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Original article

Seroprevalence, associated risk factors and clinico-pathological studies of buffalopox disease in various regions of Punjab province, Pakistan

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Abstract

Buffalopox (BPX) is a highly contagious disease that causes high morbidity and production losses in buffaloes. During this study, seroprevalence, effect of various associated risk factors, and pathological studies of BPX were recorded in the Punjab province. A total of 97 blood samples and 63 scabs were collected from clinically pox suspected buffaloes. Serum was harvested to perform single radial hemolysis to assess the seroprevalence, and scabs were subjected to PCR for BPX virus confirmation. Results revealed that, animal demographics and environmental associated factors showed significant effect ($p<0.05, 1<R^2>0$) on BPX occurrence. The overall BPX seroprevalence was recorded 4.18% in the Punjab province. The BPX was recorded 5.48% in Nili Ravi breed during winter (7.42%), aged 5-7 years (7.46%) under loose housing (5.51%) in the Faisalabad region (8.03%). Further, BPX was 5.37% in pregnant, 6.86% pregnant milking buffaloes during the 3rd lactation period (7.28%) in dairy herds (5.20%). The BPX was 5.22% in non-vaccinated buffaloes where multiple animals were reared together (4.99%) in the herds having 21-30 total number of animals. A total of 49 scab samples were found positive for the BPX virus via PCR with C18L gene amplification. Grossly, inflammatory lesions with pits in the center and wart-like nodules were seen on teats and udder of buffaloes. Increased leukocytes, especially neutrophils and lymphocytes, were seen in the blood of the infected animals. These results provide a broader window to understand the effect of associated risk factors, strengthen the diagnostic aid, and to contain the current spread of BPX in Pakistan to safeguard large ruminant-based livelihood.

Key words: seropositive, single radial hemolysis, scab samples, PCR, C18L gene, leukocytes

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Introduction

Buffalopox (BPX) is a re-emerging viral disease caused by the buffalopox virus (BPXV) and has been declared an important zoonotic disease by the FAO/ /WHO (Eltom et al. 2020). BPXV is a member of the *Orthopoxvirus* genus, belongs to the *Poxviridae* family (Gujarati et al. 2019). In 1934, BPX was first reported from India, followed by continuous sporadic outbreaks in Asian buffaloes in Russia, Egypt, Pakistan, Bangladesh, Italy and Indonesia (Essbauer et al. 2010). Clinically, lesions are mostly seen on the udder, teats, inside of the thighs, base of the ear and the inner surface of earflap in the mild form whereas generalized lesions are in severe systemic form (Goyal et al. 2013, Riyesh et al. 2014). BPXV replicates in the cytoplasm of the cell and seems like VACV in terms of shape, structure and physio-chemical properties (Singh et al. 2006).

With the passage of time, BPXV is becoming more pathogenic and vulnerable to the livestock population as a re-emerging contagious viral disease with high morbidity (Singh et al. 2007, Yadav et al. 2010). The re-emergence of BPXV occurred by gradual variation of the vaccine strain in buffaloes (Bera et al. 2012) until it transferred to pathogenic, leading to outbreaks in new hosts (Goraya et al. 2015). The product of host-range genes has been seen to alter the virus's capacity to infect cells by diverting the host's defense mechanisms (McFadden 2005, Gurav et al. 2011). Therefore, BPX outbreaks occurred frequently in many parts of Asia and Africa, affecting buffaloes, cows and humans (Bhanuprakash et al. 2010, Marinaik et al. 2018). Outbreaks of BPX infection have been reported in commercial and domestic farming in India with 23.4% to 79.4% while in the Khushab district of Punjab, Pakistan, its prevalence has been reported 50% (Khan 2010).

Furthermore, frequent epidemics and transmissibility of BPXV in buffaloes, cows and humans is an important public health concern following the cessation of smallpox vaccination globally (Singh et al. 2006). In such situation, where multiple hosts such as buffaloes and cows of same herds are involved, a rapid, and effective laboratory diagnostic tool is needed to identify and differentiate BPXV with other OPXVs (Singh et al. 2007). Molecular biological tools substantially enhanced the diagnosis of diseases and do not require isolation of infectious agents. Therefore, PCR is being used in the diagnosis of various infectious diseases as an alternative to conventional assays owing to its sensitivity, rapidity, specificity, and reproducibility (Mackay et al. 2002).

Currently, there are increasing incidences of BPXV infection as a re-emerging disease and spreading in various regions of Punjab, Pakistan. Keeping in view the economic significance of the livestock industry (about 6455$ USD annual financial losses), a comprehensive molecular investigation is essential to record the seroprevalence and the role of various associated risk factors (animal and environmental) with BPX occurrence for the implementation of accurate diagnostic tools and effective preventive managemental practices against such viral disease.

Materials and Methods

Sampling and data collection

Collection of samples and then harvesting of serum was done by adopting the standard protocol described by the OIE terrestrial manual. Along with the collection of samples, observations were recorded on the questionnaire to determine the effect of animal demographics (breed, age, lactation period, physiological stages of production and vaccination status) and environmental factors (area, season, total number of animals in a herd and production system) on disease spread. First, based on history and clinical signs, 98 herds having 1173 buffaloes were examined during September 2017 - August 2020 and a total of 97 blood samples and 63 scabs were collected from clinical cases of pox suspected buffaloes from various regions of Punjab province. In animal demographics, physiological stages of production were further divided into pregnant (milking and dry) and non-pregnant (milking and dry) categories. In environmental factors, the production system was further classified as type of housing (loose housing and conventional barns), type of herd (dairy and mixed) and rearing of animals (single spp. and multiple spp.). These samples were brought to the Department of Pathology, University of Agriculture, Faisalabad for further processing. This study was conducted following the guidelines of "Biosafety Committee and Punjab Biosafety rules – 2014" as this study was approved by "Institutional Biosafety/Bioethics Committee" of the University of Agriculture, Faisalabad, Pakistan.

