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Molecular identification and phylogenetic analysis of emerging foot-and-mouth disease virus type O, topotype ME-SA, in the southwestern region of Pakistan[1]

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ABSTRACT.- Khan D, Sheikh IS, Kasi KK, Samad A, Naeem M, Din Z, Roonjho MJ, Tareen MI. **Molecular identification and phylogenetic analysis of emerging foot-and-mouth disease virus type O, topotype ME-SA, in the southwestern region of Pakistan.** *Pesquisa Veterinária Brasileira 46:e07775, 2026.* Center for Advanced Studies in Vaccinology and Biotechnology, University of Balochistan, Quetta, 87300 Pakistan. E-mail: drdaudpk@gmail.com

Foot-and-mouth disease virus (FMDV) is among the most contagious and economically significant viral pathogens of livestock worldwide. In Quetta, Balochistan, recurrent FMDV outbreaks continue to affect cattle and buffalo populations, yet genomic data on circulating strains remain scarce. This study investigated the molecular characteristics and phylogenetic relationships of FMDV serotypes and topotypes detected during 2021 surveillance in Quetta, Pakistan. Twenty epithelial tissue samples, previously confirmed as FMDV-positive through sandwich ELISA, were analyzed for viral RNA using real-time reverse transcription polymerase chain reaction (RT-PCR). Positive samples underwent *VP1* gene sequencing, followed by phylogenetic analysis. FMDV RNA was detected in 80% (16/20; 95% CI: 56%–94%) of samples. Among infected animals, 87.5% were female, 69% were cattle, and most (63%) were aged 1–4 years. The highest number of cases (25%) originated from the Jan Muhammad Road area. Phylogenetic analysis revealed all isolates belonged to FMDV serotype O, topotype Middle East-South Asia (ME-SA). These findings confirm FMDV serotype O (ME-SA) as the dominant strain causing endemic outbreaks in Quetta. Strengthening active surveillance and molecular monitoring systems is crucial for early detection, effective vaccination planning, and regulation of cross-border livestock movement with Afghanistan, Iran, and other regions of Pakistan.

INDEX TERMS: Buffalo, cattle, FMDV serotype O, foot-and-mouth disease, Pakistan, phylogenetic analysis, RT-PCR.

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RESUMO.- [**Identificação molecular e análise filogenética do vírus emergente da febre aftosa tipo O, topotipo ME-SA, na região sudoeste do Paquistão**] O vírus da febre aftosa (FMDV) está entre os patógenos virais mais contagiosos e economicamente significativos para o gado em todo o mundo. Em Quetta, Balochistão, surtos recorrentes de FMDV continuam a afetar populações de bovinos e búfalos, porém dados genômicos sobre as cepas circulantes ainda são escassos. Este estudo investigou as características moleculares e as relações filogenéticas dos sorotipos e topótipos de FMDV detectados durante uma vigilância realizada em 2021 em Quetta, Paquistão. Vinte amostras de tecido epitelial, previamente confirmadas como FMDV-positivas por ELISA sanduíche, foram analisadas quanto ao RNA viral usando reação em cadeia da polimerase com transcrição reversa em tempo real (RT-PCR). As amostras positivas foram submetidas ao sequenciamento do gene *VP1*, seguido de análise filogenética. RNA do FMDV foi detectado em 80% (16/20; IC 95%: 56%–94%) das amostras. Entre os animais infectados, 87,5% eram fêmeas, 69% eram bovinos e a maioria (63%) tinha entre um e quatro anos de idade. O maior número de casos (25%) se originou da área da Jan Muhammad Road. A análise filogenética revelou que todos os isolados pertenciam ao sorotipo O do FMDV, topotipo Middle East-South Asia (ME-SA). Esses achados confirmam que o sorotipo O do FMDV (ME-SA) é a cepa dominante que causa surtos endêmicos em Quetta. Fortalecer a vigilância ativa e os sistemas de monitoramento molecular é crucial para a detecção precoce, planejamento eficaz da vacinação e regulamentação do movimento transfronteiriço de animais com o Afeganistão, Irã e outras regiões do Paquistão.

