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RESEARCH ARTICLE

Epidemiological Survey, Molecular Characterization and Subtyping of BoHV-1 from Healthy and Sick, Cattle and Buffalo from Okara, Pakistan

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ABSTRACT

Bovine herpesvirus 1 (BoHV-1) is an evident respiratory and reproductive pathogen in bovines. It causes infectious bovine rhinotracitis (IBR) and reproductive disorders. Present study evaluated the prevalence of BoHV-1 among the healthy and sick cattle and buffaloes. Two hundred blood samples were randomly collected from the animals of district Okara, Pakistan. To detect BoHV-1 antibodies, indirect ELISA was performed. Out of 200 samples from both species (cattle and buffalo), an overall prevalence of BoHV-1 of 124/200 (62%) was obtained. Samples from buffalo had higher species-specific prevalence 64/98 (65%) than samples from cattle 60/102 (58.8%). Overall, BoHV-1 was more prevalent in female (73.35%) animals as compared to male animals (48.5%). Present study depicted that 84/124 (67.70%) animals from positive results were apparently healthy and 40/124 (32.25%) animals from seropositive results were sick. Identification and molecular characterization was performed on sixty seropositive blood samples, PCR was performed by using glycoprotein E gene primers on isolated DNA from these samples, six were positive 6/60 (10%), and gene sequencing and phylogenetic analysis had shown close similarities with other BoHV-1 gE gene sequences world widely. The accession number assigned to these samples in GenBank were OQ656376.1 and OQ656377.1. Subtyping of BoHV-1 was conducted by multiplex PCR following HindIII enzyme activity, three samples were identified as BoHV-1.1 that indicated BoHV1.1 prevalence in study area. In this study, Multiplex PCR proven as efficient, economical and rapid technique for diagnosis and subtyping. This study will be helpful in vaccine development and in devising measures to control the spread of BoHV-1.

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INTRODUCTION

Bovine herpesvirus-1 is an economically important infectious pathogen of livestock that spread world widely and causing infectious bovine rhinotracitis (IBR) and abortion in cattle and buffalo (Dagalp *et al*., 2020; Hashemi *et al*., 2022). It is a large enveloped double stranded positive sense DNA enclosed in icosahedral capsid virus belonging to the family Herpesviridae. It contains 135,000 bases encoding for 75 proteins (Chase *et al*., 2017). In host cell, its replication is intranuclear, however its final processing arises in the cytoplasm of cells (Pellett, 2007; Zhang *et al*., 2016).

There are three subtypes of BoHV-1 (BHV-1.1, BHV-1.2a, and BHV-1.2b) (Benaissa *et al*., 2021). The subtypes 1.1 and 1.2 (a) cause respiratory disease (IBR) and abortion in cattle (Nandi *et al*., 2009). The subtype 1.2 (b) is involved in causing infection in reproductive system (Zhou *et al*., 2020). These subtypes also lead to rhinotracheitis affecting the bovines without any age

discrimination (Rhaymah *et al*., 2012). It was first reported in 1953 from feedlots of USA where it caused infectious bovine rhinotracheitis in cattle (Raaperi *et al*., 2014). Now, it has spreaded globally leading to approximately 3 billion dollars loss to the cattle industry annually (Thomas *et al*., 2022). With the advancement in vaccination, the occurrence has lowered, but the threat to livestock in Pakistan and worldwide is still a big concern (Riaz *et al*., 2021).

In Pakistan, during winter many respiratory disorders such as IBR and malignant catarrhal fever lead to animal sickness that reduce milk production as well as lead to reproductive losses (Pérez-Guiot *et al*., 2023). Abortion occur in cows between the 4th and 7th month of gestation period due to BoHV-1 (Can *et al*., 2016). Moreover, IBR casued by BoHV-1 has been documented globally (Wathes *et al*., 2020). The financial damage caused by this virus also include less dairy output, diagnostic cost, animal weight loss, and eventually death (Khaneabad *et al*., 2023). While utilizing the technique of artificial insemination, the blood progesterone level plays a major role in the survival of embryo (Nyman *et al*., 2018). BoHV-1 has been found to affect the progesterone level in the animal leading to abortion (Rodríguez *et al*., 2022; Khaneabad *et al*., 2023).