Single radial hemolysis (SRH)

The serum samples were heated at 56ºC for about half hour in a water-bath to inactivate the complement, non-specific antibodies, and agglutinins to minimize false positive results. A cell culture based live attenuated buffalopox vaccine was obtained from the Veterinary Research Institute (VRI), Lahore, Punjab, Pakistan and 100ml normal saline was added to freeze dried virus for antigen preparation. Guinea pig serum of good hemolytic titer was used as complement and the sam-

Table 1. Thermocycler conditions for the amplification of C18L gene of buffalopox virus (BPXV).

Reactions	Temperature/Time	
Initial denaturation	94°C for 3 minutes	1 cycle
Denaturation	94°C for 1 minutes	34 cycles
Annealing	50.9°Cfor 30 seconds	
Extension	72°C for 60 seconds	
Final extension	72°C for 10 minutes	1 cycle

Table 2. Comparison of blood profile of healthy and pox suspected buffaloes.

Parameters	Healthy Buffaloes	Infected Buffaloes
RBCs (10^6/µL)	7.310±0.676^{a}	7.123±0.648^{ab}
Hb. Conc. (g/dL)	13.203±1.693^{a}	12.677±1.708^{ab}
Hematocrit (%)	41.558±2.720^{a}	39.121±2.711^{ab}
MCV (fL)	50.098±2.827^{a}	48.237±2.834^{ab}
MCHC (g/dL)	34.439±3.766^{a}	31.952±3.765^{ab}
WBCs (10^3/µL)	9.88220±0.01943^{b}	12.1168±0.0166^{a}
Neutrophils (%)	37.3027±0.0482^{b}	48.0898±0.0241^{a}
Eosinophils (%)	2.80024±0.01151^{b}	3.90293±0.01230^{a}
Basophils (%)	0.74805±0.01167^{b}	0.96634±0.01374^{a}
Lymphocytes (%)	42.9795±0.0480^{b}	52.4602±0.0223^{a}
Monocytes (%)	4.92634±0.01513^{b}	6.65341±0.01931^{a}
Platelets (10^3/µL)	306.927±1.642^{a}	306.444±1.653^{a}

Mean±SE having different alphabets (superscript) in a row are significantly different ($p<0.05$)

ples were processed further with single radial hemolysis (SRH) as described by Bhanuprakash et al. (2010).

First, 1% agarose gel was prepared in physiological saline and cooled at 40°C followed by addition of 1.2 ml of BPXV coated sheep RBCs and 1.2 ml of complement with good hemolytic titer in the medium. This medium was poured on the glass slides. In addition, sterilized Whatman's filter paper no.1 (6mm) disks were soaked in the inactivated serum and absorbed about 35 µl of serum. These disks were placed on the glass slides containing the agarose gel at equal distances and incubated overnight at 37°C with humidified condition. The zone of hemolysis was observed on the next day around the positive sera.

Molecular detection of BPXV through PCR

For BPXV confirmation, DNA was extracted. Briefly, scab samples were triturated in PBS (pH=7.4) to prepare 10% (w/v) suspension and transferred to 1.5 mL microcentrifuge tube (labeled for each sample). DNA was extracted by the protocol, mentioned in the commercially available GeneJET Genomic DNA Purification Kit (catalog # K0722, Thermo Fisher Scientific Baltics UAB, Lithuania). Quantification of the extracted DNA from BPXV isolates was done through nanodrop (ND-1000, Thermo Scientific, Lithuania).

Preparation of PCR reaction mixture

The extracted DNA was amplified by using the specific set of primers (F 5′-GCGGGTATCACTGTTAT GAAACC-3′: R 5′-CATAAATACACTTTTATAGTCC TCG-3′) in a thermal cycle (Bio-Rad T100™ – Thermal cycler, USA) at the conditions described in Table 1. A single PCR reaction mixture (25 µl) was comprised of 12.5 µl master mix (catalog # K1081, Thermo Scientific, Dream Taq Green PCR master mix, 2X, Lithuania), 1µl of each primer (forward and reverse), 2 µl of DNA template, and 8.5 µl nuclease-free water. The C18L gene amplification (PCR product) was visualized via gel electrophoresis apparatus containing 1.5% agarose gel (Singh et al. 2008).