TERMOS DE INDEXAÇÃO: Búfalo, gado, sorotipo O de FMDV, vírus da febre aftosa, Paquistão, análise filogenética, RT-PCR.

INTRODUCTION

Foot-and-mouth disease (FMD) is caused by the foot-and-mouth disease virus (FMDV). It is a highly contagious disease, affecting cloven-hoofed animals, including buffalo, cattle, goats, sheep, pigs, and various wildlife animals (Jamal & Belsham 2013, Jolles et al. 2021). It has been considered a notifiable disease by the World Organization for Animal Health (WOAH). It has been prevalent across a wide geographic area worldwide, causing substantial economic losses. FMDV belongs to the Picornaviridae family and genus *Aphthovirus.* It has seven serotypes, including SAT 1-3, A, C, O, and Asia 1, with no cross-protection between different serotypes. FMDV strains evolve, posing challenges for susceptible animals worldwide, particularly in disease-free areas, leading to persistent epidemics and making the virus difficult to control (Mahapatra & Parida 2018, Santos et al. 2018). FMDV is reliant on the host cell and has a high mutation rate. It continuously evolves through mutation and recombination, and consequently changes its genome (Kustin & Stern 2021).

Pakistan is an agricultural country. Approximately eight million households in the country are either directly or indirectly involved in the livestock-related businesses, which account for around eleven percent of the national gross domestic product (GDP). Pakistan has approximately 50 million cattle and 40 million buffalo. These animals are at high risk of getting FMDV infection (Jamal & Belsham 2018). Pakistan remains in endemic pool-3 for FMDV, characterized by co-circulation of A, O, and Asia 1 serotypes (Naqvi et al. 2022). In Pakistan, FMDV outbreaks are controlled through mass vaccination with polyvalent vaccines that include antigens from the A, Asia 1 and O serotypes, along with quarantine measures. Due to limited resources, culling has not been considered as a viable option. Poor management practices often lead to the emergence and subsequent spread of new, potentially more infectious variants of FMDV (Shah et al. 2014). Moreover, this region provides a unique environment for the emergence and establishment of new variants of serotypes O, A, and Asia 1 (DiNardo et al. 2021).

FMDV serotype O is the most widely prevalent among the seven serotypes in many parts of the world, including Pakistan. More than eighty percent of the outbreaks of FMD in East Asia and South-East Asia are caused by serotype O. Historically, there are three FMDV lineages of serotype O, which have been circulating in recent years in East Asia and South-East Asian regions, including O/ME-SA/Pan-Asia, O/CATHAY, and O/SEA/Mya-98. Another lineage O/ME-SA/Ind-2001, which was first reported and restricted in the Indian subcontinent, has caused large-scale outbreaks of FMD, further complicating the epidemiological situation (Hemadri et al. 2002, Jamal et al. 2011).

Balochistan has a large livestock population and makes a major contribution to the provincial GDP. It shares long borders with Afghanistan and Iran, and there is continuous unregulated animal movement across the borders, which always remains at risk of transboundary animal diseases (TAD), including FMD. Hence, it causes major economic losses to the livestock sector. For effective preventive and control measures, it is pertinent to determine the currently circulating FMDV serotypes in the province. The last molecular study in Balochistan was conducted in

2011 (Ullah et al. 2016). Therefore, it is necessary to determine the molecular identification of the currently circulating FMDV in livestock in Balochistan province, which is performed in the current study.

MATERIALS AND METHODS

Ethical approval. The ethical approval was obtained from the Ethical Committee of Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan Quetta, under registration # 1991/UB-2019/R-665.