At present very limited information is available on molecular characterization and subtyping of BoHV-1 in Pakistan. In a recent study, BoHV-1 gE deleted vaccine has been developed locally however there is still a need to explore in vivo studies to develop vaccine containing local BoHV-1 subtype in Pakistan (Rehman *et al*., 2022). The present research work was done on samples taken from the Okara district, which is in the Punjab province in Pakistan and is at high risk due to its extreme weather. It is one of the richest districts in animal population. Huge number of large animals produce an immense milk production for province Punjab (Saeed *et al*., 2019).

The present study described Seroprevalence, risk factors, isolation, identification of BoHV-1, phylogenetic analysis and subtyping of BoHV-1 from study area. The present research also focused on the identification of the main prevalent subtype of BoVH-1. The results of the study will be helpful in deciding the best time and exact type of vaccination for herd against BoHV-1.

MATERIALS AND METHODS

Sample collection and processing: Sample collection was carried out in bovine (Cattle and Buffalo) from various farms and veterinary hospitals of district Okara in Pakistan. Two hundred samples of blood and serum were collected in EDTA tubes, clot activator tubes from healthy and sick animals, respectively. All the samples were transported to and stored in Virology laboratory, Faculty of Veterinary and animal sciences, PMAS university of Arid Agriculture Rawalpindi at -20°C.

Seroprevalence and risk factor studies of BoHV-1: Indirect ELISA was performed on two hundred serum samples for Seroprevalence of BoHV-1 using Vet Innovative Diagnostics® Elisa Kit (I.D vet). Associated risk factors like herd size and density, species, gender, health measures, geographical factors of the targeted herds were also studied as these can impact the Seroprevalence.

Identification and molecular characterization of BoHV-1: Collected blood samples were used for DNA extraction of the concerned virus via Thermoscientific DNA Extraction Kit® as per the manufacturer's instruction. The purity of extracted DNA was checked through NANO Drop technique. Approximately 100 ng/µl extracted DNA were used in PCR. The confirmation of presumptive BoHV-1 isolates carried out by targeting the Glycoprotein-E gene. The PCR was carried out using the primer sequence F: 5'-GCTTCGGTCGACACGGTCTT-3' and R: 5'-CTTTGTCGCCCGTTGAGTCG-3' by the following program: An initial denaturation of 95°C for 10 min, 35 cycles of denaturation at 95°C for 60 s, annealing at 54°C for 45 s and extension at 72°C for 60 s; Final Extension at 72°C for 10 min (Elhassan *et al*., 2015). The amplified DNA was run on 1.6% agarose gel at 70V.

Phylogenetic analysis: The amplicons were then subjected to sequencing, for this purpose positive samples were sent to the Macrogen® Korea laboratory, sequences from this study and obtained from GenBank database were assembled and aligned using MEGA-11. A phylogenetic tree was constructed to infer the evolutionary relationships among the bovine herpes virus isolates. Phylogenetic tree robustness was assessed using bootstrap analysis with 1000 replicates. The sequences were analyzed with neighbor joining and phylogenetic tree constructed. The spatial distribution patterns of BOHV-1 strains were explored by mapping the geographical origin of the isolates on the phylogenetic tree. Two sequences from this study were deposited to GenBank database. The accession numbers assigned by NCBI for BoHV-1 samples are OQ65377.1 and OQ656376.1. A comparison of sequences of this study was made among the BoHV-1 sequences of other studies (NC 055561.1, NC0755564.1, OQ669138.1, Z23068.1, HM575424.1, AF133121.1, KM258882.1, MK348039.1, KM985498.1, MK552112.1 and MH751898.1).

Viral subtyping through Multiplex PCR following *hind*-III enzyme activity: Multiplex PCR method used for detection of DNA restriction patterns associated with BoHV-1subtypes. It was conducted with two sets of primers; RS-1 F: 5′-TCGTCGAAGAGCGTCCA CACA-3′ and R: 5′-ACCGCGCTGTACCGGCAGCT-3′ and RS-2 F: 5′-TACAAATCGGCGGCGC CAAA-3′ and R: 5′-TTGTTGACGGCCAAGTATAA-3′ having an initial denaturation of 95°C for 10 min, 35 cycles of denaturation at 95°C for 60 s, annealing at 54°C for 45 s and extension at 72°C for 60 s with a final extension of 10 min at 72°C (Maidana *et al*., 2020). PCR product was then divided into two tubes. One tube was digested with *hind*-III enzyme at 37°C for 16 hours and the other tube was processed as undigested control. Digested fragments were separated by gel electrophoresis with a 2% agarose gel. The restriction patterns were analyzed based on fragment sizes, band patterns and their relative intensities.

