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Original Article

Comparative homology and complete B2L gene-based sequence analysis of ORF virus from sheep and goats in Pakistan

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Abstract

Contagious Ecthyma (CE), also known as scabby mouth disease, is caused by an epitheliotropic parapoxvirus that primarily affects the goat and sheep populations worldwide. This study focused on investigating 12 outbreaks of CE in sheep and goat herds across various regions of Punjab, Pakistan. A total of 35 samples were collected between March 2021 and May 2022, with 34 out of 35 samples testing positive for parapoxvirus through PCR. Subsequently, 24 complete sequences of the major envelope protein B2L gene were successfully obtained. The nucleotide and amino acid sequences of the ORF virus B2L gene were analyzed. The 1206bp amplicons, after Sanger sequencing revealed an open reading frame of 1137bp encoding 378 amino acids. The minimum and maximum nucleotide differences of 0 and 34, respectively, were observed, while the percentage similarity at the nucleotide level and amino acid level ranged from 97.98% to 100% and 97.62% to 100%, respectively, among the ORF strains in this research study. The results of the phylogenetic analysis revealed that all 24 ORF virus isolates from Pakistan belonged to Group-I ORF viruses. The comparative homology of Pakistani ORF virus strains with Indian, Chinese, and Turkish isolates was 99.03%, 98.59%, and 98.15%, respectively. This study contributes to understanding the circulation of Group I ORF viruses in Pakistan and their relationship with strains from neighbouring countries. Furthermore, these findings may offer insights into the genotype of the causative agent responsible for the contagious pustular dermatitis (CPD) outbreak in Punjab, Pakistan.

Keywords: B2L gene, Contagious, Contagious pustular dermatitis, ORF

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Introduction

Epitheliotropic parapoxviruses (PPVs), members of the *Poxviridae* family, infect a variety of species, including goats, sheep, camelids, deer and occasionally, human beings (Kumar et al., 2022; Zhu et al., 2022). Diseases associated with parapoxviruses are prevalent in various parts of the world, spanning Germany, New Zealand, Norway, Finland, Italy, the United Kingdom, North and South America, Western Australia, and several Asian countries (Lawan et al., 2021).

Contagious Ecthyma (CE), also known as ORF virus disease, CPD, sore mouth, infectious labial dermatitis, and scabby mouth, is caused by a prototype ORF virus. This virus belongs to the genus 'Parapoxvirus' and is closely related to other parapoxviruses, including pseudocowpox virus (PCPV), red deer parapoxvirus in New Zealand (PVNZ), and bovine papular stomatitis virus (BPSV) (Li et al., 2023; Peralta et al., 2023). The virus genome comprises a linear double-stranded DNA molecule, measuring 138 kb in size, with a G+C content of up to 64%. Morphological characteristics, such as a high G+C content, a relatively small size and a surface exhibiting a crisscross arrangement, set it apart from other parapoxviruses (Lawal et al., 2021). The ORF virus genome is composed of 132 putative genes, and among these, the B2L gene encodes for a highly immunogenic envelope protein weighing 42 kDa. The B2L gene, known for its high conservation across different strains of the ORF virus, serves as a valuable tool for virus detection, molecular characterization, and phylogenetic analysis (Shehata et al., 2022).

The disease is often neglected in tropical regions due to its self-limiting nature and a lack of proper attention. However, the zoonotic nature of the disease presents substantial health risks to veterinarians, butchers, and small ruminant holders. Although rare, there is potential for human-to-human transmission (Pang and Long, 2023; Tobler et al., 2022).

The ORF virus infects goats more severely compared to sheep. Infection typically occurs through contact, with the virus entering epidermal cells through bruised or damaged skin. This leads to the development of proliferative localized skin lesions in various areas, including the oral mucosa, nostrils, and lips. The disease has an incubation period ranging from 3 to 7 days, and clinical signs typically diminish within 3 to 5 weeks after progressing through several stages (Martins et al., 2021; Sahu et al., 2022). The lesions are multifocal, initially occurring predominantly on the oral commissures and subsequently spreading to the lips, mucocutaneous junction, muzzle, tongue, nostrils and eyelids. In severe cases, the lesions may extend to areas such as the udder, feet and genitalia. Infected animals often experience difficulty feeding properly due to the presence of painful scabs on their mouths and lips (Eilts et al., 2022; Hussain et al., 2023). CPD disease exhibits high morbidity, with variable mortality rates ranging from 10% to 90%. In cases where care is inadequate, the lesions are prone to secondary bacterial infection. Additionally, in sheep and goats, lesions on the udder skin have the potential to cause inflammation in the mammary glands (Wu et al., 2023). The virus is highly resistant to desiccation in the environment and has been recovered from dried scab material after 12 years (AlDaif et al., 2022).

