



## First Molecular Evidence and Phylogenetic Analysis of Bovine Herpesvirus-4 in Bovine Milk of Mastitic and Healthy Cows in Pakistan

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### Abstract

**B**OVINE herpesvirus-4 (BoHV-4), a member of the *Herpesviridae* family, has been increasingly associated with reproductive and mammary gland pathologies in cattle. Despite its widespread global detection, limited data is available on its presence in Pakistan, particularly in relation to bovine mastitis. This study aimed to detect the presence and estimate the prevalence of BoHV-4 in cattle milk samples collected from Pakistan. A total of 174 milk samples were obtained from clinically mastitic (n=87) and non-mastitic (n=87) cattle and were subjected to DNA extraction followed by nested polymerase chain reaction (PCR) targeting the thymidine kinase (TK) gene of BoHV-4. Positive samples were confirmed via gel electrophoresis, and selected amplicons were sequenced for phylogenetic analysis. Out of 174 samples, 7 tested positive for BoHV-4, with a slightly higher prevalence in mastitic animals (5.74%) compared to non-mastitic ones (2.29%). However, statistical analysis using chi-square tests indicated no significant association between BoHV-4 presence and mastitis ( $p > 0.05$ ). Phylogenetic analysis of local isolates revealed close genetic similarity (98–99%) to strains previously reported from Turkey, Japan, and Belgium. Risk factor analysis did not show significant associations between BoHV-4 detection and herd size, parity, vaccination status or respiratory illness ( $p > 0.05$ ). This study confirms the first molecular detection of BoHV-4 in milk of both healthy and mastitic cows in Narowal, Pakistan. This study provides baseline molecular evidence for BoHV-4 circulation in Pakistan. Although detected at a low rate, BoHV-4 may contribute to subclinical infections and complicate mastitis pathogenesis, warranting further epidemiological investigations and routine surveillance.

**Keywords:** Bovine Herpesvirus-4 (BoHV-4); Nested PCR; Milk samples; Cattle; Mastitis; Phylogenetic analysis.

### Introduction

Bovine herpesvirus 4 (BoHV-4) is an enveloped, double-stranded DNA virus classified under the family *Herpesviridae*, subfamily *Gammaherpesvirinae*, and genus *Rhadinovirus* [1]. First isolated in Europe during the 1960s, BoHV-4 has since been identified in various parts of the world, including North America, Asia, and Africa [2, 3]. Although it was initially classified under the *Betaherpesvirinae* due to its cytopathic effects and the formation of inclusion bodies, advances in

molecular studies led to its reclassification into the *Gammaherpesvirinae* subfamily, based on genome structure and sequence homology [4]. They possess a genome size of approximately 144 kilobase pairs with a guanine-cytosine Content of 41.4 percent, coding nearly 79 open reading frames, among which 17 are unique to the virus [1]. It is similar to the genetic structure of other members of the subfamily, Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS), both of which are well-known representatives of the *Gammaherpesvirinae* subfamily. The viral infection usually occurs when the virus attains a

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latent or reservoir infection in monocytes and macrophages that act as vehicles for the shedding of the virus [5].

Although BoHV-4 infection can be asymptomatic, its role has been implicated in numerous clinical diseases in cattle, such as respiratory diseases, postpartum metritis, abortions, and mastitis [6]. Particularly, it is observed that BoHV-4 has been isolated either in clinically discerned animals and those that look healthy, bringing into question its status as either a primary pathogen or a cofactor in disease development [7]. Recent matters of interest have embraced the possibility of its connection to mastitis, a common and economically important disease in the dairy sector all over the world [8]. There is a centuries-old opinion that the cause of the mammary gland inflammation (mastitis) is based on the bacterial etiology. Nevertheless, even though mass control measures are taken, and the general treatment with antibiotics (particularly, along with the use of type III mastitis protective agent) is frequent, a significant part of mastitis remains unexplained, and research into other possible causes, such as viral agents, is conducted [9]. The presence of BoHV-4 in the milk of cows with clinical and subclinical mastitis makes it possible that this virus can contribute to the increase in bacterial colonization or inflammation through immune-mediated ways [10, 11]. Experimentally, there were exhibitions that intramammary inoculation of BoHV-4 can cause subclinical mastitis, histologically following indicated tropism of the virus in the ductal epithelium, and there was destruction of several tissues [12, 13]. Furthermore, co-detection of BoHV-4 with *Staphylococcus aureus*, *Streptococcus uberis*, and *Escherichia coli* supports its role as a potential co-pathogen in mastitis pathogenesis [14, 15]. Its persistence in mammary tissue also raises public health concerns about the possible release of viral particles into raw milk [16].