Statistical analysis of BoHV-1 risk factors in cattle and buffalo: Data was analyzed by SPSS software (version

26). Chi-square test of independence, odds ratio (OR), and relative risk (RR) were applied to examine the relationships between bovine species, gender, and sero-positivity with respect to the health status of animals.

RESULTS

Seroprevalence and Risk Factors Studies of BoHV-1: Seroprevalence studies were performed on 200 Blood serum samples, which were collected from different region of Okara District of Pakistan. This study depicts that 124 samples out of 200 found positive for BoHV-1 that show 62% seropositivity in targeted area. Current study indicates that this virus is more prevalent in female [n = 91/132 (73.35%) animals as compared to male n =33/68 (48.5%)]. That results in reproduction as well as production losses in dairy sector. Furthermore, study concluded that buffalo are more susceptible to this disease as compare to cow, in this study [n =64/98(65.30%) buffalo and n = 60/102 (58.8%)] cows were seropositive. this finding reveals that specie might be a risk factor for BoHV-1 infection in Pakistan as well as other countries.

Serum sampling of this study was conducted in May and June 2021 during that time weather of this region is moderate more likely to summers, 35-45°C temperature recorded in these days. This study depicts that [n =84/124 (67.70%)] animals from positive results were apparently healthy and active, they have normal body parameters including body temperature, respiratory rate and normal heart rates while [n =40/124(32.25%)] animals from seropositive were having slight respiratory distress (Table 1). A Chi-square test of independence was conducted to evaluate the relationship between animal type and health status. The p-value (0.2725) exceeded the significance level ($\alpha = 0.05$), indicating no significant association between animal type and health status.

The Odds Ratio (OR) was used to assess the odds of being sick in cows compared to buffaloes.

OR ≈ 1.65, indicating that cows have higher odds of being sick compared to buffaloes.

The Relative Risk (RR) was calculated to compare the risk of sickness between cows and buffaloes. RR ≈ 0.711, indicating that the risk of being sick for cows is about 71.1% lower than for buffaloes.

The statistical analysis suggests that there is no significant association between animal type (cow or buffalo) and health status in relation to BoHV-1 seropositivity. However, there is a difference in the odds and relative risk of sickness, with buffaloes having a higher risk of being sick compared to cows.

Molecular characterization of BoHV-1: Molecular characterization was performed on blood samples. Total 60 samples of sick animals were subjected to this experiment. All of them were seropositive. Six samples were identified BoHV-1 positive through PCR and gel electrophoresis (Fig. 1). The positive ratio of samples [n = 6/10 (10%)] was recorded. phylogenetic analysis was conducted on positive samples and neighborhood joining test was performed to construct phylogenetic tree through software "MEGA 11" (Fig. 2). Furthermore, gene sequences of positive samples from this study were 100% similar to the isolates from China (MK348039.1), 99% to the isolates from India (KM985498.1) and 98% from Hungry (AF133121.1).

Table 1: Epidemiological survey of BohV-1 in Okara region of Pakistan

Animal Breed	Sample No.	Sero positive	Sero Negative	Seropositive Male	Seropositive Female	Seropositive Apparently healthy	Seropositive Apparently sick
Buffalo	98	64	34	20	44	40	24
Cow	102	60	42	13	47	44	16
Total No.	200	124	76	33	91	88	40

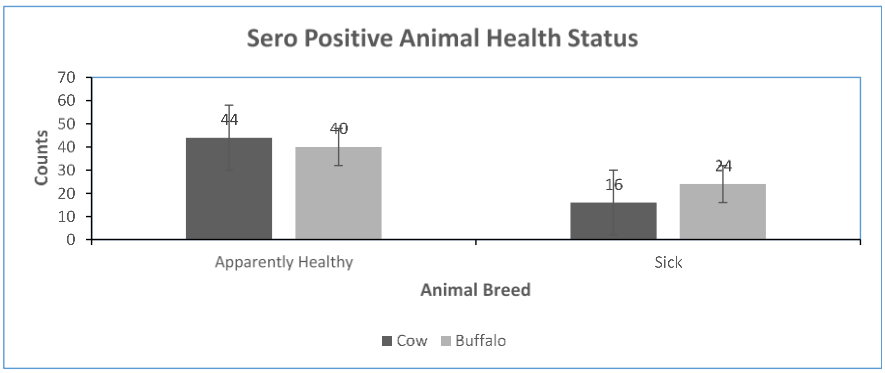

Fig. 1: shows the health status of seropositive samples. total 124 samples were seropostive. 84 out of 124 were healthy while 40 were sick animals. Dark grey indicates Cow samples while light grey bar indicates buffalo samples