Sample collection and processing. A cross-sectional study was conducted by our research team from January to December 2021 to investigate the circulating FMDV serotypes in cattle and buffaloes in the Quetta district of Balochistan, Pakistan (Khan et al. 2024) (Fig. 1). During this period, a total of 200 epithelial tissue samples were collected from animals clinically suspected of FMD infection, based on reports of outbreak events in livestock farms across the district. Suspected cases were identified by the presence of characteristic clinical signs, including vesicular lesions on the tongue, buccal mucosa, udder, and hooves.

All tissue samples were collected aseptically in sterile Falcon™ tubes containing virus transport medium comprising phosphate-buffered saline, glycerol, and antibiotics to maintain RNA integrity. Notably, from the 114 samples that tested positive by ELISA, a subset of 20 was selected for further molecular characterization, including virus isolation and VP1 sequencing (Khan et al. 2024). These 20 samples were selected using simple random sampling to ensure an unbiased representation of ELISA-positive cases for downstream analysis.

FMDV isolation. Epithelial tissue samples (at least 1 gm for each sample) were washed twice in phosphate-buffered saline (PBS). Further, each sample was triturated using a sterilized mortar and pestle in 1 mL PBS, centrifuged at 2,000 rotations per minute (rpm) for 10 minutes, and finally, the supernatant was obtained. Supernatant was diluted fivefold by the addition of 1 mL of supernatant into 4 mL sterile Dulbecco's Modified Eagle's medium-high glucose (DMEM, D7777-50L, Sigma-Aldrich, Germany), and then filtered through a 0.2 µm filter. Five hundred microliters (500 µL) of filtrate was inoculated onto a monolayer of low-passage fetal bovine kidney cell line (LFBK) in 25 cm^2 cell culture flasks obtained from the virology section of the Animal Health laboratory of Animal Science Institute at NARC, Islamabad, Pakistan. For each sample, two flasks were inoculated. For the control, sterile PBS was inoculated in a separate flask.

Furthermore, flasks were incubated for 30 minutes to absorb the virus. Then, 7 mL of DMEM was added to each flask, and the flasks were incubated at 37 °C in a CO_2 incubator for 72 hours. During this incubation period, each flask was observed after every 24 hours for the appearance of cytopathic effects (CPE). The samples with 80% specific CPE were considered positive. Those samples with no CPE after three passages were considered negative. CPE was identified as rounding/swelling of the cells, shriveling, detachment of the cells from the flask surface, and increased refractivity.

Molecular identification. Viral RNA was extracted from the supernatants of cell culture by using QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and stored at -20 °C until further processing. Furthermore, real-time reverse transcription polymerase chain reaction (rRT-PCR) was performed to detect viral RNA by using TaqMan® EZ RT-PCR kit (Applied Biosystems, California, United States of America), and the primers sequences (forward: 5' ACTGGGTTTTACAAACCTGTGA 3' and reverse: 5' GCGAGTCCTGCCACGGA 3') and probes (5' TCCTTTGCACGCCGTGGGAC) were used as described by Callahan et al. (2002). The master mix with a total volume of 22.5 µL included: 5 µL of TaqMan EZ Buffer, 3 µL of dNTPs, 2.5 µL of 25 mM $Mn(Oac)_2$, 0.1 µL (25 pm) each of forward and reverse primers, 0.25 µL (25 pm) of probe, 10.5 µL of RNase-free water, and 1 µL of rTth DNA polymerase. The rRT-PCR reactions were performed in the Applied Biosystems™ 7500 Real-Time PCR System (Thermo Fisher Scientific Inc, Waltham, Massachusetts, United States of America). Thermal cycling conditions were as follows: initial reverse transcription step at 60 °C for 10 minutes, followed by 45 cycles of 95 °C for 2 seconds and 60 °C for 60 seconds. Finally, CT values were obtained, and results were interpreted. For the sequencing, samples were sent to the Pirbright Institute, Ash Road, Pirbright, United Kingdom.