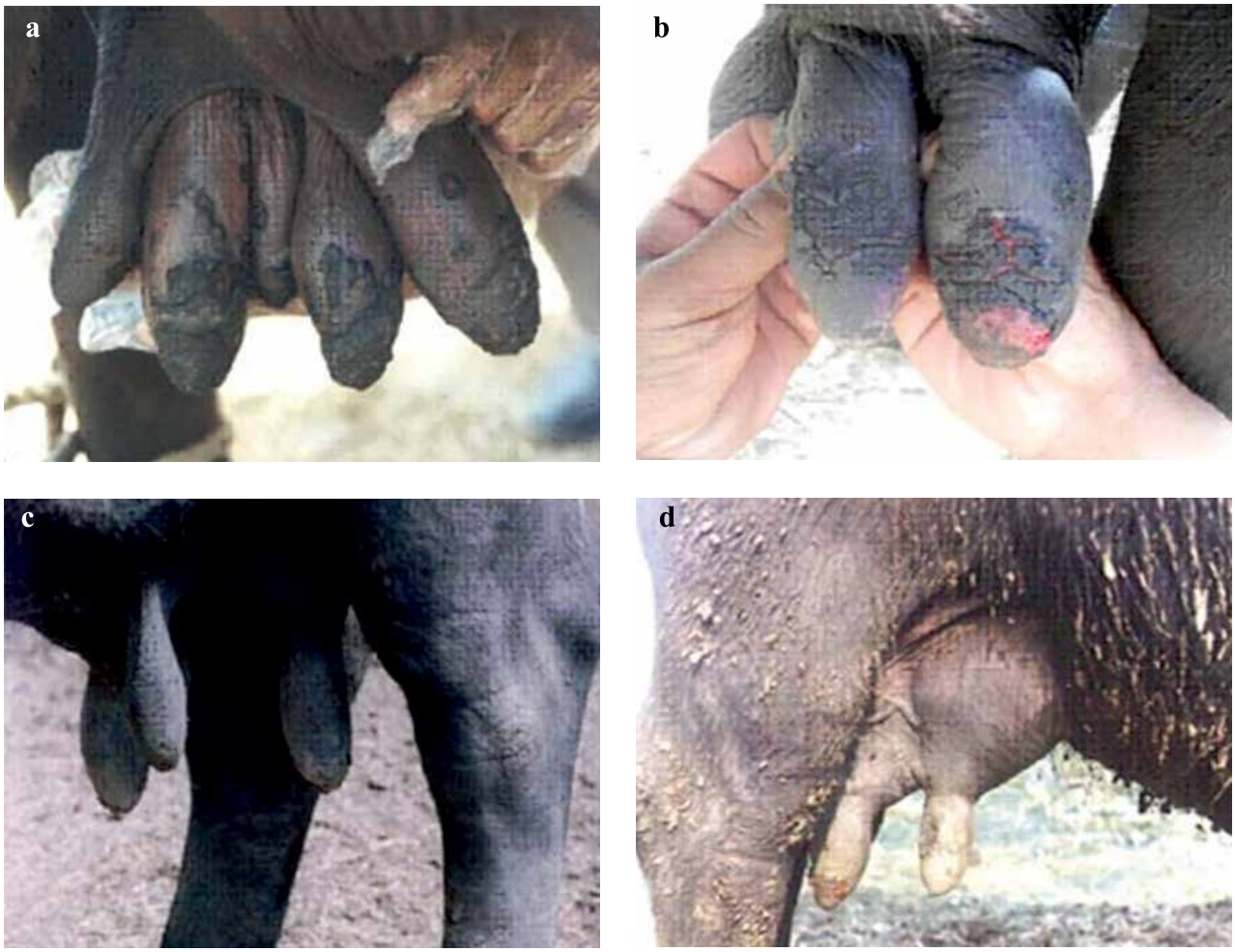

Fig. 1. Different pox lesions on the teats of buffaloes infected with BPXV. a) scab lesions with pits in the center, b) wart-like nodules and scabs, c) dry scabs, d) scab with inflammatory lesions.

Hematology

Blood samples (3-5 ml per animal) were collected from the jugular vein of the pox suspected and healthy buffaloes. These blood samples were subjected to a hematology analyzer (SN-15851, CDS Medonic M-Series Hematology Analyzer, Clinical Diagnostics Solutions, Inc. Sweden) to record the blood profile of the diseased and healthy buffaloes for comparison.

Statistical analysis

Pearson's chi-square test was applied to check the significance of animal and environment associated risk factors on BPX occurrence ($p<0.05$) and R^2 value was determined through linear regression analysis ($1<R^2>0$). Blood profile was analyzed by one-way ANOVA (analysis of variance) and group means were compared for significant results via Tukey's test ($p<0.05$) with Statistix 8.1 (analytical software).

Results

Gross pathology of BPX disease

During sampling, different pox lesions (Fig. 1) were seen on teats of buffaloes such as scab lesions with pits in the center (Fig. 1a), wart-like nodules, scabs (Fig. 1b), dry scabs on teats (Fig. 1c) and scabs with inflammatory lesions (Fig. 1d).

Seroprevalence of BPX

In the present study, the overall seroprevalence of BPX was recorded 4.18% (seropositive) through single radial hemolysis (SRH) in the Punjab province of Pakistan during September 2017 to August 2020 (Fig. 2). The animals were declared seropositive based on the results of this test.

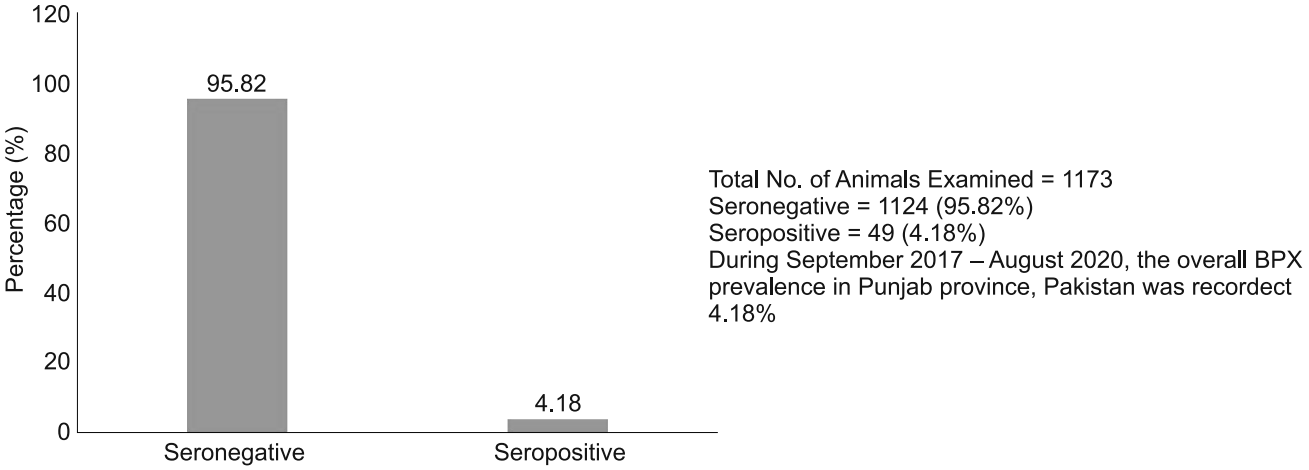

Fig. 2. Overall seroprevalence of buffalopox disease (BPX).