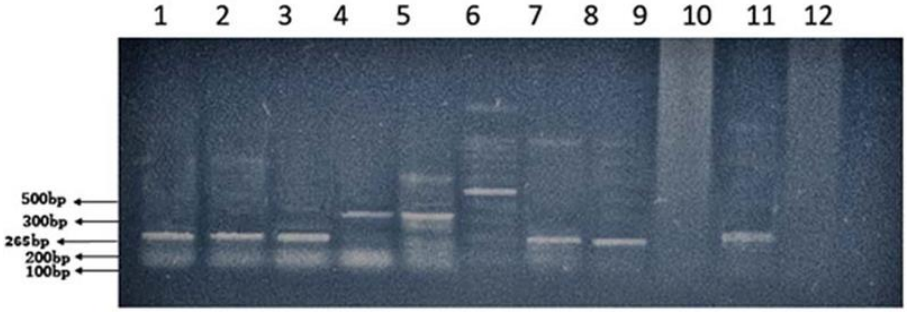

Fig. 2: Agarose gel shows positive samples of 265bp amplified from blood samples of infected animals. 1,2,3,7,8,10, were BoHV-1 positive, while 6 showing DNA ladder and 12 is of –ve control

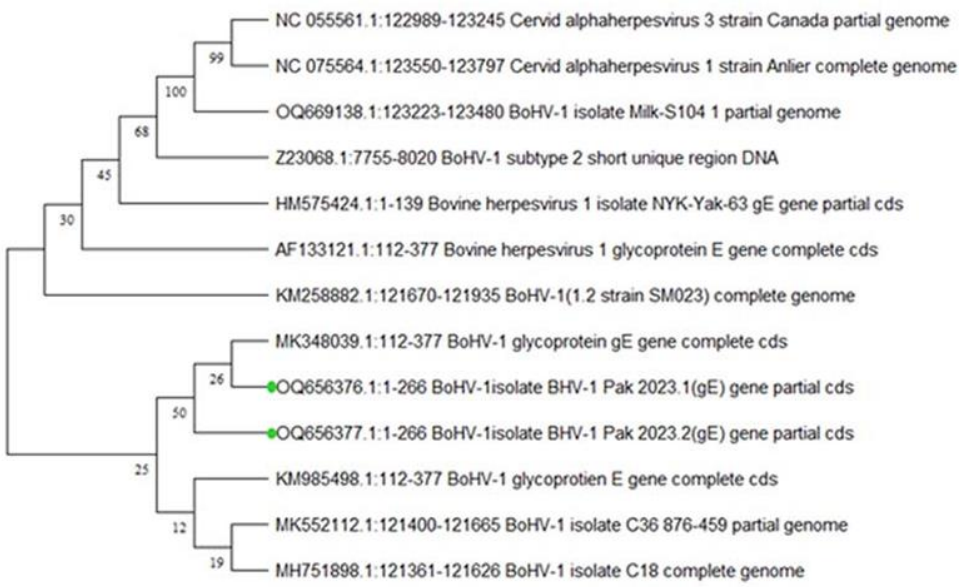

Fig. 3: phylogenetic tree for the fragements of BoHV-I glycoprotien E gene. the green dot shows the samples isolated from present study. The evolutionary history was inferred by maximum likelihood method