In Pakistan, where dairy farming is a vital economic activity, disease-related milk loss continues to affect productivity. Despite global reports of BoHV-4 data from Pakistan remain limited, and the virus's contribution to mastitis and other disorders is largely unexplored. Only one limited study has detected BoHV-4 DNA in a single milk sample from Punjab [17], underscoring a major epidemiological knowledge gap and the need for broader molecular investigations. Therefore, the present study was designed to determine the prevalence and molecular characteristics of BoHV-4 in milk samples from mastitic and healthy cattle in Narowal District, Punjab, Pakistan. Using nested polymerase chain reaction (PCR) targeting the thymidine kinase (TK) gene, followed by sequencing and phylogenetic analysis, we aimed to provide the first baseline molecular evidence of BoHV-4

circulation in the region and to evaluate potential epidemiological risk factors, including herd size, mastitis status, vaccination history, parity, and respiratory illness.

## **Material and Methods**

### ***Study Area and Sample Collection***

The study was a cross-sectional one carried out in District Narowal, a district in the Punjab province in Pakistan. It is identified with its animal farming enterprises, with large numbers of small and medium-sized dairy farms in the region. A total of 174 milk samples were collected from local indigenous cattle breeds. The sample set included 87 milk samples from clinically mastitic cows and 87 from apparently healthy, non-mastitic animals. Mastitis status was determined using the Surf Field Mastitis Test (SFMT), a widely applied cow-side screening tool. Milk samples were aseptically collected using sterile 15mL centrifuge tubes and were appropriately labeled with animal identification, sampling date, and location. The samples were transported to the laboratory under cold chain conditions, maintained below 4°C, using ice packs. Upon arrival, the samples were stored at -20°C until further processing to preserve viral nucleic acids.

### ***Sample Processing***

Prior to analysis, frozen milk samples were thawed at room temperature for 30 minutes. From each sample, 1.5 mL was transferred into a sterile microcentrifuge tube and centrifuged at 7000 rpm for 3 minutes. The supernatant fat layer was carefully removed using sterile swabs. The remaining cellular pellet was washed thrice with phosphate-buffered saline (PBS) to remove inhibitors and residual fat. After the final wash, 350 µL of supernatant was retained for DNA extraction.

### ***DNA Extraction***

Viral DNA extracted from 200 µL of processed milk supernatant using a commercial nucleic acid extraction kit (Ascend, Henan, China) following the manufacturer's protocol. DNA extracted automatically with an Auto-Extractor based on magnetic beads. The DNA was eluted using 50 µL of elution buffer and kept at -20°C awaiting extraction to carry out in the PCR.

### ***Nested Polymerase Chain Reaction (PCR)***

The gene of thymidine kinase (TK) of BoHV-4 was detected by nested PCR with two rounds of amplification using specific outer and inner sets of primers (Kalman *et al.*, [9]). The first round included primers M1 (5'-GTTGGGCGTCCTGTATGGTAGC-3') and M2 (5'-ATGTATGCCCAAACCTTATAATATGACCAG-3'), targeting a 567 bp product. The second round used primers M3 (5'-

TTGATAGTGC GTTGTGGGATGTGG-3') and M4 (5'-CACTGCCCCGGTGGGAAATAGCA-3') for a 260 bp nested product.

Nuclease-free water was added to 5 µL of primer stocks so that working dilutions of primers contained 45 µL of water and 5 µL of primer. Each PCR reaction used 25 µL of reaction volume and comprised 12.5 µL DreamTaq Green 2X PCR Master Mix (Thermo Fisher Scientific), 9.5 µL nuclease-free water, 1 µL of each primer, and 1 µL of template DNA in the first round or 1 µL of product of the first round in the second round. Each PCR run included a positive control (BoHV-4 positive DNA) and a negative control (no template). Thermal cycling conditions were as follows: initial denaturation at 94°C for 45 seconds; 30 cycles of denaturation at 94°C for 45 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 1 minute; followed by a final extension at 72°C for 10 minutes.