Putative virulence proteins of the ORF virus, possessing immunomodulatory properties, have been characterized. The OVIFNR gene, located at the left terminal of the viral genome, encodes an interferon resistance protein. This protein, homologous to the ORF virus B2L gene, plays a crucial role in the interferon-induced antiviral state within infected cells. OVIFNR functions by inhibiting a dsRNA-dependent kinase, which would otherwise suppress both virus and cell protein production. Additionally, the ORF virus B2L gene encodes a highly immunogenic envelope protein that elicits a robust antibody response (Wang et al., 2022).

Like other poxviruses, the ORF virus replicates in the presence of an active host immune and inflammatory response. Viruses, including the ORF virus, have evolved immune modulatory genes and proteins. These elements are designed to inhibit protective aspects of host immunity, providing the virus with the means to replicate in epidermal cells. This modulation is orchestrated to counteract the host's defensive mechanisms, allowing the virus to persist and replicate within the host's cellular environment (Bukar et al., 2021; Minott et al., 2022). The repertoire of immune modulatory proteins encoded by DNA viruses varies with the pathogenesis of the virus, offering insights into the nature of host immunity during these infections. In the context of ORF virus infection, the inhibition of interferon is crucial for virus survival.

Both OVIFNR and vIL-10 play roles in this process. vIL-10, in particular, inhibits inflammatory cytokines like IL-1 beta and TNF alpha, as well as chemokines such as IL-8. This inhibition impacts the recruitment and subsequent activation of immune and inflammatory cells, influencing the local immune response at the site of infection (Tong et al., 2020).

Several seasonal outbreaks of the disease are observed annually in Pakistan and the CPD is considered endemic in many areas of Punjab, Pakistan. Despite its recurrent occurrence, CPD is categorized as one of the neglected viral diseases of livestock in Pakistan (Iqbal et al., 2020). As of now, no vaccine against CPD is available for traditional and commercial farmers in Pakistan. Developing a vaccine hinges on understanding the genetic diversity of local ORF virus strains, but data regarding ORF virus strains currently circulating in sheep and goat population of Pakistan is limited.

Considering the aforementioned factors, this study was conducted to molecularly characterize and perform a phylogenetic analysis based on the complete B2L gene sequence of ORF virus strains. The strains were isolated from outbreaks in the sheep and goat population across various regions of Punjab, Pakistan, encompassing Cholistan, Multan, and Faisalabad.

Material and methods

Study area

In Southern Punjab, Pakistan, which houses 32% of the province's human population and encompasses 52% of its agro-ecological land, the desert region of Cholistan spans three districts: Bahawalpur, Rahim Yar Khan, and Bahawalnagar. Livelihoods in Cholistan heavily rely on livestock, including substantial numbers of small ruminants such as sheep, goats, cattle and camels. Cholistan's small herds, including camels are managed in nomadic, non-humanitarian, and sedentary farming systems. Livestock farmers face challenges like hot and humid climates, inadequate water sources, poor husbandry practices, fodder scarcity, seasonal migration and a lack of animal health monitoring services. These challenges not only impact farmers but also contribute to the occurrence of diseases in small ruminants. Despite the significance of small ruminants in desert communities, there is a dearth of proper husbandry methods, promoting the rapid multiplication and transmission of diseases in sheep and goats, resulting in severe outbreaks. A substantial knowledge gap exists regarding disease outbreaks and diagnosis in small ruminants in these areas.