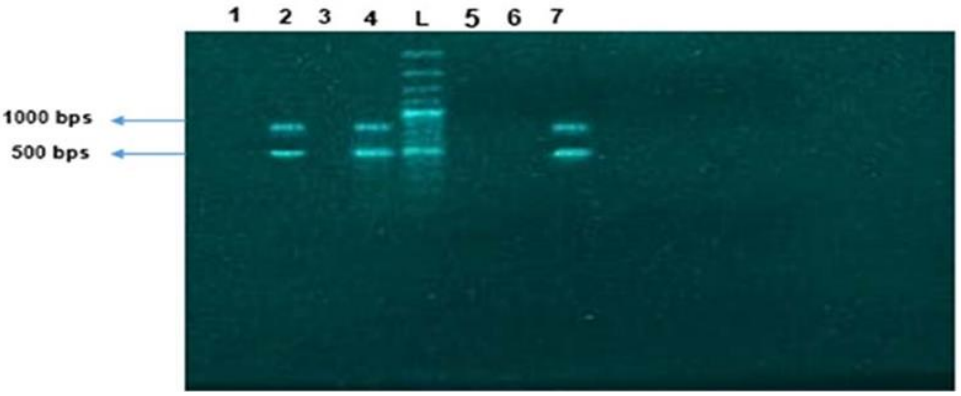

Fig. 4: Agrose gel shows the pattern of BoHV-I subtype. 1: negitive control, 2,4,7 showing BoHV-I subtype 1.1 while L; ladder and 3,5,6 were negitive samples

Sub typing of BoHV-1 positive samples by multiplex PCR: Multiplex PCR were performed for subtyping of BoHV-1. Multiplex PCR proved affective and significant for subtyping of BoHV-1 subtyping. Three positive samples from PCR and ELISA were subjected in this experiment. In this study all PCR product has shown amplification of RS-1 and RS-2 fragments. Furthermore, both fragments were shown without cleavage hence all 3 samples were considered as BoHV-1.1 sub type (Fig. 3; Fig. 4). Gene sequencing of PCR product and phylogenetic analysis also showed these strains similarly with subtype 1.1 from other countries.

DISCUSSION

Bovine herpesvirus is the causative agent of clinical conditions like IBR, abortion, infectious pustular vulvovaginitis and systemic infections in fetus (Muylkens *et al*., 2007). It effects severely the livestock sector results in economic losses. IBR outbreak in cattle farm can cause production losses, weight losses, reproduction losses and can impose ban on international livestock trade. IBR effected animals remain lifelong carrier, immunosuppressive conditions may re activate the virus replication and leading to disease outbreak in herd (Biswas *et al*., 2013). In Pakistan, most of the population's earning depend on livestock sector and diseases like IBR cause animal health problems. First time this virus was isolated in 1958, after that it was reported globally by many countries including Pakistan, America, India, turkey, Italy, and many others (Dagalp *et al*., 2020; Hashemi *et al*., 2022). A study was conducted in 2009 in the UK that has shown 83% antibodies presences in unvaccinated herd (Woodbine *et al*., 2009). Similarly, a study in Pakistan also has shown 69% prevalence of this virus, (Rehman *et al*., 2020). Furthermore, studies conducted in several parts of world showed high prevalence of BoHV-1. BoHV-1 seroprevalence was recorded 36% in China (Yan *et al*., 2008), 36 to 48% in Central and South America (Pospíšil *et al*., 1996), 63 to 86% in Egypt (Mahmoud *et al*., 2009), 14 to 60% in African countries and, 60.1% in India (Mallick *et al*., 1994). Several factors including animals breed, herd size, management, geographical conditions and different techniques of diagnoses can be the reason of varied prevalence rate of BoHV-1 in different countries (Biswas *et al*., 2013).

In Present study, Seropositivity of BoHV-1 was determind by using indirect ELISA technique. The ELISA results revealed that the prevalence of BoHV-1 in the targeted area was 62% The result of this study closely related to recent serological study of BoHV-1 in area of Lahore, Pakistan that shown 69% Seropositivity (Rehman *et al*., 2020). However, molecular characterization revealed low prevalence of BoHV-1 in seropositive animals, reason for this may be due to its latency in sensory ganglia, so that' virus presence in blood samples lower (Silva *et al*., 2007).

BoHV-1 causes abortion, endometritis and oviduct infection. A study showed that oviduct, uterus and ovarian tissues can be the target of viral replication in female animals and a potential source of virus spread to embryo. 100 % detection of virus found in the uterus of seropositive animals (Queiroz-Castro *et al*., 2019).

Current study indicated that this virus was more prevalent in female animals (73.35%) as compared to male animals (48.5%). Similar results were also found all over the world in which female animals were more susceptible to this virus, a study in Algeria found more female cattle positive against this virus in 2019 (Kaddour *et al*., 2019). The present study depicted that Buffalo were more susceptible to this disease as compared to cattle in the targeted area. In this study (65.30%) buffalo and (58.8%) cows were found seropositive. Similar findings were also reported with higher prevalence in buffalo (38.14%) as compared to cows (26.78%) (Thakur *et al*., 2017). In contrary to this study, other study found more seropositive cattle (72.88%) as compared to buffalo (63.41%) in Pakistan. The possible factors to these disagreements could be different breeds, livestock health management and import of the animals (Rehman *et al*., 2020).

