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Incidence of brucellosis in aborted animals and occupationally exposed veterinary professionals of Bannu, Khyber Pakhtunkhwa, Pakistan

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Original Article

Incidence of brucellosis in aborted animals and occupationally exposed veterinary professionals of Bannu, Khyber Pakhtunkhwa, Pakistan

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Abstract

Brucellosis in animals is a serious global health concern mainly due to its zoonotic nature and association with heavy economic losses. Brucellosis has been reported randomly from different parts of Pakistan; however, the prevalence of brucellosis in animals and humans in Bannu District, as well as overall epidemiological data of the country, is missing. The present study reports on the occurrence of brucellosis in domestic animals and its cross-circulation among occupationally exposed human population in Bannu District, Khyber Pakhtunkhwa, Pakistan. A total of 223 (animals=100, animal handlers=73, veterinary staff=50) serum samples were collected and subjected to serum plate agglutination test (SPAT), Rose Bengal plate test (RBPT) and direct polymerase chain reaction (PCR) specific for *Brucella melitensis* and *B. bovis*. Results indicated overall prevalence of 27% (27/100) by SPAT, 10% (10/100) by RBPT and 11% (11/100) by PCR. Prevalence in animal handlers was 24.6% (18/73) by SPAT, 6.84% (5/73) by RBPT and 12.3% (12.3% (9/73) by PCR, while prevalence in veterinary professionals was 30% (15/50) by SPAT, 4% (2/50) by RBPT and 18% (9/50) by PCR. Age, sex, introduction of new animals, contact with infected animals, breeding methods, and hygienic conditions were statistically significantly ($p < 0.05$) associated with the occurrence of brucellosis. Taken together, brucellosis is a persistent public health threat in Bannu District of Pakistan. Therefore, proper control management and mass education are imperative.

Keywords: brucellosis, PCR, risk factor of brucellosis, brucellosis in animal handlers, prevalence of brucellosis

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Introduction

Brucellosis, a worldwide zoonotic disease of economic and public health importance, is a highly contagious disease that predominantly infects animals. Brucellosis is enlisted as the second leading zoonotic infection around the globe by the Office International des Epizooties (OIE), followed by rabies, due to its nature of high transmissibility rate (http://www.oie.int/en/for-the-media/animal-diseases/animal-disease-information-summaries/). Particularly, brucellosis is a risk to human beings occupationally exposed to animals such as butchers, veterinarians, animal handlers and laboratorians. However, people in general can also be infected by ingestion of contaminated products, such as milk and meat, of infected animals or close physical contact with infected animals or secretions. Improper disposal of contaminated remains such as aborted foetal contents may contaminate the environment, increasing the spreading of infection (Pappas G et al., 2006; Muma J et al., 2006). Regular epidemiological investigation and surveillance are performed in countries where the disease is endemic. For regular screening of brucellosis in animal herds, serological tests such as Rose Bengal test (RBT), serum plate agglutination test (SPAT), complement fixation test (CFT), and enzyme-linked immunosorbant assay (ELISA) are performed. In addition, recently, molecular based such as detection based on DNA by PCR is widely implemented for screening, surveillance and diagnostic purposes. PCR-based detection performed directly on infected specimens is preferred, although expensive, because of its ability to reveal the status of brucellosis, such as clinical form or previous exposure.

The causative agent of brucellosis is a Gram-negative coccobacillus, *Brucella* species, which can infect virtually all ruminants (Cloeckaert A et al., 2001; Cloeckaert A et al., 2003). Although *B. melitensis* is considered to be genetically the parental species of single variant for all *Brucella* species, for practical reasons they exist in different names, such as *B. abortus*, which infects cattle and buffalo; *B. ovis*, which causes brucellosis mainly in sheep; and *B. melitensis*, which infects both goats and sheep. Of note, these three *Brucella* species are also popular zoonotic species and can infect human, causing undulant fever or malta fever and, therefore, holding its importance due to its zoonosis. Animals infected with brucellosis go through a complex condition with clinical manifestation of decreased milk production and severe complications of reproductive track, such as abortion, placenta retention and infertility. *B. melitensis* infection mainly affects ewes, compared to rams, and causes late term abortion in pregnant animals. However, abortion is less common in ewes compared to birth of weak offspring. Furthermore, in endemic cases, retained placenta is often seen during infection of brucellosis and rams infected with brucellosis often show clinical features of orchitis and epididymitis, and in some cases polyarthritis in endemic flocks (Radostits OM et al., 2006).

Brucellosis remains relatively higher in the subcontinent, particularly, in Pakistan, India and Bangladesh. Although random reports on the incidence of brucellosis from different regions of Pakistan suggest higher prevalence of brucellosis, unfortunately, overall countrywide system surveillance, strategic planning and control measures are totally missing (Abubakar M et al., 2012). The prevalence of *B. abortus* in bovines has been reported in different regions of Pakistan as high as 4.7% (Adnan et al., 2017), 3.25% (Ahmad R et al., 1990) and 4.4% (Naeem K et al., 1990). Mukhtar & Kokab (Mukhtar F and Kokab F, 2008) demonstrated cross-circulation of *Brucella* to human by diagnosing seropositive employees working in abattoirs in Lahore and Kurram Agency of Pakistan. Additionally, other reports from Punjab province of Pakistan indicated higher overall prevalence (5.05%) of brucellosis in cattle using a serum agglutination test (Ahmed R and Munir M, 1995), whereas lower prevalence of 1.46% and 1.93% in sheep and goat, respectively, was documented (Nasir AA t al., 2000). Bannu District is located in the south-east of Khyber Pakhunkhwa with around 1.07 million of human and 1.966 million of livestock population (Statistics of Khyber Pakhtunkhwa Government). Livestock and agriculture are the main source of income and food for the inhabitants of Bannu. Literature regarding seroprevalence of brucellosis in animals and its cross-circulation among animal handlers and veterinary practitioners in Bannu District is not available. This is the first report on the prevalence of brucellosis in animal and human population of Bannu District. Particularly, the study applied PCR directly on specimens revealing active status of the disease, consequently eliminating ambiguity of previous exposure.