#### *Gel Electrophoresis*

Amplified PCR products were resolved on 1.2% agarose gel prepared in 1× TAE buffer with and 3 µL of ethidium bromide added after gel cooling. Gels were poured into casting trays with wells molded using combs and allowed to solidify for 30–40 minutes. The GeneRuler 1 kb DNA Ladder (Thermo Scientific, MA, USA) was used to assess the size of PCR products. Samples (10 µL each) were loaded into wells and electrophoresis was carried out at 100 V for 30 minutes. Bands were visualized under ultraviolet (UV) transillumination using a gel documentation system.

#### *DNA Sequencing and Phylogenetic Analysis*

To confirm BoHV-4 detection and assess genetic relationships, four of the seven PCR-positive samples were selected for sequencing. The amplicons were purified and submitted to a commercial sequencing service. The obtained sequences were subjected to BLAST analysis using the NCBI GenBank database to identify homology with known BoHV-4 isolates. Phylogenetic relationships were analyzed using MEGA X software version 10.2.6. Sequences were aligned using the ClustalW algorithm, and a phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura 3-parameter model. Bootstrap analysis with 1000 replicates was used to assess tree reliability. The local isolates were compared with BoHV-4 TK gene sequences retrieved from GenBank. The confirmed sequences were submitted to the NCBI GenBank database and assigned accession numbers: OR485305, OR485306, OR485307, and OR485308.

#### *Statistical Analysis*

A structured questionnaire was used during sampling to collect metadata on individual animals and herd-level management practices. Variables included mastitis status, herd size, respiratory illness

history, vaccination status, and parity. These were evaluated as potential risk factors for BoHV-4 infection. Data were compiled in Microsoft Excel and analyzed using SPSS software (version 23.0). Associations between BoHV-4 presence and risk factors were tested using the Chi-square test. A  $p < 0.05$  was considered statistically significant.

### **Results**

#### *Sample Overview and BoHV-4 Detection*

A total of 174 milk samples were analyzed in this study, comprising 87 samples from cows diagnosed with mastitis and 87 from apparently healthy, mastitis-negative animals. DNA extracted from all samples was subjected to nested PCR targeting the thymidine kinase (TK) gene of Bovine Herpesvirus-4 (BoHV-4). The expected amplicon size of 260 base pairs (bps) was successfully obtained from 7 samples, indicating the presence of BoHV-4 DNA. Among the BoHV-4 positive samples, 5 were from mastitis-positive cows (5.74%) and 2 from mastitis-negative cows (2.29%), yielding an overall prevalence of 4.02% in the sampled population (Table 1).

#### *PCR Visualization and Band Confirmation*

PCR products were visualized through agarose gel electrophoresis. Distinct bands at the 260 bp region were observed in the positive samples, confirming successful amplification of the BoHV-4 TK gene. The banding pattern was absent in negative controls, validating the specificity of the nested PCR assay (Fig.1).

#### *Sequencing and Phylogenetic Analysis*

Four PCR-positive samples were selected for nucleotide sequencing to confirm BoHV-4 identity and assess their genetic relatedness to global strains. The sequences were submitted to GenBank under the accession numbers: OR485305, OR485306, OR485307, and OR485308. BLAST analysis revealed that the local isolates shared 98-99% identity with BoHV-4 strains previously reported in Turkey, China, and Belgium. This suggests a high degree of evolutionary conservation of the TK gene among BoHV-4 strains from different regions. A phylogenetic tree was constructed using the Maximum Likelihood method in MEGA X. The tree showed that the Pakistani isolates clustered closely with BoHV-4 strains ANK-Teat-TR2018 and ANK-Br-TR2015 from Turkey, as well as with Belgium isolate 1981 and China 2022 (Fig. 2).

#### *Risk Factor Analysis*

To explore potential epidemiological determinants of BoHV-4 infection, various herd and animal-level factors were analyzed using the chi-square test. The evaluated factors included herd size, mastitis status, respiratory illness history, vaccination status, and parity (Table 2).

### Herd Size

Samples were stratified into three herd size categories: <50, 50–100, and >100 animals. Although 3 positive cases were observed in herds with >100 animals, no significant association was found between herd size and BoHV-4 positivity ( $p = 0.251$ ).

### Mastitis Status

Among mastitis-positive animals, 5 samples tested positive (5.74%) compared to only 2 positives (2.29%) from mastitis-negative cows. However, the difference was statistically non-significant ( $p = 0.247$ ), indicating no clear association between BoHV-4 presence and clinical mastitis.