Sample collection

In this study, a total of twelve outbreaks of CPD were investigated, with seven occurring in Bahawalpur, two in Multan and three in Faisalabad from March 2021 to May 2022 (Figure 1). Clinical signs in animals infected with the ORF virus were meticulously observed. Typically, three representative animals were sampled per outbreak, except for the 9th outbreak, where only two animals were sampled. Sampling was based on the observed clinical features of the disease and skin scrapings from the muzzle, lips and oral commissures of goats and sheep were collected from different herds (Figure 2). A total of 35 animals were sampled across these 12 outbreaks. The collected samples were placed in viral transport media, labeled and shipped to the virology laboratory in the Department of Microbiology, Faculty of Veterinary and Animal Sciences, The Islamia University of Bahawalpur. Here, the samples were stored at -40°C until further laboratory analysis.

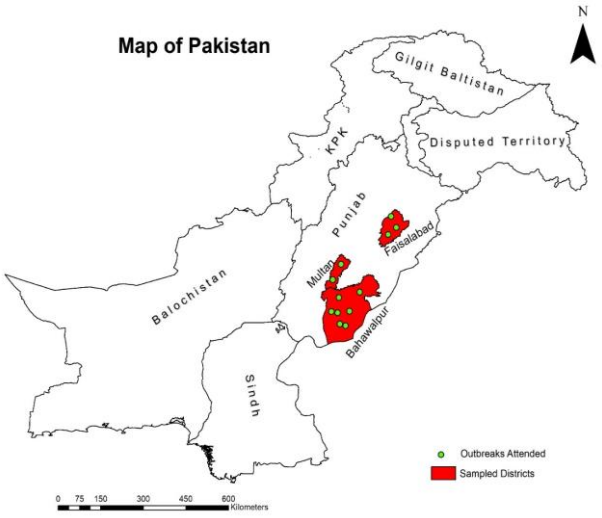

Figure-1. Map of Pakistan showing outbreaks attended in three districts of Punjab

DNA isolation

First of all, dried scabs (35 in total) were washed with 1X PBS solution to remove the dust and debris. The samples were then triturated in 1X PBS with the help of an electronic tissue homogenizer (Heidolph slientcrusher, Netherlands). Following

homogenization, the samples were sonicated (Sonicator, Thermo fisher scientific, USA) in order to release the virus particles from the cells. After sonification the suspension was then clarified by centrifugation at 8000x g for 10 min at 4 °C and clarified supernatant was used for viral DNA extraction using Alphagen viral nucleic acid extraction kit (Cat. # APVNK200, Alphagen Biotech Ltd., Taiwan). The DNA was resuspended in nuclease free water. All the steps for extraction were performed as per manufacturer's instructions.

Figure-2. Sampling of ORF positive animals (A&B)

Polymerase chain reaction (PCR)

The concentration of extracted genomic DNA was determined using spectrophotometer (Thermo Fisher scientific Nano drop UV-vis spectrophotometer). DNA concentration was determined by calculating the absorbance value at 260nm wavelength (A260) while purity of DNA samples was determined by calculating (A_{260}/A_{280}) ratio. ORFV specific B2L gene encoding highly immunogenic major envelope glycoprotein was amplified using full length gene primers. The nucleotide sequence of forward and reverse primers used for amplification of B2L gene were OVB2LF1: TCCCTGAAGCCCTATTATTTTTGTG (25mer) and OVB2LR1: GCTTGCGGGCGTTCGGACCTTC (22mer). These primers were expected to amplify 1206 nucleotide (nt) long ORFV-specific gene (B2L) segment. The primer stock solution 100µm was prepared by adding amount of nuclease free water in lyophilized primers as per manufacturer's guidelines. For PCR 10µM working solution was prepared by diluting the primer at 1/10 ratio (1µL of primer stock was added in 9µL of nuclease free water). Ready to use premix (Vazyme 2x Taq Plus Master mix), primers, nuclease free water and DNA template were used to prepare PCR reaction mixture. Each reaction tube contained 10µL premix, 1µL of 10µM forward and reverse primer, 4µL nuclease free water and 4µL DNA template. The PCR conditions for the reaction were as follows: Pre-denaturation at 95°C for 3 minutes followed by 35 cycles of final denaturation (95°C for 15 seconds), annealing (57°C for 15 seconds) and extension (72°C for 70 seconds) and at the end final extension step at 72°C for 5 minutes. The annealing temperature was calculated using online NEB Tm calculator whereas remaining PCR reaction conditions were provided by the manufacturer of premix.