Phylogenetic analysis. A phylogenetic tree was constructed for the VP1 region of 639 nucleotides (nt). Sequences of the current study were entered in the Basic Local Alignment Search Tool (BLAST)[5] at the National Center for Biotechnology Information (NCBI)[6] to identify regions of similarity between the current study sequences and GenBank sequences. These were further imported into MEGA 11 (Tamura et al. 2021) and aligned by the

[5] Accessed Dec 13, 2022. https://blast.ncbi.nlm.nih.gov/Blast.cgi

[6] Accessed Dec 13, 2022.https://www.ncbi.nlm.nih.gov/

ClustalW method. The evolutionary relationship was inferred using the neighbor-joining method with an output of a phylogenetic tree (Saitou & Nei 1987). The evolutionary distances were computed by using the Kimura 2-parameter model (Kimura 1980). A bootstrap test with 1,000 replicates was performed to assess the robustness of the topology of the tree (Felsenstein 1985).

Statistical analysis. The data were entered into Microsoft Excel 2016 (Microsoft, Washington, United States of America) and further transferred to R and R-studio software (R-Studio 2016) for statistical analysis. The exact 95% Clopper-and-Pearson interval method was used to estimate binomial confidence intervals (CIs) for proportions by using the binom test function in the binom package (Dorai-Raj 2014). The study area map was developed by using the QGIS geographic information system software version 3.38.1[7].

RESULTS

A total of 200 epithelial tissue samples were collected from suspected FMD cases. Of these, 114 (95% CI: 50%–64%) tested positive for FMDV by enzyme-linked immunosorbent assay (ELISA) (Khan et al. 2024). From these 114 positive samples, a random subset of 20 was selected for further molecular analysis. Of these 20 samples tested, 80% (16/20; 95% CI: 56%–94%) were confirmed positive for FMDV using RT-PCR. The demographic analysis was based on these 16 confirmed positive animals. Among the FMDV-positive animals, 87.5% (14/16) were female, while 12.5% (2/16) were male. Regarding species distribution, 69% (11/16) of the positive cases were detected in cattle, and 31% (5/16) in buffaloes. Analysis by age group revealed that FMDV positivity was highest in animals aged 1–4 years (10/16, 63%), followed by animals over four years old (5/16, 31%), and the lowest prevalence was observed in animals under one year of age (1/16, 6%) (Fig. 2).

Geographical distribution showed that the highest number of FMDV-positive cases were recorded in the Jan Muhammad Road area (4/16, 25%), followed by Aghburg (3/16, 19%), Panjpai and Qambrani Road (2/16, 13% each), and one positive case (6.25%) each in Killi Almas, Killi Almo, Rasani Road, Samali Salam Killi, and Miaghundi (Table 1).

Phylogenetic analysis of the *VP1* gene region revealed that all sequences obtained in this study belonged to FMDV serotype O, specifically the Middle East-South Asia (ME-SA) topotype (Fig. 3). Comparative sequence analysis demonstrated a high nucleotide identity with FMDV strains reported in neighboring and regional countries. The isolates showed:

- 99.84% identity with strains circulating in the United Arab Emirates in 2021 (NCBI Accession Numbers: OR425052, OR425051),
- 99.69% identity with Indian strains from 2021 (NCBI Accession Numbers: OQ847009, OQ847008, OQ847002, OQ847003),
- 99.65% identity with Pakistani strains from 2021 (NCBI Accession Number: ON014775),
- 97.81% identity with Iranian strains reported in 2022 (NCBI Accession Number: OR354929) (Table 2).

DISCUSSION

Balochistan, a geographically expansive province in southwestern Pakistan, is home to a substantial livestock population, making the livestock sector a critical contributor to the provincial economy (Ullah et al. 2016). The province's strategic location, bordering Afghanistan and Iran, facilitates continuous, largely unregulated cross-border animal movement. This dynamic significantly heightens the risk of transboundary animal diseases (TADs), with FMD being the most prominent and persistent threat. Despite frequent outbreaks causing significant production losses and economic damage (Kanwal et al. 2014, Ullah et al. 2016), data on the genomic diversity of circulating FMDV strains in Balochistan have remained limited. This study addresses this gap by characterizing the FMDV serotypes and topotypes identified during routine surveillance in Quetta district in 2021, providing data to inform more effective control, prevention, and vaccination strategies.

