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Patho-Epidemiology of Bovine Brucellosis in Aza-kheli Buffalo in Pakistan

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Abstract

Buffalo is main dairy animal with 32.7 million populations in Pakistan. Azi-Kheli breed is newly discovered breed of buffalo, localized in Swat valley of Khyber Puktunkwa (KP). In this study seroprevalence and gross as well as histopathological lesions of reproductive tract were observed in seropositive buffaloes for brucellosis. Considering PCR a gold standard test for detection of Brucella antigen, detecting 14% samples positive out of 100, the sensitivity and specificity was higher for RBPT and indirect ELISA revealing overall seropositivity of 11% and 13% respectively. The most prominent lesions were mild to severe endometritis followed by cervicitis, inflammation of uterine horns, edema, necrotic foci, yellowish exudates and ulceration in uterus. The cervix showed accumulation of whitish exudate of mild to moderate degree and severe cervicitis while uterine horns had mild to moderate Inflammation mild edema, mild necrotic foci, mild pus, exudates and mild ulceration. The histopathological examination was in uterus showed mild to severe increase of the fibrous connective tissues in the glandular region with slight hypertrophy in the endometrial glands, mild mononuclear inflammatory cells infiltration, moderate to severe congestion along with degeneration of endometrial glands and myometrium, mild to severe necrosis, degradation of the mucosa, atrophy of the endometrial glands and hemorrhages in the blood vessels. The cervix showed mild to moderate fibrosis, infiltration of mononuclear inflammatory cells, congestion with necrosis along with degeneration of secretory glands, mild atrophy of secretory glands while uterine horns showed mild to moderate fibrosis, infiltration of mononuclear inflammatory cells, congestion, atrophy of secretory gland and degeneration.

Keywords: Brucellosis, Azi-Kheli, Slaughter house and Pathology

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Introduction

The buffalo is world's second most important dairy animal and considered as black gold of Pakistan [1], with population of 33.7 million (Economic survey of Pakistan 2012-2013) and contributed 70% milk production in Pakistan [2]. Azi-Kheli breed is a newly discovered Pakistani buffalo breed, used for dual purpose, known for its adaptation to mountain environment and mainly localized in Swat valley of Khyber Pakhtunkhwa (KP) province.

The name Azi-Kheli is given due to its origin home tract Azikhel (village name at Tehsil Khwazakhela district Swat) and after a local tribe Aziz Khell [3]. Indigenous breeds of livestock are known for disease resistance, required little inputs for survival and adoptability in local envirment [4]. Therefore it is believed that indigenous breeds are more economically efficient than other breeds when raised in their home tract [5]. Brucellosis is an infectious endemic disease and a major constraint for animal production and performance [6]. Bovine brucellosis is caused by *Brucella abortus* and is transmitted to animals and humans mainly by direct contact between infected hosts or through oral, respiratory, cutaneous, ocular and sexual routes [7]. It can also be transmitted from herd to herd by movement of carrier animal, by exposure to birth materials, ingestion of unpasteurized infected milk and contaminated feed or water and through fomites [8], with 25% lost in milk production by the carrier animal [9].

It is reported that prevalence of brucellosis is affected by many factors such as species, age, sex, diagnostic tool, environment, geological area, number of animals reared in the locality and genetic makeup of the animal which act as tools for difference in results [10]. There is scarcity of data available regarding to brucellosis in Azi-Kheli buffaloes. Therefore, the present study is providing baseline data brucellosis in Azi-Kheli buffaloes. The aim of present study was to study the seropositivity of brucellosis in slaughtering Azi-Kheli buffaloes at slaughter house of Mingora district Swat and examination of gross and histopathalogical lesions of reproductive tract tissues of brucellosis carrier Azi-Kheli buffaloes.

Materials and methods

Study design and location

A cross section survey was conducted in district swat, Khyber Pakhtunkhwa. Convenience based

sample of 100 Azi-kheli buffaloes were randomly selected from Jan to May 2013 at local slaughter house. Azi-kheli is the local buffalo breed of KPK. Study was conducted in swat district as it was convenient to locate the Aza-kheli buffalo breed here.

