistan*

[5]*National Institute of Genomics and Advanced Biotechnology (NIGAB), Islamabad, Pakistan*

[6]*Forman Christian College, Ferozepur Road, Lahore, Pakistan*

[*]**Corresponding author:** Ali A, Animal Health Research Laboratories, Animal Sciences Institute, National Agricultural Research Centre (NARC), Park Road Islamabad, Islamabad, Pakistan, Tel: + 972-883-6360; E-mail: microbiologist_hu@hotmail.com

Received Date: 04 July 2018; **Accepted Date:** 17 December 2018; **Published Date:** 04 January 2019

Abstract

Foot and mouth disease (FMD) is a major Transboundary Animal Disease (TAD) which causes major economics losses to the developing countries. In Pakistan the disease is considered endemic and outbreaks are still being reported. Rapid diagnosis of the disease and Isolation of FMD virus is important to confirm viral subtyping and allow for the development of effective vaccines against the specific subtypes. Carrier animals are the major source of current outbreaks of FMD in Pakistan. Current study was planned and conducted for isolation of FMD virus from persistently infected animals by using *LFBK* cell line and comparison of *LFBK* and *LFBK αVβ6* for isolation of FMDV isolates from recent FMD outbreaks. A total of 120 serum samples were collected from persistently infected riverine buffaloes and examined for the presence of FMD virus Non-Structural Proteins by using NSP-ELISA. Of 120 sera samples 23 animals were found positive for NSP's. The Oro-pharyngeal fluids (OP) were collected from NSP-ELISA positive animals. The OP fluids samples were treated with Tri-cholo-Tri-flouro-Ethane (TTE) and inoculated onto *LFBK* cell line. Out of 23 OP fluid samples 11 exhibited CPE's. A total of six (06) FMD viruses were confirmed by rRT-PCR and characterized by Indirect Sandwich ELISA as type O, Asia-1 and A. The FMD virus isolates were acquired from FAO-UN project on FMD in Pakistan. All isolates were inoculated on both of the cell lines and observed for the development of CPEs. We found that the newly modified *LFBK αVβ6* cell line exhibited CPEs more rapidly after 18-20 hours, while *LFBK* cell line CPEs developed after 24 to 48 hours. TCID50 calculated on *LFBK αVβ6* was higher for the all the serotypes tested than *LFBK* cell line. Percentage of CPEs in *LFBK αVβ6* per plate resulted higher than over *LFBK* cell line.

Keywords: *FMDV*; *LFBK αVβ6*; Persistent infection; Buffaloes

Introduction

Foot and Mouth Disease (FMD) is highly infectious and contagious disease of large ruminants and wild animals with high morbidity and low motility rate. Foot and Mouth Disease (FMD is characterized by lameness, fever, vesicular lesions on feet, tongue, snout and teats [1]. Sever morbidity in young susceptible animals may lead to death [2]. Thus, FMD pose serious threats to livestock and limits their productivity [3,4]. In Pakistan, livestock contribute 12% of total Gross Domestic Product from Pakistan Economic Survey [5] and loss due to FMD may reach around 6.00 billion Rs. in Pakistan [6]. In South Asian region, FMD remains endemic and has been common in subcontinent (indo-pak) [7]. In Pakistan the disease is considered endemic and outbreaks are still being reported [8]. Documented that number of outbreaks of the FMD is higher during January-March due to Eid-ul-Azha (Muslims' religious festival) [9].

The causative agent of FMD is foot-and-mouth disease virus (FMDV), which is found in seven distinct serotypes such as O, A, C, Asia-1, SAT1, SAT2, SAT3 but within the serotypes, subtypes have been evolved [10]. In Pakistan, four serotypes of FMDV A, O, Asia-I and C were detected [6,10-13]. Type C is no longer present in Asia, because the most recent strain was detected in 1995 in Pakistan [14]. Individual contribution of serotypes A, Asia-1 and O for outbreak was 4.7%, 25% and 70% respectively [6]. Despite of several drawbacks isolation of virus remains promising technique, due to its high sensitivity and it provides viral material for further studies [15]. Moreover, isolation of virus is important to identify the viral subtyping and developing effective vaccines against the specific subtype and utility in epizootic- logical studies.