Molecular analysis of epithelial tissue samples from suspected livestock in Quetta revealed that 80% were positive for FMDV. To our knowledge, only one previous study has reported the molecular identification of FMDV in Balochistan, conducted in 2011 in the Mastung and Quetta districts, which found an 89% positivity rate in cattle (Ullah et al. 2016). In other provinces of the country, several molecular studies for the identification of FMDV have previously been reported, with Punjab province (northeastern part of the country) remaining on the top. In Punjab province, the first molecular study for the FMDV was conducted in 2005, and the latest in 2022. The molecular

[7] Accessed Nov 10, 2022. https://www.qgis.org/

prevalence ranged from 5% to 90% in different districts of the Punjab (Ali et al. 2017a, Ali et al. 2017b, Yousaf et al. 2021, Abbas et al. 2023). A previous molecular study in the Sindh province of Pakistan reported 22% FMDV positivity among livestock (Jamal et al. 2011). Another study in the Sindh and Punjab provinces reported an 89% molecular identification of FMDV in livestock (Ali et al. 2018). Other studies conducted in Khyber Pakhtunkhwa, Sindh, Punjab, Azad Jammu and Kashmir (AJK), and the Federal Islamabad Capital Territory (ICT) of Pakistan have reported FMDV molecular identification ranging from 71–83%. In these studies, only the cumulative molecular prevalence for the respective provinces was reported, not for individual provinces (Waheed et al. 2009, Ahmed et al. 2018). Studies conducted in the ICT of Pakistan have reported molecular prevalence of FMDV in livestock of 11% (Navid et al. 2018), 12% (Ahmed et al. 2017), and 55% (Ali et al. 2019).

Findings from current and previous studies indicate persistent circulation of FMDV foci across wider geographic areas of the country. Balochistan borders Afghanistan and Iran on the southwest, with continuous, uncontrolled animal movement across these borders (Waheed et al. 2011). Secondly, there is no animal identification system in the country, and animal movement is uncontrolled. This results in the persistence and transmission of FMDV to a broader geographical area within the country. Molecular analysis of epithelial tissue samples from suspected livestock in Quetta revealed that 80% were positive for FMDV. To our knowledge, only one previous study has reported the molecular identification of FMDV in Balochistan, conducted in 2011 in the Mastung and Quetta districts, which found an 89% positivity rate in cattle (Ullah et al. 2016). The persistently high detection rate over a decade highlights the virus's ongoing circulation in the region and underscores the urgent need for enhanced surveillance and control measures.

Phylogenetic analysis of the VP1 region confirmed the circulation of FMDV serotype O, specifically the Middle East-South Asia (ME-SA) topotype, among livestock in Quetta district during 2021. The sequenced viruses showed high nucleotide identity (97–99%) with previously reported FMDV serotype O ME-SA strains from the United Arab Emirates, India, Pakistan, and Iran, collected between 2021 and 2022. This close genetic relationship indicates a high level of connectivity and viral flow within the region. While serotypes A and Asia-1 were previously reported in Balochistan in 2011 (Ullah et al. 2016), this study provides the first published report confirming the circulation of the serotype O ME-SA topotype in the province. The absence of prior reports is likely attributable to the historical lack of molecular surveillance and active sampling in the area, rather than the recent introduction of the serotype. The findings of this study are consistent with the broader epidemiological picture of FMDV in Pakistan. The serotype O ME-SA topotype has been the predominant strain reported across the country from 2005 to 2022, including in Punjab, Khyber Pakhtunkhwa, Sindh, Azad Jammu and Kashmir, and the Federal Islamabad Capital Territory (Ali et al. 2017a, 2018, Ahmed et al. 2018, Abbas et al. 2023). While serotypes A and Asia-1 were previously reported in Balochistan in 2011 (Ullah et al. 2016).