Sample collection

Approximately 5 ml blood was collected in a clean plane test tube from each slaughtered Azi-Kheli buffalo at slaughter house and serum from the blood sample was collected in 3 ml eppendorf tube . Gross pathological lesions in reproductive tract were examined of positive Azi-Kheli female buffaloes and tissue samples were taken from these pathological lesions for histopathalogical examination.

Serological diagnosis

Rose Bengal Plate Test (RBPT)

The serum samples were screen out for the brucellosis by Rose Bengal Plate Test (RBPT) in the slaughter house as described by [11]. Rose Bengal antigen was obtained from Veterinary Research Institute (VRI), Lahore. The antigen was stored in refrigeration at 4 0C during the course of study as per manufacturer's recommendations. PCR confirmed negative and positive control serum was obtained from the Veterinary Research Institute Peshawar in ordered to minimize the chance of any false positive or negative sample. The RBPT was used as screening test and interpreted as positive when agglutination were observed visually, positive serum samples were stored at -20 0C for further processing.

Indirect Enzyme Linked Immunossorbant Assay (i-ELISA).

The indirect Enzyme Linked Immunossorbant Assay (i-ELISA) was carried out by Bovine Brucella antibody IgG ELISA kit number CSB-E1306B, imported from Cusabio Biotech Company Limited China. The assay was progress according to the user kit manual. The results obtained for Brucella abortus antibody (IgG) was calculated by comparing the sample well with control well according to the kit user manual. Positive results were taken in the range of optical density 0.80 to 2.5.

Molecular basis diagnosis by Polymerase Chain reaction (PCR)

The PCR was performed on the serum samples for detection of bacterial nucleic acid using procedure described by [12]. The genomic DNA was extracted from the serum samples by using QIAamp DNA Mini kit (Qiagen Inc. 28159 Avenue Stanford, Valencia, CA 91355. fax 800-718-2056, USA). The protocol adopted for DNA extraction was provided by the manufacturing company. DNA concentration and quantification was obtained by using nanodrop software on a nanodrop attached computer screen. PCR assay was performed by using already reported primers, used by [12]. The primer used was able to amplify 223 bp, which codes for 31kDa BCSP protein. The primers used were B4 forward primer 5 TGG CTC GGT TGC CAA TAT CAA 3 and B5 Reverse primer5 CGC GCT TGC CTT TCA GGT CTG 3. The PCR reaction mixture volume was 25ul. Thermal cycling for primer pairs in PCR assay were initial denaturation at 93C for 5 min, denaturation at 90C for 1 min, annealing at 58C for 30 sec, extension at 72C for 1 min and final extension at 72C for 10 min respectively. A total of 35 cycles were repeated for each PCR assay. Amplification of brucella nucleic acid in the samples of Azi-Kheli buffaloes were confirmed by Agarose Gel Electrophoresis.

5. Gross lesions

The positive animal's cervix, uterus and uterine horns were examined for gross pathological lesions by naked eye such as endometritis, pyometra, fibrosis and hemorrhages [13]. The photographs of gross pathological lesions in reproductive tract were taken with digital camera.

6. Histopathological examinations

Tissue specimen from grossly affected tissue was collected in clean container and fixed in 10% natural buffered formalin solution. The tissue samples were further processed for H& E stained histopathalogical slides were prepared from tissue specimen. The tissue samples were processed with standard techniques for fixation, dehydration, clearing, embedding, sectioning and staining [14].