Gel electrophoresis

Tris-acetate-EDTA (TAE 50X) buffer stock solution (Thermo-scientific, Cat # B49. Baltics UAB–Lithuania) was diluted to 1X by adding 1mL of TAE in 49mL of deionized water and then 1% agarose gel was prepared by dissolving 1 gram of agarose powder (Invitrogen) in 100mL of TAE buffer, heated in microwave oven to boil again and again till solution became transparent. The safe gel red stain (Zokeyo, China) was added (1uL for 10mL of gel) to the molten gel, cooled down to 55° C, poured into cassette and then immediately comb was placed to create wells. After the gel was completely solidified the comb was removed gently and placed in electrophoresis chamber (Bio-Rad) containing 1X TAE buffer. The amplicons on holding temperature (4°C) in thermal cycler were removed and using pipette about 10µL volume was poured into wells of solidified agarose gel. Positive and negative controls were added in the wells after

samples. About 4µL 100bp DNA ladder (Takara, China) was also added in wells on both sides of samples. Electrophoresis container was covered with lid, wires were attached, and voltage (100V) for 25 minutes was provided using voltage system (Bio-rad). Gel was removed after required time, placed on UV transilluminator, and observed for bands of amplified gene on 1206bp.

Nucleotide sequencing

Thirty-four samples were found positive out of 35 samples, producing bands of 1206bp size on gel. Positive bands were cut using a gel cutter, purified using a gel extraction kit (Thermo Fisher Scientific, Cat # K0691, Baltics UAB–Lithuania) as per manufacturer's guidelines. Bidirectional Sangar sequencing was performed at Lab Genetics, Lahore, Pakistan. Obtained sequences were edited using Geneious R11 software, resulting in an open reading frame of 1137bp encoding 378 amino acids. The 1137 nt long full-length B2L gene sequences from Pakistan. NCBI BLAST searches were then performed using the non-redundant database. Multiple sequence alignment was performed using Translation Align in Geneious R11. Pairwise distances were then calculated. Phylogenetic analysis was performed in MEGA-X11 using Neighbour-Joining method.

Results

Clinical findings

A total of 35 animals were sampled from 12 different CPD disease outbreaks. Among these 12 outbreaks, seven, three and two outbreaks were observed in district Bahawalpur, Faisalabad and Multan respectively. All sampled animals showed different stages of lesions including papules, vesicles, pustules and thick scabs on muco-cutaneous junction of lips and on other body parts including muzzle, tongue, around erupting incisors and oral mucosa (Figure 3). Some animals also showed lesions on feet, ears, face, teats, flank, scrotum, udder and vulva. Animals with painful scabs on mouth and lips were unable to eat and were found weak and anorexic. Some animals with pustular lesions on teat and udder showed the signs of mastitis. All the other physical parameters were found within reference range regardless of the hydration status that was below the normal.

Identification of ORF virus

ORF virus specific PCR successfully detected the ORF virus in 34 scab samples collected during this study (Figure 4). Sangar sequencing yielded complete nucleotide sequence of the complete major envelop protein B2L gene from 24 samples. The remaining samples, showing poor sequence quality, were subjected to a second round of PCR and sequencing. Unfortunately, even on the second attempt, we were not able to obtain full length gene sequence of B2L gene, this could be attributed to a low virus load in the samples**.** An open reading frame of 1137 bp encoding 378 amino acids was obtained by editing and aligning the complete major envelop protein B2L gene forward and reverse sequences in Geneious R11 software.

Figure-3. Representative pictures of animals displaying typical lesions of CPD on oral commissars