Various cell cultures *viz.*, Calf Thyroid (CTh) and Porcine Kidney, Secondary Porcine Kidney Cells, Primary Lamb Kidney (LK), Bovine kidney and Lamb Testicle LT have been used to isolate FMDV [16-19]. Established cell lines, such as BHK-21, continuous Pig Kidney Cells used for FMDV propagation [20]. But there is urgent need to find susceptible cell line to all FMDV serotypes and subtype and which can be maintained easily [21].

Some primary cells lines, such as bovine thyroid (BTY) are highly susceptible to a wide range of FMDV serotypes Snowdon et al. [16], but some drawbacks are associated with these cell lines such as low sensitivity, costly preparation and losing susceptibility after multiple passages [22]. The most sensitive cells for the isolation of FMDV are BTY which have highly levels of *αV β6* integrin expression [23]. LFBK is well documented cell line of fetal porcine kidney which has susceptibility to all FMDV serotypes and remains unchanged over several passages. Bovine thyroid (BTY) cells are more susceptible to FMDV serotypes as compare to other cells like, *IBRS-2* and Fetal Bovine Kidney Cells [24]. Furthermore, two established Cell lines *ZZ-R 127* and *LFBK αVβ6* reported by different research groups up till now to have highly sensitivity to all FMDV serotypes.

FMDV can survive at 4°C (39.2°F) on wool and in bovine feces for approximately two to three months which is the potential source of FMD infection to the susceptible animals. Hence, FMD may be spread to other FMD free countries through trade of contaminated meat, milk, semen and animal byproducts. FMDV can severely affect the economy of a country by limiting the animal and their products trade in international market. The virus must be inactivated in these products by exposure to temperature acidic pH or by using different chemical treatments without compromising nutritious value. Several environmental factors inactivate the FMD virus in animal products.

Keeping in view the above scenario the objectives of planned study were isolation and identification of FMDV from persistently infected animals by using cell culture techniques, Comparative Sensitivity of *LFBK* and *LFBKαVβ6* cell line for FMDV isolates and to check the physiochemical properties of FMDV.

Materials and Methods

The current study was planned to isolate FMDV from persistently infected buffaloes and comparison of *LFBK* and *LFBK-αVß6* cell lines for isolation of FMD viruses. The study was conducted at Animal Health Research Laboratories, Animal Sciences Institute (ASI), National Agriculture Research Centre (NARC), Islamabad, Pakistan.

Experimental animals and study plan

The animals included in the study were riverine buffaloes kept at peri-urban dairy farms of Islamabad Capital Territory (ICT). A total of one hundred and twenty (n=120) buffaloes having a history of FMD during last six months were bled for sera collection. The sera were examined for the presence of FMD virus non-structural proteins by using NSP-ELISA. The NSP positive animals were sampled for Oro-Pharyngeal (OP) fluid using probang cups. The OP fluid samples were treated with Tri-cholo-Tri-flouro-Ethane (TTE) for isolation of FMDV. The isolated FMD viruses were confirmed by reverse transcriptase Real time PCR (rRT-PCR) and characterized using indirect sandwich ELISA (Is-ELISA). For comparison of *LFBK* and *LFBK-αVß6* cell lines FMD field viruses were provided by Progressive Control of Foot and Mouth disease in Pakistan (GCP/PAK/123/USA), Food and Agriculture Organization (FAO) [6].

Non-structural protein ELISA (NSP-ELISA)

The non-structural protein antibodies of FMD virus were detected using NSP-ELISA. The NSP-ELISA kit (CHEKIT FMD-3ABC bo-ovIDEXX, USA) according to the manufacturer's protocol [25], the standardized reagents, assay protocol and manual were supplied with the kit.

Oro-Pharyngeal (OP) samples were collected from the NSP positive animals by using probang cups form the anterior part of oral cavity and specialized treatment called Tri-cholo-Tri-flouro-Ethane (TTE) treatment was done of OP samples to emulsify and neutralize antibodies for virus liberation. With TTE treatment of suspected samples for virus, the titer of virus increased 10-100 folds. The end product of the TTE treatment was stored at -80°C and used as inoculum for virus isolation.

Isolation of FMD viruses

LFBK cells were cultured in Dulbecco Modified Essential Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics Amphotericin B (100 IU), penicillin (100 IU) and streptomycin (100 µg/ml). The *LFBK* cells were maintained using DMEM supplemented with 2% fetal bovine serum at 37°C in the presence of CO_2 and pH ranging from 6.5 to 7.5.