### Respiratory Illness

A non-significant association ( $p > 0.05$ ) was observed between BoHV-4 detection and a history of respiratory signs in animals. This suggests that BoHV-4 may not play a contributing role in respiratory disease pathology.

### Vaccination Status

No significant difference was found in BoHV-4 positivity between vaccinated (1/44) and unvaccinated animals (6/130), with a  $p = 0.275$ .

### Parity

Samples were categorized based on lactation parity: 0–2, 3–4, and 5–6. No statistically significant difference was observed in BoHV-4 detection across these groups ( $p = 0.772$ ).

## Discussion

The present study provides molecular evidence for the presence of Bovine Herpesvirus-4 (BoHV-4) in the milk of dairy cattle from District Narowal, Pakistan. Using nested PCR targeting the thymidine kinase (TK) gene, BoHV-4 DNA was detected in 7 out of 174 milk samples (4.02%) (95% CI: 1.63–8.09%), confirming its circulation within the local dairy population. These findings contribute to the limited data available on BoHV-4 epidemiology in Pakistan and suggest that this gammaherpesvirus, although detected at a low rate, may influence udder health and broader disease dynamics in cattle herds. The detection rate observed in this study is comparable to earlier work conducted in Canada, where BoHV-4 DNA was identified in 2 out of 176 milk samples using PCR [14]. Similarly, a previous study reported one positive case among 50 milk samples collected from cattle and buffaloes in Rawalpindi, indicating a low but detectable prevalence of the virus in Pakistan [17]. Conversely, studies from Europe have reported higher detection rates. For example, a previous study detected BoHV-4 in 37 out of 118 milk samples from Hungarian dairy cattle, particularly in those with subclinical mastitis. Such variability in prevalence likely reflects

differences in geographical distribution, sample types, herd management, animal health status, and diagnostic methodologies across studies [9]. A slightly higher proportion of BoHV-4-positive cases was observed among cows with clinical mastitis (5.74%; 95% CI: 1.89–12.79%) compared to apparently healthy cows (2.29%; 95% CI: 0.28–8.00%), though this difference was not statistically significant ( $p > 0.05$ ). This trend aligns with previous findings suggesting a potential association between BoHV-4 and mammary gland pathology.

A previous study demonstrated that intramammary and intranasal inoculation of BoHV-4 in lactating cows could induce subclinical mastitis, characterized by elevated somatic cell counts and localized inflammation. Histopathological studies have confirmed the virus's affinity for ductal and sinus epithelial cells, supporting the hypothesis that BoHV-4 can infect mammary tissue and contribute to udder dysfunction [11, 13].

Although BoHV-4 not considered a primary mastitis pathogen, its role as a cofactor in polymicrobial infections has garnered increasing attention. Several studies have highlighted strong epidemiological links between BoHV-4 infection and co-infections with *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus uberis*, which are well-known mastitis-causing bacteria [10, 15]. It is postulated that BoHV-4 impairs innate immune defences in the mammary gland, thereby facilitating secondary bacterial invasion. This hypothesis is supported by in vitro studies where BoHV-4 infection modulated cytokine expression in bovine endometrial and mammary epithelial cells [18]. Consequently, even though the virus was infrequently detected, its immunomodulatory and synergistic role in mastitis pathogenesis warrants consideration. A notable observation in this study was the apparent association between BoHV-4 detection and a history of respiratory illness, although the number of animals with respiratory signs was small and none tested positive. Therefore, this apparent association should be interpreted cautiously, as the  $p$ -value obtained from a small subgroup ( $n = 13$ ) lacks statistical reliability. Nonetheless, previous studies have detected BoHV-4 DNA in nasal secretions, bronchoalveolar lavage fluid, and respiratory epithelium, supporting its broader tissue tropism beyond reproductive organs [7]. Experimental studies have shown that BoHV-4 can infect respiratory epithelial cells from the basolateral surface, potentially evading mucosal immune barriers. Future research involving larger sample sizes and diverse specimen types (milk, nasal, vaginal, and respiratory secretions) could clarify the role of BoHV-4 in respiratory disease complexes.