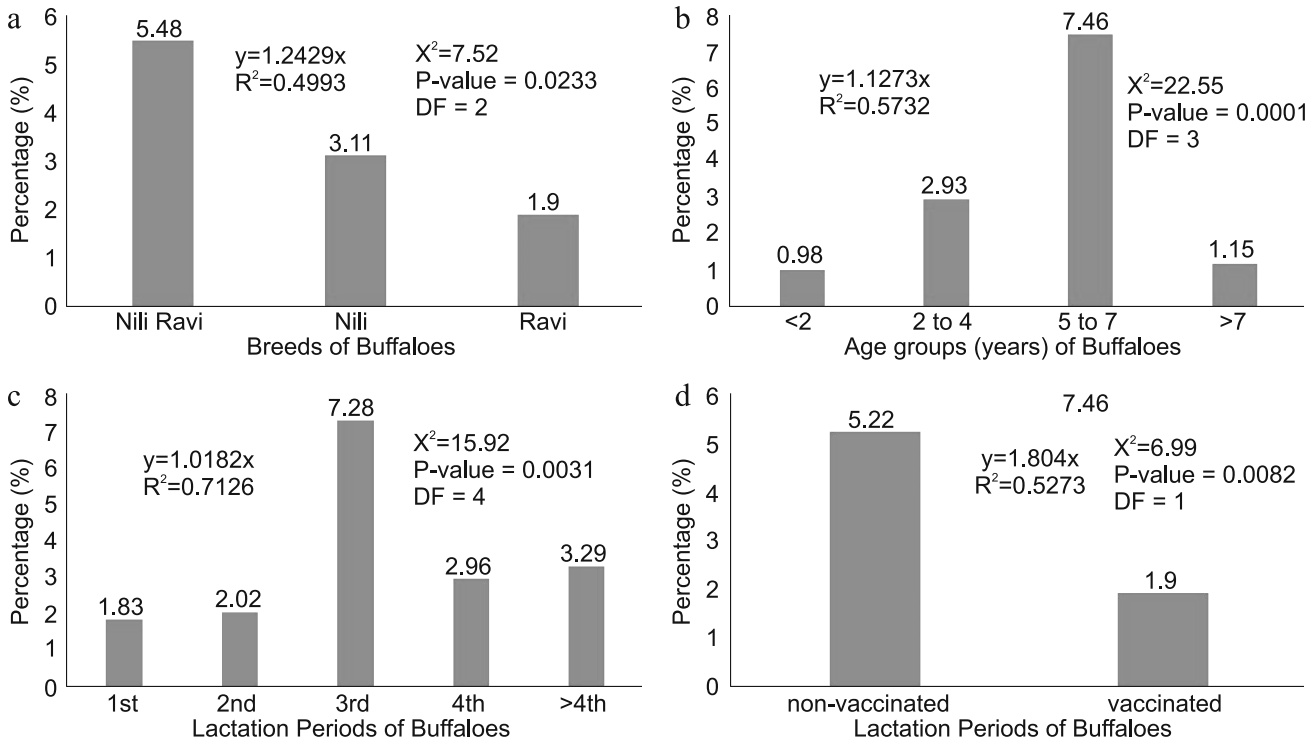

Fig. 3. Seroprevalence of BPX; a) in different breeds of buffalo, b) among different age (year) groups, c) during different lactation periods, d) vaccination status of buffaloes.

Effect of animal demographics on BPX occurrence

Breed

The buffalo breeds showed significant effect on BPX occurrence ($p<0.05$, $1<R^2>0$) as shown in Fig. 3(a). The BPX prevalence was 5.48% (OR: 3.082) in Nili Ravi breed having 3.88%-7.65% disease chance followed by Nili (3.11%, OR: 1.659) and Ravi (1.90%) breeds.

Age

The significant effect of age was seen on BPX occurrence in buffaloes ($p<0.05$, $1<R^2>0$) as shown in Fig. 3(b). The BPX prevalence was 7.46% (OR: 8.145) in buffaloes aged 5-7 years having 5.32%-10.32% disease chance followed by 2-4 years of age (2.93%, OR: 3.051), above 7 years of age (1.15%, OR: 1.174) and below 2 years of age (0.98%) groups.

Lactation period

The lactation period showed significant effect on BPX occurrence in buffaloes ($p<0.05$, $1<R^2>0$)