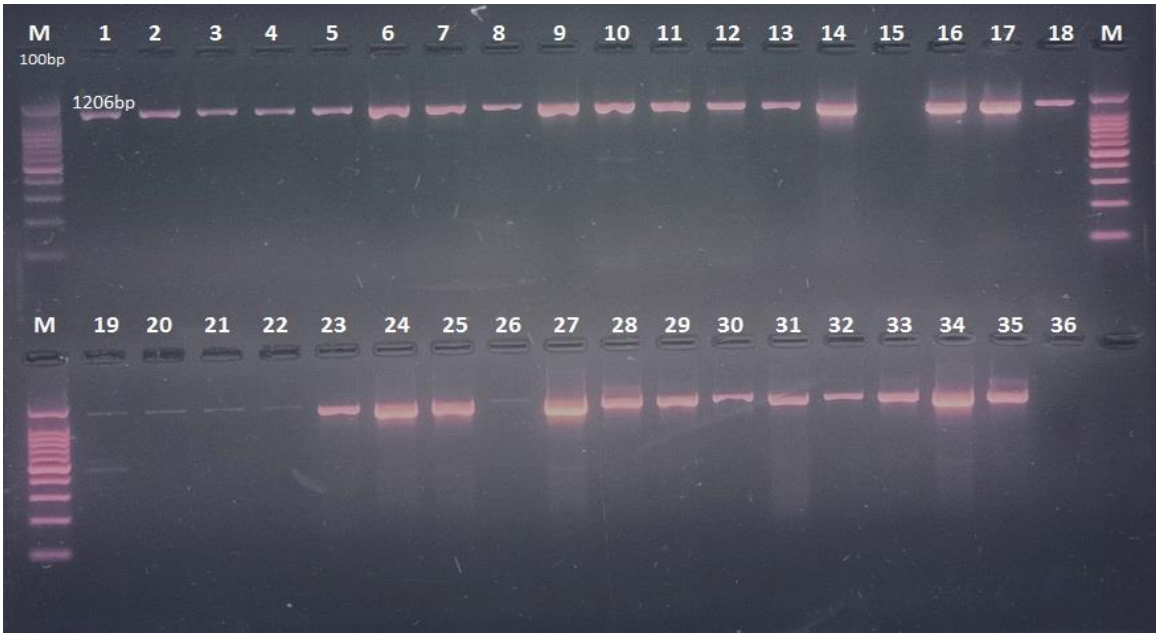

Figure-4. Amplification of the ORF virus complete major envelop protein B2L gene segment (1206bp) *via* PCR

All 24 sequences obtained from this study showed close homology with the ORF virus strain, further confirming the identity of the virus as ORF virus. A translation alignment of nucleotide sequences was then performed, and pairwise distances were computed. Similarly, amino acid-based alignments were also performed, and pairwise distances between amino acid and nucleotide sequences were calculated. The percentage similarity among nucleotide sequences ranged from 97.98% to 100%, while the similarity of amino acid sequences ranged from 97.62% to 100%, respectively.

Phylogenetic analysis

A phylogenetic tree was constructed based on the deduced amino acid sequences of the B2L gene. To determine the phylogeny of the 24 Pakistani ORF virus strains, 57 representative sequences were retrieved from GenBank. Translation alignment of these 81 sequences was performed and a neighbor-joining tree (NJ) was constructed. All 24 sequences from Pakistan clustered in group I from (OP744469 to OP744492). The ORF virus strains representing countries India, China and Turkey also belong to group I, while those from North America, Brazil, Finland and South Korea belong to group II. The Pseudocowpox virus (PCPV) (Finland) and ORFV strains from camels (India) belong to group III (Figure 5).