LFBK cells were sub-cultured using split ratio method following Burleson in class II biosafety cabinet. Flasks with complete monolayer of *LFBK* cells were washed with Phosphate Buffer Saline (PBS) by gentle shaking to remove all cell debris, after washing PBS was discarded. About 1 ml of trypsin (0.05%) solution was added into the cell culture flask and incubated for 10-15 min. Flask was observed for complete detachment of cells from flask. About 21 ml growth media (DMEM with 10% fetal bovine serum) was added and shacked gently to break up clumps and made a homogenized suspension. This suspension medium was equally distributed into 3 tissue culture flask (25 cm^2) each of the flask contained 7 ml of suspension culture. The flask was incubated at 37°C containing 5% CO_2.

Infection of LFBK cells for isolation of FMDV

Tissue culture flasks (25 cm^2) which are 80% confluent was used for infection. The growth media was discarded and cells were washed with PBS. The cell culture flask was inoculated with 500 µl of inoculums (TTE treated OP fluid samples) filtered through 0.22 µm filter (Sartorious). The inoculated flask was incubated for 40 min till the adsorption of virus. About 7-10 ml DMEM medium was added for maintenance of cells. Negative control flask was inoculated with Sterile PBS. The flask was incubated at 37°C containing 5% CO_2.

Flask was examined regularly for appearance of Cythopathic Effects (CPE) after every 24 hours for 72 hours. The sample was considered positive when 80% of CPE cells were observed in the flask, Samples showed no CPE after third passage considered as negative. The same procedure was followed for all the TTE treated OP fluid samples.

Detection of FMD virus isolates

Each FMD virus isolate was confirmed by Real-time Polymerase Chain Reaction (rRT-PCR) as described by (Challens et al, 1997). RNA was extracted using QIAamp Viral RNA Mini Kit following the manufacturer instructions (Qiagen, GmBh, Germany). Reagents and washing buffers were prepared according to the kit manual. To elute the extracted RNA 40 µl of RNA's free water was used. The extracted RNA was places at -200°C until further used. The quantity and purity of extracted RNA was determined using Nano drop (Nano Drop 1000, Thermo scientific, Wilmington, DE, USA).

Real time RT-PCR

For the detection of FMD virus, real time PCR was performed as described by Challahan et al. (2002). Taq Man core reagents kit was used (Taq Man, EZ-RT-PCR CORE REAGENTS). The reaction mixture having 5X TaqMan EZ Buffer, 25 mM Mn (Oac), 10 mM dNTPs Mix, 50 µM of each primers, 10 µM Probe, 2.5 U/µl rTth DNA Polymerase, 10.5 µl of RNAse-free water. Template RNA was used in real time polymerase chain reaction. Reaction was carried out in a machine ABI Prism 7500 real time PCR machine (Applied Biosystem). All conditions were maintained as described by Challahan et al. [26].

For the detection of Serotype of FMDV Indirect Sandwich ELISA was performed following the kit manual (BDSL-UK), the standardized reagents, assay protocol and manual were supplied with the kit.

Preparation of 96 well cell culture plate of LFBK and LFBK-αVß6 cell line

LFBK and *LFBK-αVß6* flask were prepared as described earlier. A flask with confluent monolayer was taken. Flasks were sub-cultured (trypsinized) as mentioned earlier. Took a 96-well, flat bottom micro titer plate. About 100 µl of cells from sub-cultured flask contained cells count of 4 × 105 cells/ml were added to each well of the plate.

For virus titration 180 µl infectious DMEM media (without serum) was added to each well from row A–H in U bottom 96 well micro titer plate thus made virus serial dilution 4-8 replicate of each virus mostly been used. Six (06) replicates were made for each of the virus, the side wells of the plate were not used to avoid contamination, 20 µl original virus was added to first well of all rows A1-H1 properly mixed it with multichannel pipeter, done serial dilution to column 11^{th} and lift 12^{th} column as control.

According to plate layout each well of the plate was inoculated with 50 µl of virus dilution. Precaution was taken not to touch tip to the walls or the bottom of each well to avoid contamination. Plate was covered with the lid, marked both lid and plate with a marker for plate identification. Incubated at 37°C for 48-72 hours in CO_2 (5%) incubator and examined the CPE after every 24 h for 72 h under an inverted microscope [26].