This study provides what seems to be the first published report confirming the circulation of the serotype O ME-SA topotype in the province. The absence of prior reports is likely attributable to the historical lack of molecular surveillance and active sampling in the area, rather than the recent introduction of the serotype. The presence of this topotype in Balochistan is epidemiologically significant due to the province's extensive border with Afghanistan, where the serotype O ME-SA topotype is also known to circulate (Jamal et al. 2011, Waheed et al. 2011). The continuous, unregulated movement of livestock across this border, coupled with the absence of quarantine measures and a national animal identification system, facilitates the frequent introduction, emergence, and subsequent adaptation of FMDV strains. This transboundary circulation perpetuates the virus and leads to major economic losses for the regional livestock industry (Munir et al. 2023). The findings from this study confirm that the FMDV serotype O ME-SA topotype is a significant and persistent threat to livestock in Balochistan, mirroring its endemic status in the rest of the country and the broader region.

CONCLUSION

In conclusion, this study confirms the active circulation of foot-and-mouth disease virus (FMDV) serotype O among livestock in the Quetta district, with a high positivity rate among suspected cases. Molecular analysis of a subset of samples confirmed the presence of serotype O. The demographic analysis revealed a higher detection rate in cattle, females, and animals aged 1–4 years. These findings provide baseline data on the current epidemiological situation and highlight the need for continued, targeted surveillance to inform evidence-based control strategies in Balochistan.

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Conflict of interest statement.- The authors declare that they have no conflicts of interest.

Credit author statement.- DK: Writing – review & editing, writing – original draft. ISS: Supervision, methodology, investigation, funding acquisition, formal analysis, data curation, conceptualization. KKK: Software, resources, project administration, investigation. AS, MN**,** ZD, MJR: Visualization, resources, investigation. DK & KKK: Writing – review & editing, validation.

Data availability statement.- The data sets analyzed during the current study are available from the corresponding author (BE) on reasonable request.

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Figures

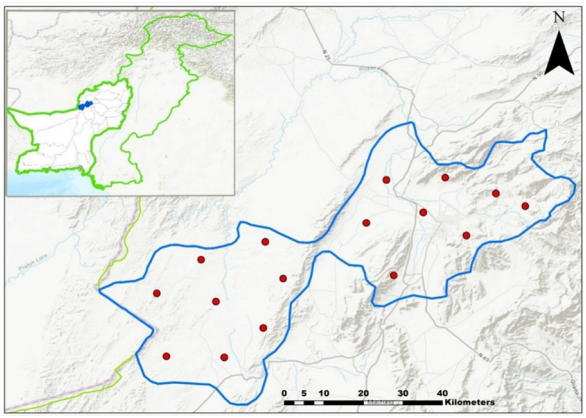

Fig. 1. Geographical distribution of foot-and-mouth disease virus (FMDV) positive animals (red circles) in Quetta district, Balochistan, Pakistan.

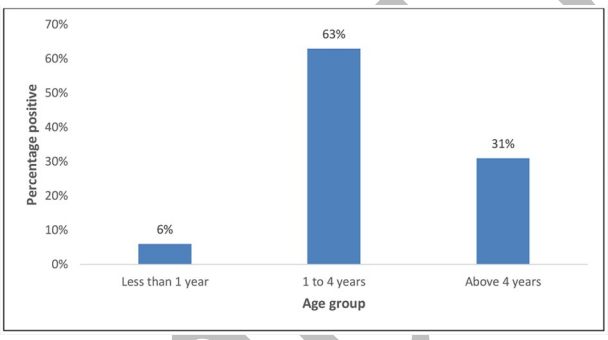

Fig. 2. Age-group-wise animals positive for foot-and-mouth disease virus (FMDV).