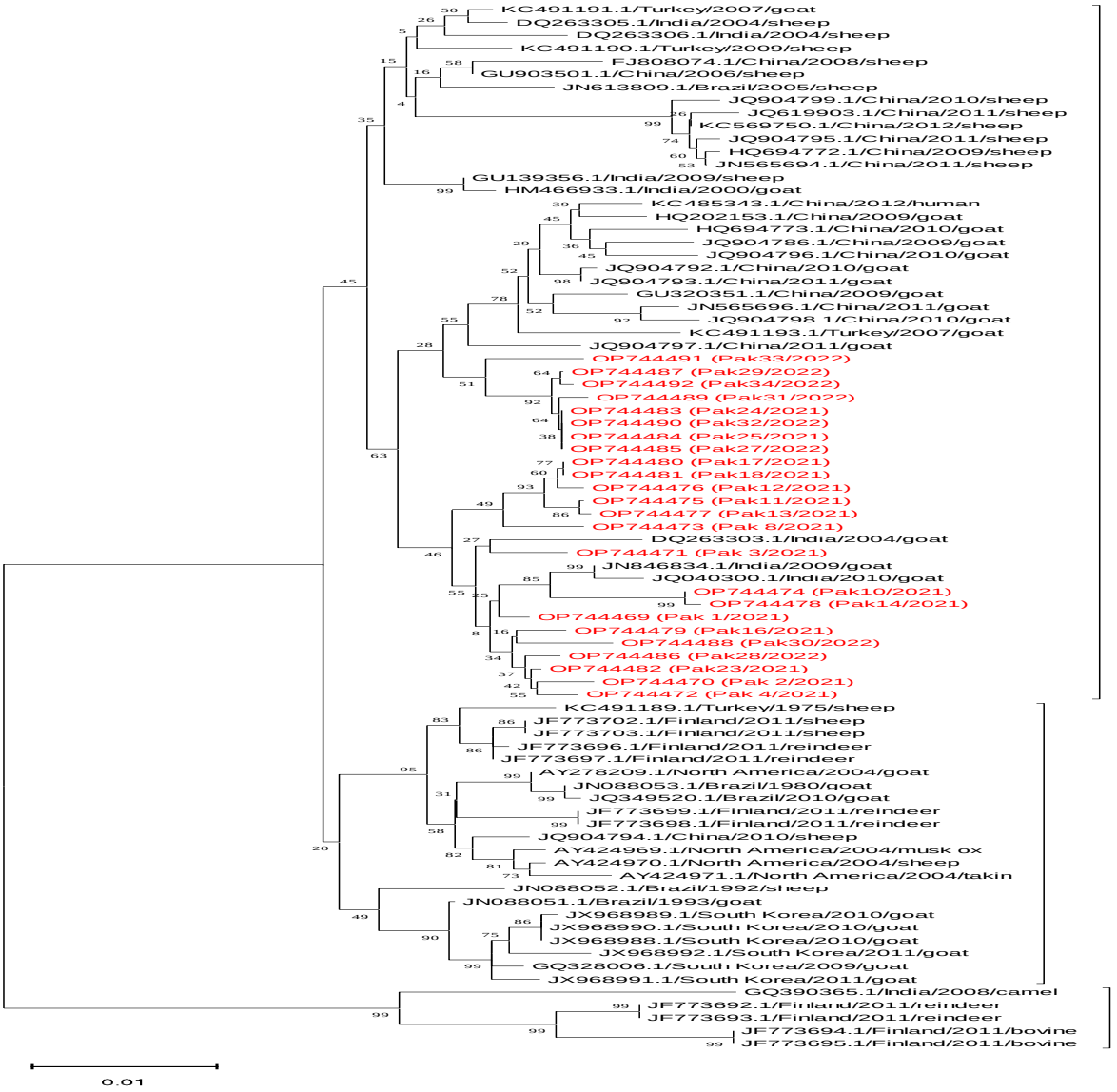

Figure-5. Phylogenetic analysis of complete major envelope protein gene (B2L). 57 nucleotide sequences representing all three groups of ORFV along with 24 sequences identified in this study were subjected to translational alignment in Geneious R11 software and phylogenetic analysis was then performed in MEGA X11 using Neighbor-Joining method

Discussion

CPD is a zoonotic disease caused by ORF virus (Inoshima et al., 2000). Typical clinical symptoms of infection in goats and sheep include vesicles, papules and crusty, rapidly forming scabs on the skin of the infected animals' lips and nostrils (Esmaeili et al., 2021). Even though the mortality rate is relatively low, the high prevalence of this disease usually leads to significant economic losses (Li et al., 2023; Abu et al., 2023). CPD is considered endemic, but the magnitude of the problem is still not fully understood. The most probable reason is that CPD is not included in the list of infectious diseases by the Office International des Epizooties (OIE) (Yamada et al., 2023). To the best of our knowledge, this study presents the first report on the complete genome sequence and phylogenetic analysis based on the major envelope protein gene (full-length B2L gene) of

ORF virus isolates from Pakistan.

A total of 35 animals were sampled from 12 outbreaks and 34 samples were found positive by the PCR method. The primers used in this study were specific for ORF virus and did not amplify sheep pox and goat pox viruses. We successfully obtained the complete nucleotide sequence of the B2L gene from 24 samples.