Calculation of Tissue Culture Infective Dose (TCID50)

Results were interpreted by the observation of CPEs under the microscope, marked and noted the number of wells showed CPEs. The Tissue Culture Infective Dose (TCID50) was calculated by Spearman-karber method

$$Log\ of\ most\ \ concentrated\ dilution\ - \frac{Total\ No.of\ Cells\ with\ CPE - 0.5}{No.of\ wells\ per\ dilution} \times Log\ of\ dilution\ factor$$

Results

NSP-ELISA

A total of n=120 sera were analyzed for the detection of FMD virus NSP antibodies. 23/120 sera were found positive by NSP-ELISA with a positive percentage of 19.1%. NSP positive animals (n=23) were sampled for OP fluid using Probang Cups. A total of 23/23 NSP positive sera were subjected to TTE treatment for isolation of FMD virus.

Isolation of FMD Viruses

A total of n=23 TTE treated OP fluid samples were subjected to isolation of FMDV using LFBK cell line. A total of 11/23 samples showed CPE's on *LFBK* cell line. The CPE's were characterized by detachment of cell, cell rounding and clumps formation of cells.

Real time RT-PCR

A total of 11/11 CPE showed samples were analyzed by Real time PCR and 6/11 found positive for FMD viral RNA.

Indirect Sandwich ELISA for Sero-typing

Real-time PCR positive sample were further tested by sandwich ELISA for serotyping of FMDV.

Comparative sensitivity of LFBK and LFBK-αVß6 cell lines

A total of n=8 FMD virus isolates were provided by UN-FAO project on FMD Islamabad. These all isolates were subjected to TCID50 using *LFBK* and *LFBK-αVß6* cell lines showing the FMD isolates received.

While TCID50 of different serotypes of FMD viruses on *LFBK* cell line in comparison with *LFBK αVβ6* cell line, results expressed in percentage of positive well of both cell lines, represents percentage of positive wells on *LFBK αVβ6.*

Discussion

Foot and Mouth Disease (FMD) is highly contagious disease of large ruminants including wild animals. It is characterized by high morbidity and low motility rate. The clinical signs associated are lameness, fever, vesicular lesions on feet, tongue, snout and teats [1]. Isolation of FMD virus is important to confirm the viral subtyping and allow for the development of effective vaccines against the specific subtype and amplify live virus from original tissue samples suitable for subsequent studies. Although new advance techniques have been developed and designed but virus isolation is irreplaceable.

In this study efforts were directed to isolate FMD viruses on *LFBK* cell line using Oro-pharyngeal (OP) fluid samples from persistently infected buffaloes. During this study it was found that 19% sera were found positive for NSP antibodies against FMD virus collected from persistently 120 infected animals. Our results showed that out of 23 OP fluid samples 11 exhibited CPE's, out of which only six (54.5%) were confirmed by Real-time RT-PCR and Is-ELISA. These results were complete concurrence with Verin et al. [27].

Interestingly, *LFBK αVβ6* was found more sensitive cell line for the isolation of FMD virus. BHK21 cell line was documented the most commonly used cell line for the propagation and isolation of FMDV worldwide but now a days the most sensitive cell line for the isolation of FMDV is *LFBK* cell line after the transduction of *αVβ6* receptors. Our results for *LFBK αVβ6* are complete agreed with LaRocco et al. [28].

Finally, comparative studies for sensitivity of *LFBK* and *LFBK-αVβ6* were investigated for the isolation of FMDV (Table 1). Results have depicted the *LFBK-αVβ6* cells were more susceptible to all FMDV serotypes derived from persistently infected animals as well as field isolates compare to commonly used cells for FMDV isolation (Table 2). Virus titer was higher with *LFBK-αVβ6* cell line when compared with *LFBK* cell line and these observations corroborated with (*LFBK* and *LFBK-αVβ6*) as reported by Zabal et al. [29] for BHK21 (6.74 OVK (7.25) and BFK (6.6,) TCID50/ml but the cell line in our study exhibited CPEs more rapidly and we can get results within 48 hours while they reported results after 72 h.

S.No	Sample I.D	Ct Values	Results
1	Cell Culture 1	16.23	Positive
2	505/1	26.6	Positive
3	21/1	36	Weak Positive

4	22	15.5	Positive
5	27	18	Positive
6	29	39	Negative
7	31	37	Negative
8	32	37	Negative
9	33	36	Negative
10	34	17	Positive
11	36	38	Negative

Table 1: Result of real time RT-PCR.