Sequencing and phylogenetic analysis of local isolates revealed 98–99% identity with BoHV-4 strains from Turkey, Japan, and Belgium, indicating

high conservation of the TK gene and limited genomic variation among global strains [19]. The submission of local sequences to GenBank (accession numbers OR485305–OR485308) expands the molecular reference data available for BoHV-4 and supports its genetic stability across regions. Risk factor analysis did not show significant associations with herd size, vaccination status, or parity, suggesting that BoHV-4 transmission dynamics may not depend strongly on these parameters. While larger herds theoretically present higher opportunities for viral spread, our results did not support this assumption. Likewise, the absence of an association with vaccination likely reflects the absence of a BoHV-4-specific vaccine. The lack of a parity effect also distinguishes BoHV-4 from other bovine herpesviruses, such as BoHV-1, which show parity-related patterns of susceptibility.

The relatively small number of BoHV-4-positive cases reduced the power of statistical analysis, as reflected by the wide 95% confidence intervals across risk factor categories. Moreover, sampling only milk may underestimate true prevalence, as BoHV-4 can also be excreted in nasal, vaginal, and uterine secretions [20, 21]. Despite these limitations, this study provides baseline molecular evidence for BoHV-4 circulation in Pakistan and highlights its potential role in bovine mastitis and respiratory health. Although detected at a low rate, BoHV-4 could contribute to subclinical infections and complicate mastitis pathogenesis, warranting further epidemiological investigation and routine surveillance to understand its distribution, co-infection dynamics, and economic implications in the dairy industry.

## Conclusion

This study represents the first molecular evidence of Bovine Herpesvirus-4 (BoHV-4) in the milk of dairy cattle from District Narowal, Pakistan. The virus detected in both mastitis positive and mastitis negative cows. Phylogenetic analysis revealed a close genetic relationship between local BoHV-4 isolates and those circulating globally, confirming the virus's genetic conservation across regions. These findings highlight the need to include BoHV-4 monitoring as part of routine herd health surveillance and encourage further studies to elucidate its virulence, impact on milk production, and potential effects on reproductive performance.

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## Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

## Authors Contributions

Conceptualization, S. Raza; methodology, U. Irshad; formal analysis, U. Irshad, Sohail Raza, I. Altaf, A. Riaz, Muhammad Ilyas Riaz and Sehrish Firyal; writing original draft preparation, S. Raza, I. Altaf; writing, review and editing, All authors.

## Ethical of approval

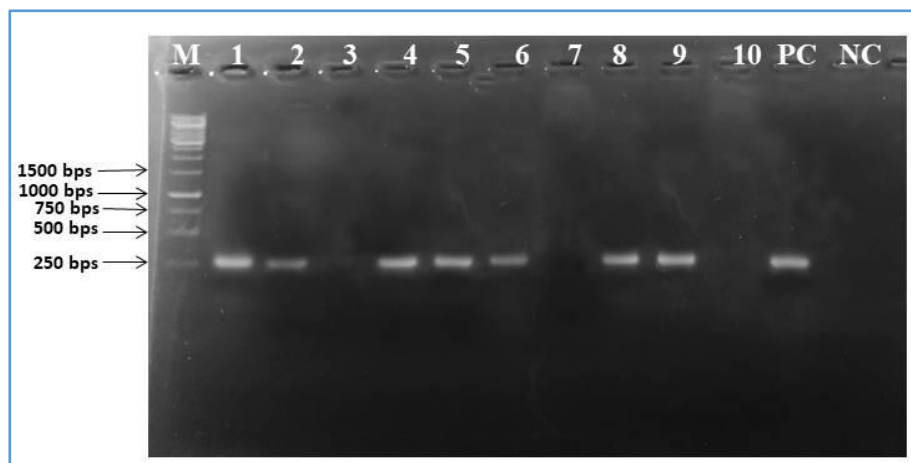
Sample collection and handling procedures were according to the guidelines and approved by the Institutional Animal Ethics Committee of the University of Veterinary and Animal Sciences, Lahore (Approval No.DR:221).