In all three analyses performed, including phylogenetic tree, percentage of nucleotide and amino acid sequence, the ORF virus strains under our study are genetically diverse but share close homology with Indian, Chinese and Turkey isolates. The results of this study revealed that ORF virus isolates currently circulating in sheep and goat population of Punjab, Pakistan belong to group-I. The calculated percentage homology between nucleotide sequences ranged from 97.98% to 100% and on the amino acid level, the homology ranged from 97.62% to 100%, respectively. OP744469 (Pak/1/2021) shared highest nucleotide similarity to the Indian isolate JQ040300 with 99.03% percentage, OP744490 (Pak/32/2022), OP744491 (Pak/33/2022), and OP744492 (Pak/34/2022) showed 98.59% percentage nucleotide similarity with Chines isolate JQ904797, OP744484 (Pak/25/2021) and OP744485 (Pak/27/2022) also revealed a high nucleotide identity of 98.15% percentage with Turkish isolate KC491193. These results may provide useful information regarding ORF virus genotype currently circulation in small ruminants population of Pakistan.

The nucleotide similarity between ORF viruses based on complete major envelop protein B2L gene sequences dedicated in this study ranged from 97.98% to 100%. These results align with Khalafalla et al. (2020), who used the B2L gene to characterize ORF virus from scabs of goats and sheep, alignment results revealed 100% identical virus isolates. These similarities between viral isolates detected at the same study level may be attributed to the uncontrolled movement of sheep and goats (Shehata et al., 2022). On the other hand, ORF virus from China and Turkey exhibited homology percentages (98.59% – 98.15%) based on the complete major envelop protein B2L gene, while the ORF virus isolate from Pakistan showed higher homology with the Indian ORF virus isolates.

It is difficult to differentiate among the foot-mouth disease, Pox, ORF virus disease, ulcerative dermatitis and staphylococcal dermatitis based on clinical symptoms. The PCR method was developed to efficiently diagnose ORF virus infection in infected animals. Traditionally, clinical symptoms of the ORF virus were believed to be observed around the oral cavity. However, in the present study, we observed ORF virus lesions not only around the oral cavity but also around the valves and hoofs of the affected sheep and goats.

Given the fact that awareness of ORF virus infections is increasing, and many outbreaks and cases are reported, this suggests that the actual diversity of ORF virus strains circulating in Pakistan may be even higher. Therefore, a country wide molecular epidemiological investigation of ORF virus disease in Pakistan is needed to understand the distribution, genetic diversity and economic losses associated with the disease. The molecular characterization of sheep and goat ORF virus strains from various regions of Pakistan is presented in this work. The gathered information improves our understanding of the molecular epidemiology of the ORF virus strains that are present in the area and highlights the need for both healthcare professionals and exposed employees to receive information on CE in the area.

Vaccination is the most simple and economical tool to control CPD in endemic areas. However no ORF virus vaccine is currently available in Pakistan and the understanding of viral genetic diversity is limited. In order to develop and effective vaccine country wide epidemiology investigation should be performed so that in depth understanding of genetic diversity of ORF virus in Pakistan can be obtained (Sahu et al., 2020). The vaccine was prepared grinding the infected scab in glycerin suspension (Abu et al., 2023). The vaccination was administered through scarification. This type of autogenous vaccine is still used by the farmers especially in countries like Pakistan where no other vaccine is available.

Conclusion

In this report CPD was confirmed in goats and sheep population of Pakistan, showing the clinical picture of ORF virus disease. We describe 12 different outbreak of CPD caused by the ORF virus, effecting sheep and goat's population in different district of Punjab. We successfully obtained 24/34 complete B2L gene sequences of ORF virus involved in these outbreaks. Sequence analysis of the B2L gene from Pakistan revealed a close homology with isolates circulating in representing countries i.e. India, China and Turkey.

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Contribution of Authors

Mansoor MK: Conceived idea, designed research methodology, supervised the research work and edited the manuscript
Iqbal K and Hassan A: Collected, analyzed data and interpreted data, and wrote first draft of manuscript
Saqib M: Designed research methodology, interpreted data and co-supervised the research work
Zohaib A: Literature review and manuscript write-up
Masood S: Contributed to manuscript write-up, editing and proofreading

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