S.No	Sample I.D	Ct Values	Results
1	Cell Culture 1	16.23	Positive
2	505/1	26.6	Positive
3	21/1	36	Weak Positive
4	22	15.5	Positive
5	27	18	Positive
6	29	39	Negative
7	31	37	Negative
8	32	37	Negative
9	33	36	Negative
10	34	17	Positive
11	36	38	Negative

Table 2: Result of Sandwich ELISA.

According to King et al. [23] the most sensitive and supreme primary cells for the isolation of FMDV are bovine thyroid cells (BTY) which have highly levels of *αVβ6* integrin expression. In current we didn't examined bovine thyroid cells (BTY) as reported by LaRocco et al. [28] that BTY is less susceptible than *LFBK αVβ6* on the basis of their results. Brehm et al. [30] claimed the most sensitive cell line fetal goat tongue cell line for the isolation of FMDV but the main drawback in this cell line is that several days are needed to become confluent as comparison with *LFBK αVβ6* and while current study showed LFBK was ready to be used after 24-30 h. LoRacco et al. [28], reported that *BHK-21* cell are not highly susceptible for FMDV isolation as compared to *LFBK αVβ6*. However in our study, *LFBK-αVβ6* cells showed higher sensitive than *LFBK* and *BHK-21* cells. This clearly indicates that *LFBK αVβ6* cell line can be useful in rapid isolation and for the high yield isolation (Table 3).

S.No	Sample I.D	Serotype	Genotype
1	RYK-282	Asia-1	Sindh08
2	HFZ-480	O	Pan Asia 2 Ant10
3	OKR-520	A	Iran 05 sis-12
4	SG-559	Asia-1	Sindh 08
5	MRP-580	A	Iran 05 sis-12
6	RHK-618	O	Pan Asia 2 (unnamed)
7	AJK-628	A	Iran 05 FARS-11
8	SG-699	A	Iran 05 sis-12

Table 3: *Virus isolates received from UN-FAO project on FMD in Pakistan.*

Current study showed that LFBK and LFBKαVβ6 cell lines are susceptible to all FDMV serotypes. There are many cell culture system have been used for the propagation and isolation of FMDV. Some strains of FMDV do not grow properly in primary bovine thyroid cells (BTY). Its isolation requires swine cells like IB-RS-2 [31]. But it is reported that IB-RS-2 cell less sensitive to some FMDV serotypes as compare to *BTY* cells [32], due to *αVβ6* low level on cell surface [23]. Also, *LFBK* cell line is susceptible to all FDMV serotypes.

For the first time this comparative study of *LFBK* and *LFBK αVβ6* cell line for the isolation of FMDV was designed to identify the capabilities of *LFBK αVβ6* for rapid and high yield isolation of FMDV in comparison with *LFBK* cell line. Moreover, we have standardized the chemical requirement of *LFBK* and *LFBK-αVβ6* cell line by providing the different quantities of serum to the cells in growth maintenance media (DMEM). It was observed that the growth cell was optimal when growth medium is supplemented with 10% serum and for maintenance of cells 2% serum medium showed good results. Both of cell lines showed same kind of CPEs but CPEs were appeared on *LFBK αVβ6* more rapidly as compared with *LFBK* cell (Table 4).

Although the reason is still unknown that how *LFBK* and *LFBK αVβ6* support the growth of FMD virus but as reported by La Rocco et al. [28] that it might be these cell lines lake factor which restricted by other bovine origin cell line (Table 5). Further studies are needed to explore the underlying mechanism [33].

S.No	Sample I.D	Serotype	Genotype	TCID50
1	RYK-282	Asia-1	Sindh08	2.8
2	HFZ-480	O	Pan Asia 2 Ant10	5.80
3	OKR-520	A	Iran 05 sis-12	4.80
4	SG-559	Asia-1	Sindh 08	4.47
5	MRP-580	A	Iran 05 sis-12	4.13
6	RHK-618	O	Pan Asia 2 (unnamed)	4.3
7	AJK-628	A	Iran 05 FARS-11	ND*
8	SG-699	A	Iran 05 sis-12	2.8

Table 4: *TCID50 of FMD virus serotypes on LFBK cell line, Result observed after 48 hours of infection. *ND=Not Detected.*

S.No	Sample I.D	Serotype	Genotype	TCID50
1	RYK-282	Asia-1	Sindh08	5.97

2	HFZ-480	O	Pan Asia 2 Ant10	6.47
3	OKR-520	A	Iran 05 sis-12	6.63
4	SG-559	Asia-1	Sindh 08	6.30
5	MRP-580	A	Iran 05 sis-12	6.30
6	RHK-618	O	Pan Asia2(unnamed)	6.47
7	AJK-628	A	Iran 05 FARS-11	ND
8	SG-699	A	Iran 05 sis-12	5.30

Table 5: *TCID50 of different serotypes on LFBKαVβ6 CELL LINES.*

Conclusion

Our results are highly Expressive that persistent infection in riverine buffaloes can serve as the potential source of new FMD outbreaks (Table 6).