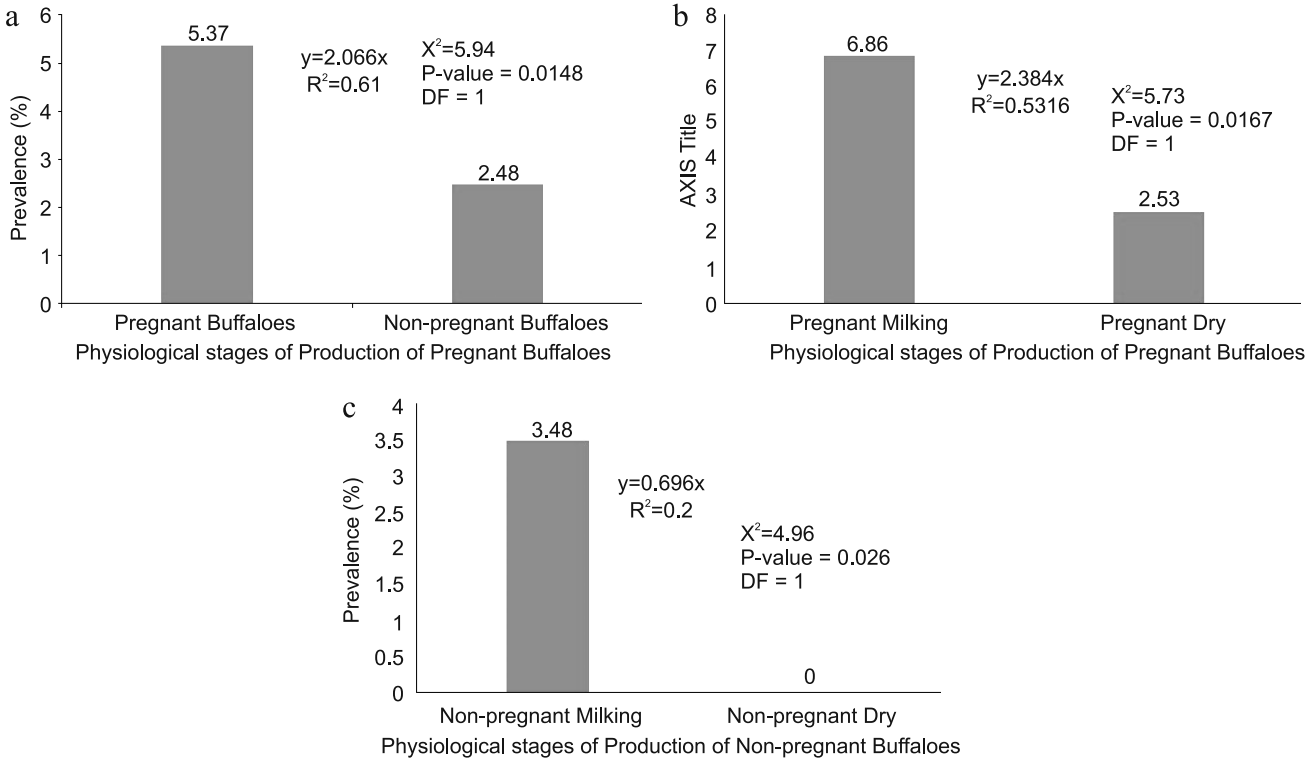

Fig. 4. Seroprevalence of BPX in physiological stages of production; a) in pregnant and non-pregnant buffaloes, b) in pregnant milking and pregnant dry buffaloes, c) in non-pregnant milking and non-pregnant dry buffaloes.

as shown in Fig. 3(c). The BPX prevalence was 7.28% (OR: 4.202) during 3rd lactation period having 5.05%-10.34% disease chance followed by above 4th lactation (3.29%, OR: 1.820), 4th lactation (2.96%, OR: 1.629), 2nd lactation (2.02%, OR: 1.103) and 1st lactation (1.83%) periods.

Vaccination status

The vaccination status of buffaloes showed significant effect on BPX occurrence ($p<0.05$, $1<R^2>0$) as shown in Fig. 3(d). The BPX prevalence was 5.22% (OR: 5.192) in non-vaccinated buffaloes having 4.85%-9.34% disease chance as compared to vaccinated (1.90%) buffaloes.

Physiological stages of production

The physiological stages of production i.e., pregnancy, milking and dry status showed significant effect on BPX occurrence in buffaloes ($p<0.05$, $1<R^2>0$) as shown in Fig. 4. The BPX prevalence was 5.37% (OR: 5.07) in pregnant buffaloes having 3.86%-7.40% disease chance as compared to non-pregnant buffaloes (2.48%) as shown in Fig. 4(a). Pregnant as well as milking buffaloes showed 6.86% prevalence (OR: 3.064) having 4.78%-9.70% disease chance as compared to pregnant dry buffaloes (2.53%) as shown in Fig. 4(b). Non-pregnant milking buffaloes showed 3.48% prevalence (OR: 2.232) having 1.90%-6.16% disease chance as compared to non-pregnant dry buffaloes as shown in Fig. 4(c).

Effect of environmental factors on BPX occurrence

Area

A significant effect of area was seen on BPX occurrence ($p<0.05, 1<R^2>0$) as shown in Fig. 5(a). The BPX prevalence was recorded 8.03% (OR: 9.432) in Faisalabad region having 5.10%-12.31% disease chance followed by Multan (6.72%, OR: 7.776), Sahiwal (3.42%, OR: 3.823), Rawalpindi (3.31%, OR: 3.692), Lahore (2.74%, OR: 3.042), Sargodha (2.70%, OR: 3.00), Bahawalpur (2.13%, OR: 2.348), DG Khan (1.79%, OR: 1.964) and Gujranwala (0.92%).

Season

Season showed significant effect on BPX occurrence ($p<0.05$, $1<R^2>0$) as shown in Fig. 5(b). The highest BPX prevalence (7.42%, OR: 8.372) was seen during winter season having 5.11%-10.60% disease chance followed by spring (4.58%, OR: 5.010), autumn (1.51%, OR: 1.602) and summer (0.95%).