Results

The RBPT showed 11%, i-ELISA 13% and PCR 14 % positive cases of brucellosis in Azi-Kheli

buffaloes.The gross pathological lesions of brucellosis positive Azi-Kheli buffaloes were summarized in table 1. The most prominent gross pathological lesion observed in uterus was endometritis with mild to severe degrees in six animals. The remaining gross pathological lesions include in uterus were moderate edema of the caruncles in one buffalo, Mild to moderate necrotic foci in two buffaloes, mild to moderate whitish to yellow exudates in five buffaloes and mild to moderate ulceration of the endometrium in two buffaloes. The prominent gross lesion observed in cervix was accumulation of whitish exudate of mild to moderate degree in two buffaloes and severe cervicitis in one buffalo while in uterine horns mild to moderate Inflammation was observed in five buffaloes and other gross pathological lesions were mild edema in three buffaloes, mild necrotic foci in one buffalo, mild pus and exudates in three buffaloes and mild ulceration in one buffalo.The histopathalogical examination was summarized in table1.2 which revealed that in uterus five animals showed mild to severe increase of the fibrous connective tissues in the glandular region with slight hypertrophy in the endometrial glands. One animal uterus showed mild mononuclear inflammatory cells infiltration. Moderate to severe congestion along with degeneration of endometrial glands and myometrium was observed in the endometrium of four animals. Three animals showed mild to severe necrosis, degradation of the mucosa, atrophy of the endometrial glands and hemorrhages in the blood vessels. The histopathalogical examination of the cervix showed that mild to moderate fibrosis, infiltration of mononuclear inflammatory cells, congestion with necrosis along with degeneration of secretory glands in three animals in each respectively. Two animals cervix showed mild atrophy of secretory glands. The uterine horns also showed mild to moderate fibrosis, infiltration of mononuclear inflammatory cells, congestion, atrophy of secretory gland and degeneration in three, three, two, three and three animals respectively.

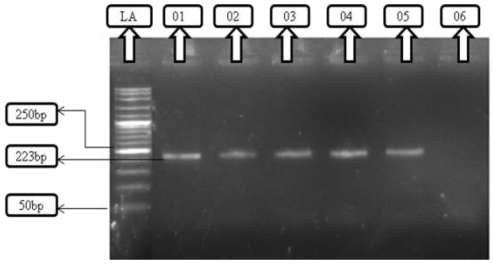
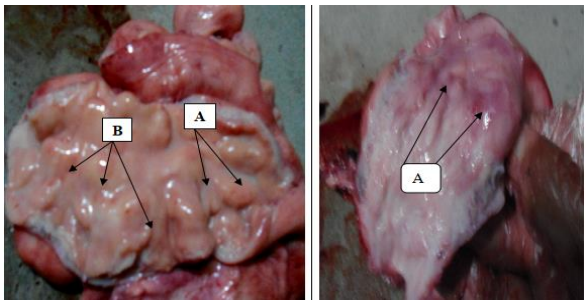


Figure 1: Agarose gel electrophoresis pattern of Brucella bcsp31 gene 223bp specific PCR product amplified. LA: DNA molecular weight ladder 50bp. 01 to 06 : Samples of Azi-Kheli buffaloes



Left: Whitish exudates present in the endometrium (headed by arrows A), along with hemorrhages (Headed by arrows B). Right: Severe hemorrhages in the cervix (Arrows A).

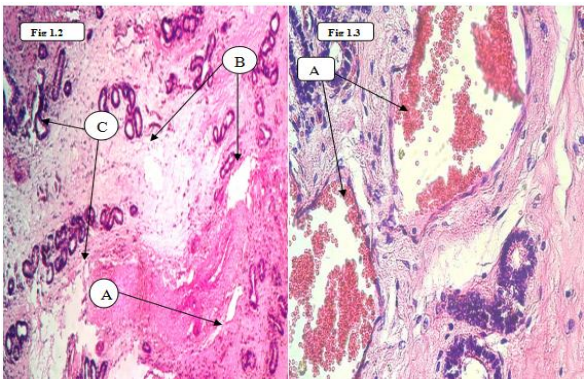


Fig 1.2: Necrosis (Arrow A) and degeneration (Arrows B) of endometrium along with atrophy of endometrial glands (Arrows C). Fig 1.3: Shows congestion mussels and blood vessels dilation (Arrows A) (H&E, 40X)