The results of current study concluded that use of *LFBK-αVβ6* harvest high yield of FMDV. Our results strongly recommend *LFBK-αVβ6* is the sensitive most cell line for rapid FMDV diagnostic purposes due to high level of *αVβ6* (Table 7).

S. No	Sample I.D	Serotype	Genotype	LFBK/ TCID50	LFBKαVβ6/ TCID50
1	RYK-282	Asia-1	Sindh08	2.8	5.97
2	HFZ-480	O	Pan Asia 2 Ant10	5.80	6.47
3	OKR-520	A	Iran 05 sis-12	4.80	6.63
4	SG-559	Asia-1	Sindh 08	4.47	6.30
5	MRP-580	A	Iran 05 sis-12	4.13	6.30
6	RHK-618	O	Pan Asia2 (unnamed)	4.3	6.47
7	SG-699	A	Iran 05 sis-12	2.8	5.30

Table 6: *TCID50 on LFBK cell line in comparison with LFBK αVβ6 cell lines.*

S. No	Sample I.D	Serotype	Genotype	LFBK/ TCID50	LFBKαVβ6/ TCID50
1	RYK-282	Asia-1	Sindh08	22.23%	50%
2	HFZ-480	O	Pan Asia 2 Ant10	55%	62.97%
3	OKR-520	A	Iran 05 sis-12	44.45%	64.8%
4	SG-559	Asia-1	Sindh 08	40.5%	61%
5	MRP-580	A	Iran 05 sis-12	37%	61%

Table 7: *Percentage of positive well of both cell line.*

Acknowledgment

Authors are very thankful to the UN-FAO Project on FMD "Progressive Control of FMD in Pakistan GCP/PAK/123/USA" for providing recent isolates of FMD viruses for this study.

References

1. Depa PM, Dimri U, Sharma MC, Tiwari R (2012) Update on epidemiology and control of Foot and Mouth Disease-A menace to international trade and global animal enterprise. Vet World 5: 694-704.
2. Grubman MJ, Baxt B (2004) Foot-and-mouth disease. Clin Microbiol Rev 17: 465-493.
3. Awan FN, Khan MA, Muhammad K, Rabani M, Younas M et al. (2009) Epidemiological investigation of foot and mouth disease in districts of Punjab, Pakistan. Pak J Zol Supl Ser 9: 179-185.
4. Zahur AB, Irshad H, Hussain M, Anjum R, Khan MQ (2006) Transboundry animal Disease in Pakistan. J Vet Medicine Series B 53: 19-22.
5. Pakistan Economic Survey (2102-2013) Government of Pakistan, Finance Division, Economic Advisor Wing, Islamabad, Pakistan.
6. Zulfiqar M (2003) Draft Report for Development of National Disease Control Policy for Foot and Mouth Disease in Pakistan under the FAO Project-Support for Emergency Prevention and control of main transboundary animal diseases in Pakistan, Food and Agriculture Organization, Rome, Italy.
7. Fisher JR (1984) Foot-and-mouth disease in Australia. Aust Vet J 16: 158-161.
8. Abubakar M, Manzoor S (2013) Epidemiological Aspects of Foot and Mouth Disease Outbreak in a Sheep Herd. Pak J Life Soc Sci 11: 244-246.
9. Jamal SM, Ahmed S, Hussain M, Ali Q (2010) Status of foot and mouth disease in Pak. Arch of Virol 155: 1487-1491.
10. Domingo E, Baranowski E, Escarmis C, Sobrino F (2002) Foot-and-mouth disease virus. Comp Immunol Microbiol Infect Dis 25: 297-308.
11. Anjum R, Hussain M, Zahoor AB, Irshad H, Farooq U (2006) Epidemiological analyses of foot and mouth disease in Pakistan. Intl J Agri Biol 8: 648-651.
12. Fida HA, Hamid A, Bhutta I (1965) Some epidemiological information on FMD in West Pakistan. Proceedings of 17th Pakistan Scientific Conference, Islamabad, Pakistan.
13. Klein J, Hussain M, Ahmad M, Afzal M, Alexandersen S (2008) Epidemiology of foot and mouth disease in Landhi dairy colony, Pakistan, the world largest buffalo colony. Virol J 5: 53.
14. Rweyemamu M, Roeder P, Mackay D, Sumption K, Brownlie J, et al. (2008) Epidemiological patterns of foot-and-mouth disease worldwide. Trans Bound Emerg Dis 55: 57-72.
15. OIE (2012) Foot and Mouth Disease. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, World Organization for Animal Health, Paris, France.
16. Snowdon WA (1966) Growth of foot-and- mouth disease virus in monolayer cultures of calf thyroid cells. Nature 210: 1079-1080.