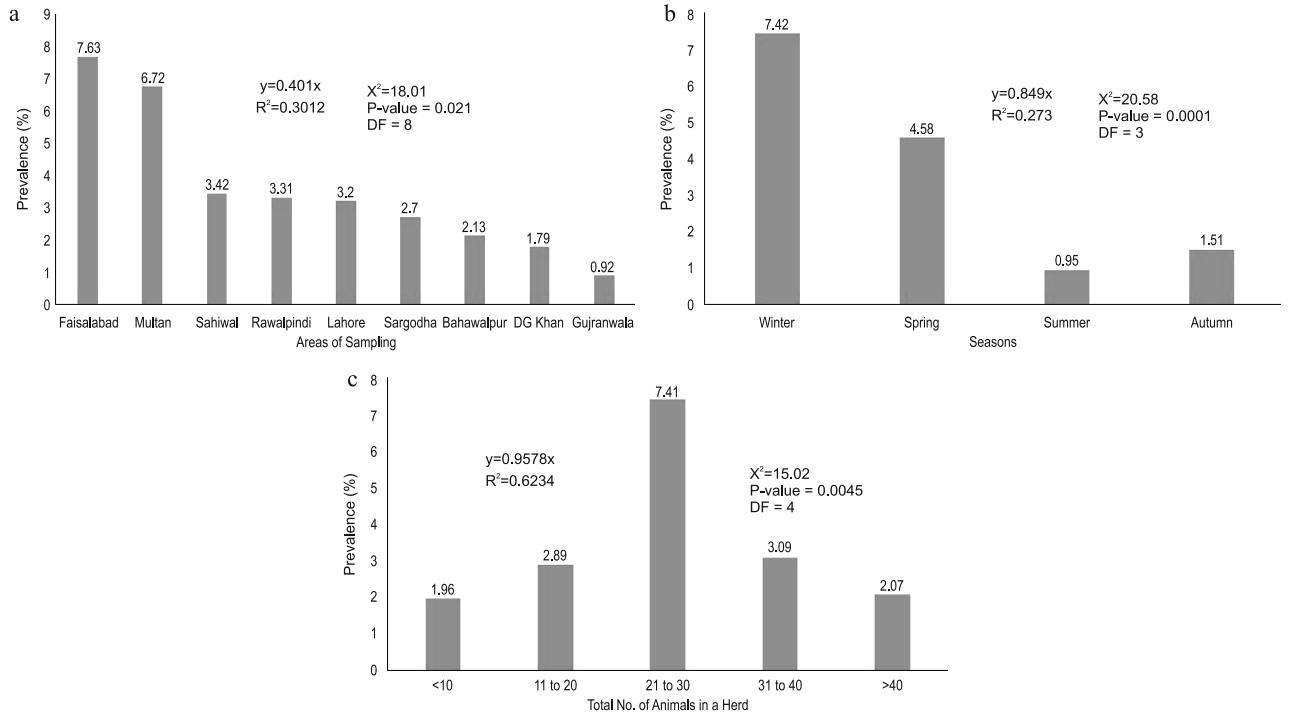

Fig. 5. Seroprevalence of BPX; a) in various areas (regions) of sampling in Punjab province, b) during different seasons, c) based on total number of animals in a herd.

Total number of animals in a herd

The number of animals in a herd had significant effect on BPX occurrence in buffaloes ($p<0.05$, $1<R^2>0$) as shown in Fig. 5(c). The BPX prevalence was recorded 7.41% (OR: 4.00) in those herds having 21-30 animals with 5.07%-10.65% disease chance followed by the herds having 31-40 animals (3.09%, OR: 1.592), herds having 11-20 animals (2.89%, OR: 1.488), herds having above 40 animals (2.07%, OR: 1.056) and herds having less than 10 animals (1.96%).

Production system

Production system i.e., type of housing, type of herd and rearing of multiple species in same herds had significant effect on BPX occurrence in buffaloes ($p<0.05$, $1<R^2>0$) as shown in Fig. 6. The prevalence of BPX was 4.99% (OR: 2.111) in those herds where multiple animals were reared together with 3.63%-6.79% disease chance while those herds where only single spp. i.e., buffalo was reared showed 2.43% prevalence as shown in Fig. 6(a). Further, a prevalence of 5.20% (OR: 2.576) was recorded in those buffaloes kept as dairy herd with 3.80%-7.05% disease chance as compared to a mixed herd with 2.08% prevalence as shown in Fig. 6(b). The prevalence of BPX was seen 5.51% (OR: 2.897) in those buffaloes kept under loose housing with 4.17%-8.06% disease chance as compared to conventional barns (2.39%, OR: 1.383) as shown in Fig. 6(c).

Molecular identification of BPXV

For BPXV confirmation, primer pair BPXV/C18L-F, BPXV/C18L-R, with DNA ladder (100bp) were carried out in 1.5% agarose gel (gel electrophoresis apparatus) and the 368bp product size was visualized through gel doc system (imaging). Sterile ultrapure water was used as negative control and no band was observed in this lane indicated the accuracy of the procedure as shown in Fig. 7. Out of 63 scab samples, 49 were found positive for BPXV.

Effect of BPXV infection on blood profile

Hematological examination revealed non-significant difference in RBCs count, Hb. conc. hematocrit, MCV and MCHC between healthy and infected buffaloes ($p>0.05$). On the contrary, infected buffaloes showed a significant increase in WBCs count and differential leukocytic counts (DLC) as compared to healthy buffaloes as shown in Table 2 ($p<0.05$).

Discussion

Buffalopox (BPX) is a highly contagious and infectious zoonotic disease that affects buffaloes, cows and humans (Bhanuprakash et al. 2010, Eltom et al. 2020). During this study, infected animals showed different pox lesions i.e., vesicles, wet scabs with serous exudate, ulcers, oozing of blood after removal of scabs.