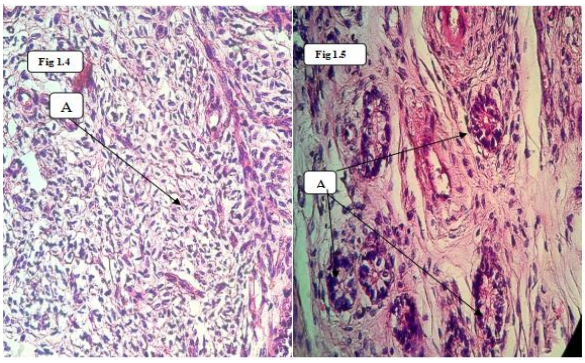


Fig 1.4: Chronic endometritis characterized by fibrosis (Arrow A). Fig 1.5: Endometritis characterized by periglandular infiltration of mononuclear inflammatory cells mainly lymphocytes (Arrows A) along with necrosis.

Discussion

The sero prevalence of brucellosis at slaughterhouse in Azi-Kheli buffaloes were 11%, 13% and 14% by RBPT, i-ELISA and PCR respectively. The RBPT base seroprevalence showed similarity with the studies of [15-16] who reported 12.75%, 12%, and 12.33% positivity by RBPT respectively. Patel [17] reported higher seropositivity by RBPT that is 18.61%. The variation in results may be due the use of different procedure adopted and different number of animals examined as well as a marked difference between geography. The present study showed contrast with the studies of [18] who reported lower seroprevalence of brucellosis in buffalo population in Swat valley 4.85% and 5.62% by RBPT and iELISA respectively. This may be suggestive that higher seroprevalence in present study was due to including of only slaughtering animals. The results obtained of seropositivity by i-ELISA showed similarity with study of [13] and reported 14.5%. Higher seropositivity by i-ELISA was reported by [16] and [15] 38.46%, 26.03% and 26.63% and lower seropositivity was reported 6.92 percent by [19]. This variation in results was due to use of different i- ELISA kits, proper vaccination protocol following by the owners and number of serum screened by i-ELISA. The PCR detected more positive cases then RBPT and i-ELISA. The results were supported by the studies of [12] who used the same primers sequence, BCSP 31 kDa protein base primer and reported 20.6 percent more positive results then serological tests. This may be suggestive because the uses of universal BCSP 31 kDa primers of brucellosis were used for the amplification of DNA. Results of the present study showed contrast with the study of [20] who reported low positivity of 11.4% by the same primers.[21] reported 26% positivity in brucella carrier animal's blood samples. The contrast in result was due different sample size, species and different protocol for DNA quantification. The gross lesions examinations results in present study indicating more animals had endometritis, followed by pus or exudates, Odema, necrotic foci, ulceration in uterus of brucellosis screened positive buffaloes respectively.

Table 1. Scoring card of gross lesions in Reproductive tract of brucellosis positive Azi-Kheli buffaloes.

Azi-Kheli buffalo Number	Uterus					Cervix					Uterine Horns				
	INF	OD	NF	P/E	UL	INF	OD	NF	P/E	UL	INF	OD	NF	P/E	UL
Azi-Kheli 09	+++	-	-	+	-	-	+	-	+	-	+	-	-	+	-
Azi-Kheli 25	-	++	-	+	-	-	-	-	-	-	-	+	-	+	-
Azi-Kheli 32	+	-	-	-	-	+++	-	-	-	-	+	-	-	-	-
Azi-Kheli 43	++	-	+	-	-	-	-	-	-	-	+	-	-	-	-
Azi-Kheli 55	-	-	++	-	-	-	-	-	-	-	-	-	+	-	-
Azi-Kheli 67	+	-	-	++	+	-	-	-	-	-	+	-	-	+	-
Azi-Kheli 74	-	-	-	+	-	-	-	-	+++	-	-	-	-	-	-
Azi-Kheli 83	++	-	-	-	-	-	-	-	-	-	-	+	-	-	+
Azi-Kheli 89	-	-	-	+	-	-	-	-	++	-	-	-	-	-	-
Azi-Kheli 97	-	-	-	-	++	-	-	-	-	+	-	-	-	-	-

−: No lesions; +: Mild; ++: Moderate; +++: Severe, INF: Inflammation, OD: Odema, NF: Necrotic Foci, UL: Ulceration.