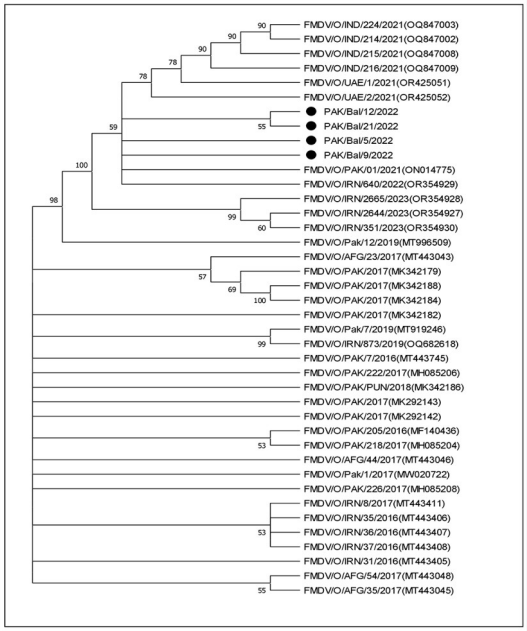

Fig. 3. Phylogenetic tree of foot-and-mouth disease virus (FMDV) serotype O of the VP1 region of 639 nucleotides by using the neighbor-joining method. Sequences with dark circles (●) are the samples of the current study and are positive for serotype O.

Table 1. Area-wise frequency of Foot-and-mouth disease virus (FMDV) positive animals in Quetta district, Balochistan

Geographical area	Frequency	Percentage
Jan Muhammad Road	4	25%
Aghburg	3	19%
Panjpai	2	13%
Qambrani road	2	13%
Killi Almas	1	6%
Killi Almo	1	6%
Rasani road	1	6%
Samali Salam Killi	1	6%
Miaghundi	1	6%
TOTAL	16	100%

Table 2. Percentage identity of current study sequences with the sequences in the NCBI GenBank[8]

Country	Year	Serotype	Percentage of identity	Accession number in NCBI GenBank
United Arab Emirates	2021	O	99.84%	OR425052
United Arab Emirates	2021	O	99.84%	OR425051
India	2021	O	99.69%	OQ847009
India	2021	O	99.69%	OQ847008
India	2021	O	99.69%	OQ847003
India	2021	O	99.69%	OQ847002
Pakistan	2021	O	99.65%	ON014775
Iran	2022	O	97.81%	OR354929
Iran	2023	O	97.65%	OR354927
Iran	2023	O	96.4%	OR354928
Pakistan	2019	O	96.37%	MT996509
Iran	2023	O	96.24%	OR354930
Pakistan	2016	O	95.6%	MT443745
Pakistan	2017	O	95.44%	MK342179
Pakistan	2017	O	95.44%	MK292142
Pakistan	2016	O	95.44%	MF140436
Iran	2017	O	95.44%	MT443411
Pakistan	2017	O	95.28%	MH085204
Iran	2016	O	95.28%	MT443407
Pakistan	2018	O	95.11%	MK342186
Pakistan	2017	O	95.11%	MK292143
Pakistan	2017	O	95.11%	MH085208
Pakistan	2017	O	95.11%	MH085206
Pakistan	2017	O	95.11%	MW020722
Pakistan	2019	O	95.11%	MT919246
Iran	2016	O	95.11%	MT443408
Iran	2016	O	95.11%	MT443406
Iran	2016	O	95.11%	MT443405
Afghanistan	2017	O	94.95%	MT443048
Afghanistan	2017	O	94.95%	MT443046
Afghanistan	2017	O	94.95%	MT443045

[8] Accessed Dec 13, 2022. https://blast.ncbi.nlm.nih.gov/Blast.cgi

Afghanistan	2017	O	94.95%	MT443043
Pakistan	2017	O	94.79%	MK342188
Pakistan	2017	O	94.79%	MK342184
Pakistan	2017	O	94.79%	MK342182
Iran	2019	O	94.79%	OQ682618