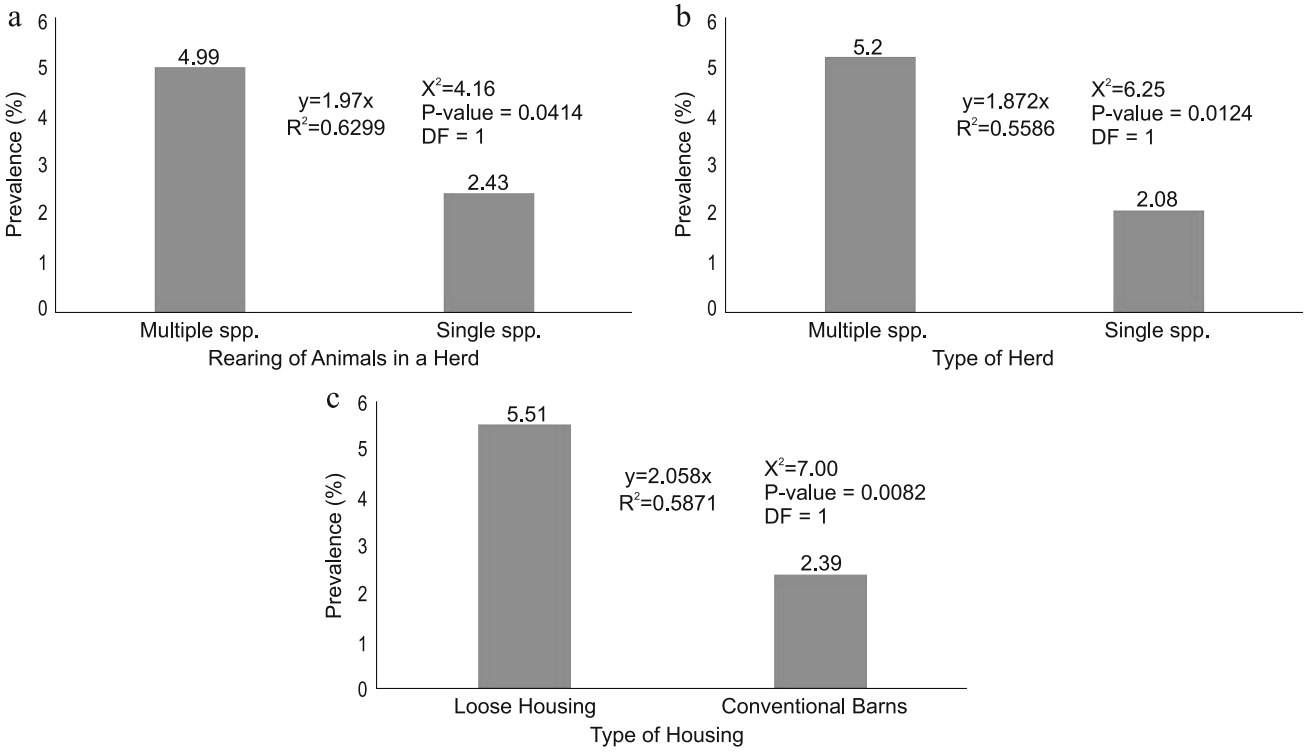

Fig. 6. Seroprevalence of BPX in production system of buffaloes; a) in the herds where multiple animals were reared to gathered, b) in two different types of herds of buffaloes, c) in two different types of housing of buffaloes.

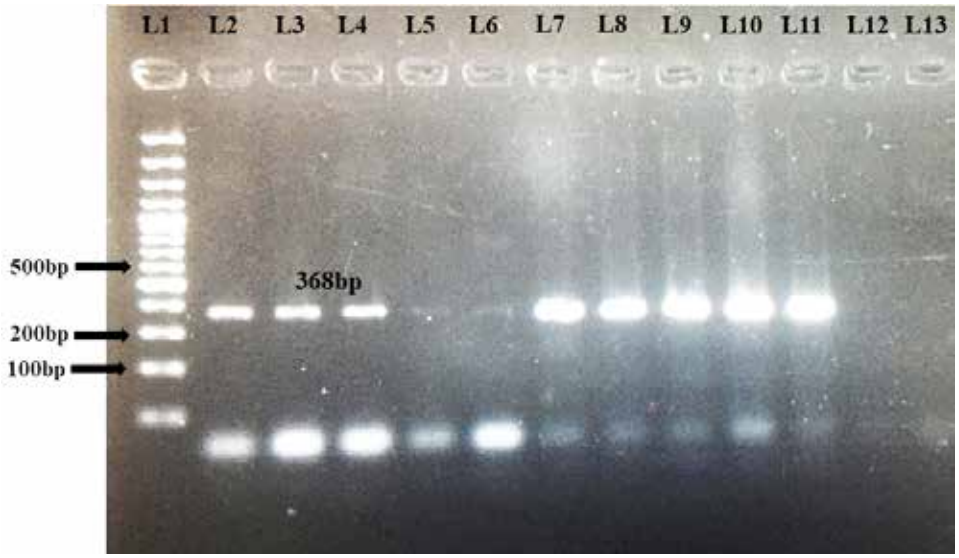

Fig. 7. Photograph of PCR result indicating positive BPXV samples, L1 (Ladder, 100bp), L2 (positive control), L3, L4, L7, L8, L9, L10 and L11 positive for BPXV showing band at 368bp, L5, L6 and L12 negative for BPXV, L13 (negative control).