This finding revealed that species might be a risk factor for BoHV-1 infection in Pakistan as well as other countries. Further comprehensive studies by providing same conditions separately to cattle and buffalo required to analyze it.

Many studies indicated, prevalence rate of BoHV-1 varies not only between the countries but also in the different regions of same country. It might be due to environmental and climate changes. Geographical conditions such as location, temperature, and humidity can affect the survival and transmission of BoHV-1, this virus is known to survive better in colder temperature. Regions with colder climate or other areas during low temperature season may find high prevalence of its diseases (Adeli *et al*., 2017). Serum and blood. Present study was conducted in summers, this study showed that (67.70%) animals from positive results were apparently healthy and active, they have normal body parameters and no lesion were found in them, they might be effected in previous winter's cycle and immunized against BoHV-1.

Further studies required to conduct in other regions of Pakistan that will help out to find better time duration for vaccination against this virus.

Only 6 samples out of 60 were identified BoHV-1 positive through PCR in present study. The positive ratio of samples (10%) was recorded. As BoHV-1 mostly found in trigeminal ganglion, it remains only 2-3 days in blood, our research plan was conducted on live animals in which only blood and swabs sample were used, it made difficulty in BoHV-1 identification in blood as there is fewer chances of this virus in the blood. In 2015 research was also conducted in Sudan on BoHV-1 in dairy cattle in which researchers conducted the study through blood samples and found (10.075%) positive samples through PCR (Elhassan *et al*., 2011).

Disease diagnosis and causative agent detection is more important for an accurate and effective treatment. Detection of BoHV-1 can be done by many methods, including nested PCR, and real-time PCR and immunoassays using monoclonal antibodies (Marin *et al*., 2016). few of these methods also useable for differentiation of BoHV-1 type from BoHV-5. PCR sequencing assays, complete genome sequencing, single-nucleotide polymorphism pattern analysis and (RAPD), are used to genotype BoHV-1. These methods, are more accurate but high cost and more lab facilities are required. Therefore, in the present study the detection and differentiation of BoHV-1 subtypes were analyzed by multiplex PCR. This technique was found as an economical, easier to conduct. It can be helpful for those lab where limited facilities are available.

Multiplex PCR was performed followed by *Hind*III restriction endonuclease enzyme digestion. The results of Multiplex PCR and *Hind*III restriction endonuclease enzyme digestion was found in accordance accordance of a previous study where all subtypes of BoHV-1 and BoHV-5 were compared. The specific two fragments contain one HindIII restriction site (sequence AAGCTT), known as RS1 and RS2, found in the UL39 open reading frame and the US3 upstream intergenic region by in-silico analysis using the complete genome of BoHV-1 (Maidana *et al*., 2020). All products from this study had two bands, furthermore first band of PCR products from this study were digested in two bands while first band appeared near 500bp while second band was near 700bp. Gene sequencing of positive samples were 100 % similar to BoHV-1.1 subtype in phylogenetic analysis. The prevalent subtypes in this study is similar to those subtypes that were reported recently in Pakistan and also in the subcontinent (Rehman *et al*., 2020).

Further studies such as molecular characterization on BoHV-1 isolates from different regions of Pakistan are required to identify prevalent subtypes to develop an effective vaccine against it. This would be helpful to avoid economic losses in livestock sector of Pakistan.

Conclusions: BoHV-1 is more prevalent in buffalo as compared to cattle in Okara, Pakistan. Furthermore, Female animals are highly susceptible to it.

In context of subytyping of BoHV-1, Multiplex PCR is significant, easier and economical method for BoHV subtyping. BoHV-1.1 is more abundant in Pakistan. Mass vaccination against BoHV-1.1 will prevent economic losses in livestock sector of Pakistan.

Author's contribution: XM contributed to the conception and design of the study and ciritical revision of the manuscript. MH conducted the experiments and data collection. AR supervised the research work and provided technical support. NB and IU performed the enzyme linked Immuno Assay. ZH analysed the research results. YQ provided intellectual input during the writing process. MB and MS collected the study samples. MF performed the statistical analysis. All authors read and approved the final version of the, manuscript.

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