17. House C, House JA (1989) Evaluation of techniques to demonstrate foot-and-mouth disease virus in bovine tongue epithelium: comparison of the sensitivity of cattle, mice, primary cell cultures, cryopreserved cell cultures and established cell lines. Vet Microbiol 20: 99-109.
18. Bachrach H, Hess WR (1955) Foot-and-mouth disease virus: Its growth and cytopathogenicity in tissue culture. Science 122: 1269-1270.
19. Hess WR, May HJ, Patty RE (1963) Serial cultures of lamb testicular cells and their use in virus studies. Am J Vet Res 24: 50-64.
20. Clarke JB, Spier RE (1980) Variation in the susceptibility of BHK populations and cloned cell lines to three strains of foot-and-mouth disease virus. Arch Virol 63: 1-9.
21. Ruiz-Sáenz J, Goez Y, Tabares W, López-Herrera A (2009) Cellular receptors for foot and mouth disease virus. Intervirology 52: 201-212.
22. House JA, Yedloutschnig RJ (1982) Sensitivity of seven different types of cell cultures to three serotypes of foot-and-mouth disease virus. Can J Comp Med 46: 186-189.
23. King DP, Burman A, Gold S, Shaw AE, Jackson T, et al. (2011) Integrin sub-unit expression in cell cultures used for the diagnosis of foot and mouth disease. Vet Immunol Immunopathol 140: 259-265.
24. Swaney LM (1988) A continuous bovine kidney cell line for routine assays of foot-and-mouth disease virus. Vet Microbiol 18: 1-14.
25. Brocchi E, Bergmann IE, Dekker A, Paton DJ, Sammin DJ, et al. (2006) Comparative evaluation of six ELISAs for the detection of antibodies to the non-structural proteins of foot-and-mouth disease virus. Vaccine 24: 66-79.
26. Challahan JD, Brown F, Osorio FA, Sur JH, Kramer E, et al. (2002) Use of a portable real-time reverse transcriptasepolymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. J Am Vet Med Assoc 220: 1636-1642.
27. Verin B, Edward J, Ellis T (2012) The role of Asian Buffaloe in Epidemiology of FMD. Scientific development and technical challenges in the progressive control of FMD in south Asia, Washington DC, USA.
28. LaRocco M, Krug PW, Kramer E, Ahmed Z, Pacheco JM, et al. (2013) A continuous bovine kidney cell line constitutively expressing bovine αvβ6 integrin has increased susceptibility to foot-and-mouth disease virus. J Clin Microbiol 51: 1714-1720.
29. Zabal O, Fondevila N (2013) Selection of Highly Susceptible Cell Lines to Foot and Mouth Disease Virus Infection. Open J Vet Med 3: 263.
30. Brehm KE, Kumar N, Thulke HH, Haas B (2008) High potency vaccines induce protection against heterologous challenge with foot-and-mouth disease virus. Vaccine 26: 1681-1687.
31. Dunn CS, Samuel AR, Pullen LA, Anderson J (1998) The biological relevance of virus neutralisation sites for virulence and vaccine protection in the guinea pig model of foot-and-mouth disease. Virology 247: 51-61.
32. Ferris NP, Abrescia NGA, Stuart DI, Jackson T, Burman A, et al. (2005) Utility of recombinant integrin V-6 as a capture reagentin immunoassays for the diagnosis of foot-and-mouth disease. J Virol Methods 127: 69-79.
33. Callens M, De Clercq K (1997) Differentiation of the seven serotypes of foot- and-mouth disease virus by reverse transcriptase polymerase chain reaction. J Virol Methods 67: 35-44.