Dry large size scabs also indicated the unhygienic living environment, served as a predisposing factor to bacterial complications. In localized form, this disease mostly affects the udder and teats of the buffaloes, produces various lesions, reduces the milk and growth potential (Gurav et al. 2011, Haller et al. 2014). Chai-square and regression analysis revealed that animal demographics (breed, age, lactation period and physiological stages of production) and environmental (area, season, number of animals and type of farming) risk factors have significant effect on BPX occurrence in buffaloes ($1<R^2>0$, $p<0.05$). At animal level, the BPX prevalence was 3-times higher in Nili Ravi buffaloes, aged 5-7 years as compared to Nili and Ravi breeds. The odds ratio indicated that BPX occurrence was 4-times higher during 3rd lactation period. Previously, it has also been reported that all breeds vary in their ability to cope with different diseases. Furthermore, animal related risk factors has great influence on disease production as emergence and re-emergence

of disease in non-vaccinated animals, occurred by gradual adaptation of the causative agent in the host until it converted to pathogenic leading to outbreaks (Singh et al. 2007, Fentie et al. 2017). Mostly, animal species have diseases peculiar to them because pathogens have preference to host as this BPXV is specific to buffaloes but rarely infects cows and humans (Yadav et al. 2020).

This study also revealed that the physiological stages of production of buffaloes especially pregnancy and milking have great influence over disease susceptibility. The BPX incidence was higher in pregnant and milking as compared to non-pregnant and dry buffaloes. The BPX prevalence was observed 5-times, 3-times, and 2-times higher in pregnant, pregnant milking and non-pregnant milking buffaloes, respectively, as compared to non-pregnant, pregnant dry and non-pregnant dry buffaloes ($p<0.05, 1<R^2>0$). Continuous exposure to various infectious agents, nutritional instability, body conditions and extreme climate changes lead to immunosuppression and animals become more susceptible to diseases. Furthermore, previously exposed animals attained natural acquired immunity while native animals, which are exposed for the first time, have equal chance to get infected. Environmental factors, causative agent and host relationship have significant role in disease outcome. These results agreed with previous studies from Pakistan (Numan et al. 2016) and neighboring countries described by (Yadav et al. 2010, Gurav et al. 2011).

In our study, BPX was seen throughout the year, but its incidence risk and morbidity rate were quite higher during December to May months in the Faisalabad region. The BPX was observed 8-times more frequently during winter season as compared to spring, autumn and summer seasons. The prevalence of this disease was 4-times higher in those herds where numbers of animals ranged between 21-30. This virus usually transmits through aerosol, direct contact with the abraded skin and mucosa of infected animals or indirectly by vectors (mechanical transmission). Second, movement of these infected animals is also a major source of disease transmission into new areas (Lobato et al. 2005, Babiuk et al. 2009, Das et al. 2017). The odds ratio showed that, BPX prevalence was 2-times, 3-times, and 2-times higher in those herds where buffaloes were reared together with multiple species (cows, sheep, goats etc.) as dairy animal under the loose type of housing, respectively. The impact of environmental factors such as seasonal variations and climatic conditions in pox disease occurrence is still not defined but a variable disease pattern was observed during different seasons of the year. Kasem et al. (2018) reported that, the cattle production systems, especially in backyard smallholdings where animals were kept mostly in loose spaces with common grazing areas, showed higher morbidity rate (38.34%) compared to an intensive cattle farm with lower morbidity rate. Şevik and Doğan (2017) reported from Eastern Europe that the higher morbidity occurred in cattle in small farms with fewer animals than in medium and large farms.

Based on HA genes and intracytoplasmic inclusion bodies, PCR is more accurate and specific molecular tool to identify and differentiate different poxviruses such as VAVC, VARV, MPXV BPXV and CPXV (Damaso et al. 2000, Kumar et al. 2016). In our study, PCR was used to amplify C18L gene. This C18L gene of BPXV is a homologue of VACV, present at the terminal region of the poxvirus genome with 453 nucleotide long open reading frame (ORF) that encodes a 150-aa ankyrin repeat protein for the determination of hosts range (Singh et al. 2008). Chandranaik et al. (2011) and Yadav et al. (2020) reported similar results of buffalopox virus (BPXV) from different geographical regions by amplifying ATI gene. In our study, a total of 49 scab samples were detected BPXV positive through PCR out of 63 samples. The remaining 14 negative cases suspected for BPXV might be infected with milker's nodule, ORF or other scab producing bacterial or viral diseases. These negative cases need to be further verified by amplifying specific genes via respective nucleotide sequences or with culturing for bacterial isolation. It is evident from these findings that BPX is present in the highest percentage (77.78%) among the other scab producing diseases (22.22%) in the studied population. Our study strengthen the findings of Khan, (2010) who reported high prevalence of BPX i.e. upto 50% in the district Khushab, Punjab of Pakistan. However, this figure was not based on scientific grounds and investigation was done through conflation of data based upon participatory appraisal and scanning surveillance. Though the true picture may be quite different if accurately described on laboratory findings.

Hematological examination revealed non-significant difference in RBCs count and its indices between healthy and diseased buffaloes. However, significant increase in WBCs count and DLC was observed in diseased buffaloes as lymphocytes number was high in infected buffaloes. These leukocytes appear to have the primary function of defending the host from foreign substances through leukocytosis and antibodies production (Rehfeld et al. 2013). The significant increase in leukocytes, especially neutrophils and lymphocytes, indicated rapid cell proliferation followed by virus replication and cell lysis which induces immune response. Previously, Numan et al. (2016) and Rehfeld et al. (2013) reported findings similar to those obtained in the present study.

Conclusion

Buffalopox is a re-emerging problem in the livestock population of those countries where restriction on animal movement is difficult and climate severity puts a significant influence over animal growth, production, and disease control programs. Such occurrence poses a direct zoonotic threat to animal handlers and milk consumers of that area as well. Furthermore, rearing of multiple animals together under uncontrolled environment is also a major source of disease spread. Therefore, such results may be valuable in recognizing BPXV genotypes and its relationship with various animal and environmental risk factors. This study is also helpful in designing vaccines and adopting prophylactic measures to reduce the circulation of viral pathogens.

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