TABLE 1. Prevalence of BoHV-4 in milk samples

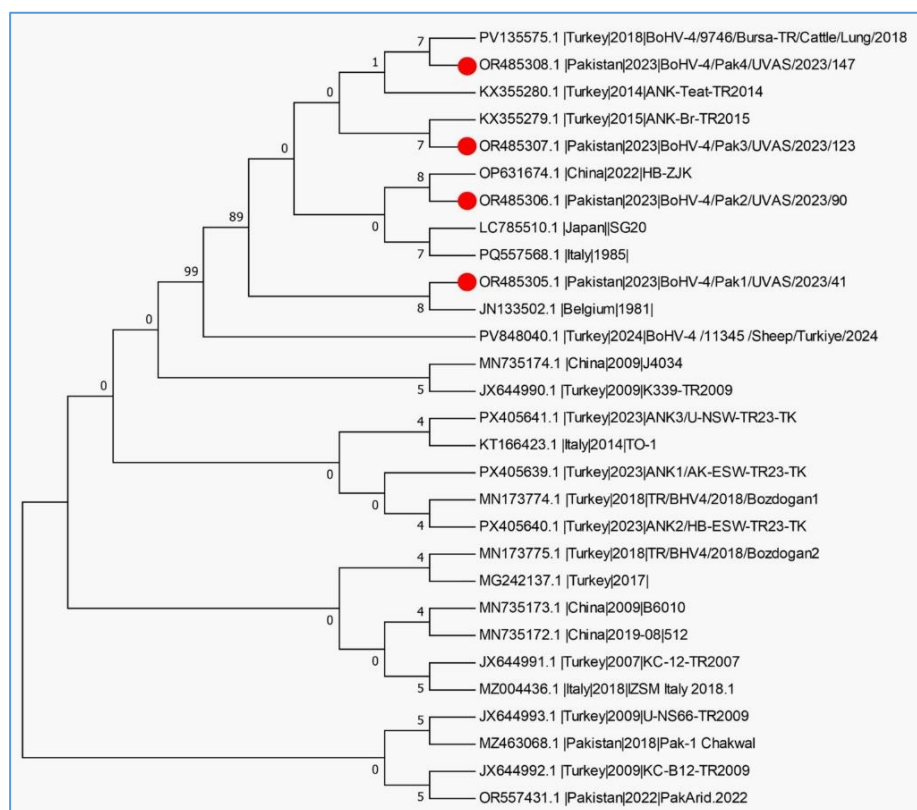
| Sample Type       | Positive (n) | Negative (n) | Total (n) | Prevalence (%) |
|-------------------|--------------|--------------|-----------|----------------|
| Mastitis-Positive | 5            | 82           | 87        | 5.74%          |
| Mastitis-Negative | 2            | 85           | 87        | 2.29%          |
| Overall           | 7            | 167          | 174       | 4.02%          |

TABLE 2. Statistical analysis of risk factors associated with BoHV-4 infection

| Variable            | Categories | n   | Positive | Prevalence (%) [95% CI] | Negative | p-value |
|---------------------|------------|-----|----------|-------------------------|----------|---------|
| Herd Size           | <50        | 49  | 2        | 4.1 (0.5–13.9)          | 47       | 0.251   |
|                     | 50–100     | 51  | 2        | 3.9 (0.5–13.5)          | 49       |         |
|                     | >100       | 74  | 3        | 4.1 (0.9–11.5)          | 71       |         |
| Mastitis            | Positive   | 87  | 5        | 5.7 (1.9–12.8)          | 82       | 0.247   |
|                     | Negative   | 87  | 2        | 2.3 (0.3–8.1)           | 85       |         |
| Respiratory Illness | Yes        | 13  | 0        | 0.0 (0.0–24.7)          | 13       | 1.000   |
|                     | No         | 161 | 7        | 4.3 (1.8–8.7)           | 154      |         |
| Vaccination         | Yes        | 44  | 1        | 2.3 (0.1–12.3)          | 43       | 0.275   |
|                     | No         | 130 | 6        | 4.6 (1.7–9.8)           | 124      |         |
| Parity              | 0–2        | 59  | 2        | 3.4 (0.4–11.7)          | 57       | 0.772   |
|                     | 3–4        | 101 | 4        | 4.0 (1.1–10.0)          | 97       |         |
|                     | 5–6        | 14  | 1        | 7.1 (0.2–33.9)          | 13       |         |



**Fig. 1.** Agarose gel electrophoresis of nested PCR products targeting the TK gene of BoHV-4, with a distinct 260 bp band. Lanes 1–10 represent field milk samples, several showing positive amplification. Lane M: DNA Marker; PC: positive control; NC: negative control confirming assay specificity



**Fig. 2.** Phylogenetic tree based on TK gene sequences showing genetic relatedness between Pakistani BoHV-4 isolates (in Red) and other strains. Bootstrap values indicated at branch points.

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