Table 2: Scoring card of histopathalogical lesions in reproductive tract of brucellosis positive Azi-Kheli buffaloes.

Azi-Kheli buffalo Number	Uterus					Cervix					Uterine Horns				
	FIB	IMI	CON	AGL	DEG	FIB	IMI	CON	AGL	DEG	FIB	IMI	CON	AGL	DEG
Azi-Kheli 09	+++	-	-	++	-	+	-	+	-	-	++	-	-	+	-
Azi-Kheli 25	-	++	++	-	-	-	++	-	-	++	-	-	-	-	-
Azi-Kheli 32	+++	-	-	++	-	+	-	-	-	-	+	-	-	+	-
Azi-Kheli 43	++	-	-	-	-	-	-	+	-	+	-	-	-	-	++
Azi-Kheli 55	-	-	++	-	-	++	-	-	-	-	-	+	++	-	-
Azi-Kheli 59	-	-	-	-	++	-	-	-	-	-	-	++	-	-	-
Azi-Kheli 67	+	-	+++	-	-	-	++	-	+	-	-	-	-	++	-
Azi-Kheli 74	+++	-	-	++	-	-	-	-	+	++	-	-	-	-	-
Azi-Kheli 83	-	-	-	+	+	-	-	++	-	-	+	-	-	-	+
Azi-Kheli 89	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Azi-Kheli 97	-	-	++	-	+++	-	+	-	-	-	-	+	+	-	+

−: No lesions; +: Mild; ++: Moderate; +++: Severe, FIB: Fibrosis, IMI: Infiltration of in mononuclear inflammatory cells, CON: Congestion, AGL: Atrophy of glands, DEG: Degeneration.

Cervicitis and whitish exudates in cervix and in uterine horns inflammation and whitish exudates were observed. These results were collaborated with the studies of [22] who reported more cases of endometritis along with its different forms. [23] also investigate the high number of endometritis cases along with erosion and sloughing of epithelium of endometrium. Similarly [24] investigated cases of multifocal to diffuse endometritis characterized by layers of green-tinged, hemorrhagic exudate covering caruncles and multifocal ulceration of the superficial endometrium and cellular debris on the endometrial surface with oedema. The present finding showed contrast with the finding of [25]. They reported that the infected uterus contains high ratio of fetid yellow to brownish exudates containing fibrin and necrotic debris. This may be suggestive because of acute stage of the infection in pregnant cows. The histopathology examination results of the present study revealed that in uterus mild to severe increase of the fibrous connective tissues in the glandular region with slight hypertrophy in the endometrial glands followed by mild to severe congestion along with degeneration of endometrial glands and myometrium, necrosis and degradation of the mucosa, atrophy of the endometrial glands and hemorrhages in the blood vessels. The cervix showed that mild to moderate fibrosis, infiltration of mononuclear inflammatory cells, congestion with necrosis along with degeneration of secretory glands respectively and mild atrophy of secretory glands. The uterine horns also showed mild to moderate fibrosis, infiltration of mononuclear inflammatory cells, congestion, atrophy of secretory gland and degeneration respectively. [23] studies collaborated with present study who reported mild to moderate stromal, diffused and multifocal accumulations of lymphocytes, machropages and plasma cells along with necrosis, calcification and surrounded by inflammatory cells. Similar study was carried by [24] showed the neutrophilic and inflammatory infiltrate, associated with necrosis, oedema, fibrin deposition, and vasculitis. [26] also reported that brucella positive cows revealed marked changes, in the form of mononuclear cell infiltrations, fibrosis, and calcification of tissue. Similar observations were found in cows naturally infected with Brucella melitensis by [22] reported infiltration of the mononuclear inflammatory cells may provide a clue to the nature of localization of the infection in the uterine tissue. [22] also reported that the evidence of disease in its chronic stage was suggested by the presence of fibrosis and calcification. Blood vessels were thickened, dilated, congested and prominent myometrium changes in all Brucella positive cases.

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