Materials and Methods

Ethical approval: The study was approved by the ethical committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan and Veterinary Research Institute, Peshawar, Pakistan. All the procedures were performed in accordance with the local and national ethical guidelines of animal handlings. The study was also approved by the ethical board of health division of the Government of Khyber Pakhtunkhwa. Blood samples from human were collected by assigned medical nurses.

Study area and population: The study was performed from January 2016 to May 2016. Bannu is the district headquarter, located at 32° 59' 22" North, 70° 36' 21" East, in the south of Khyber Pakhtunkhwa province, sharing a border with tribal area and Afghanistan in the north-west. Agriculture depends upon canal irrigation (45%) while the rest of the area depends upon rain. Bannu District has a total animal population of approximately 1.99 million, of which 0.168 million are cattle. More than 43% of the population depend upon livestock rearing and agriculture. Samples were obtained from animals with previous history of abortion, humans who handled animals during feeding and milking, and veterinary professionals who handled sick animals or performed artificial insemination. A total of 223 serum samples were collected from domestic animals (n=100), animal handlers (n=73, 50 males and 23 females) and

veterinary health professionals (n=50) from Bannu District, Khyber Pakhtunkhwa, Pakistan.

Sample collection: Blood was collected aseptically using a sterile syringe and serum was separated by centrifugation at 3,000 rpm for 4 minutes and stored at -20ºC until use.

Primary screening by SPAT and RBPT: All the serum samples were subjected to serum plate agglutination test (SPAT) and Rose Bengal plate test (RBPT) for detection of anti-*Brucella* antibodies. SPAT was performed by placing a drop of serum onto a glass slide. A drop of *Brucella abortus* and *Brucella melitensis* antigens was added to each serum drop and mixed gently with a tooth stick. Any agglutination on the slide was considered as positive test. Antigens for RBT and SPAT were bought from Veterinary Research Institute, Lahore, Pakistan.

DNA extraction and PCR: All SPAT-positive samples were subjected to confirmation by PCR. DNA from the blood/serum samples was extracted using a 50 µl extraction buffer (Shangi ZJ Biotech, China) followed by high speed centrifugation according to the manufacturer's instructions. The supernatant was used as source of antigen. Known antigens of *Brucella abortus* and *Brucella melitensis* were used as positive control. Reaction was performed in a total of 50 µl reaction mixture containing 5 µl DNA, 35 µl PCR-master mixture, 0.4 µl Taq enzyme and 9.6 µl molecular grade water. The master mixture was bought from Shangi ZJ Biotech, China; it contained primers specific for detection of *Brucella abortus* and *Brucella melitensis*. A band of 306 bp PCR product indicated *Brucella abortus*, while 312 bp showed *Brucella melitensis*. The PCR cycling conditions were optimized as follows: initial denaturation at 94ºC for 2 min, denaturation at 93ºC for 15 sec, annealing at 55ºC for 30 sec and final extension at 72ºC for 30 sec in 35 cycles. The PCR product was visualized in 1.2% agarose gel using gel documentation system.

Statistical analysis: Data collected were analyzed by Chi-square test using (SPSS Version 16) to compare the disease in different population.

Results

Overall incidence of brucellosis in animals and humans: The seroprevalence of brucellosis in dairy animals with previous history of abortion was evaluated with SPAT, RBPT and PCR; the results are mentioned in Table 1. Out of the 100 serum samples of dairy animals, 27% (27/100) and 10% (10/100) were found positive against *Brucella* antibodies by SPAT and RBPT, respectively, while PCR confirmed 11% (11/100) of the samples positive for brucellosis. Similarly, 18 out of 73 (24.65%) and 5 out of 73 (6.84%) samples of animal handlers were found positive by SPAT and RBPT, respectively, while PCR confirmed 9 out of 73 (12.3%) samples. Moreover, out of the 50 serum samples of veterinary staff (veterinary doctors, veterinary assistants and AI technicians), 15 (30%) were found positive by SPAT, 2 (4%) by RBPT and 9 (18%) by PCR (Fig.1, Tables 1-3).

Table 1 Prevalence of brucellosis in serum samples of dairy animals with previous history of abortion using SPAT, RBPT and PCR as confirmatory test and association of different risk factors with the disease

Sr. No	Variables	Category	SPAT*			RBPT**		PCR***		
			n	+ ive	P value	+ ive	P value	N	+ ive	P value
1	Breed	Buffalo	23	7	0.017	3	0.43	7	3	0.047
		Native cattle	14	1		1		1	0	
		Cross-bred cattle	46	18		6		18	8	
		Holstein Friesian	17	1		0		1	0	
		Total	100	27 (27%)		10 (10%)		27	11 (11%)	
2	Age	3-7 (years)	46	6	0.004	1	0.05	6	2	0.09
		8-13 (years)	54	21		9		21	9	
3	Breeding method	AI	34	16	0.001	9	0.000	16	6	0.001
		Natural	66	11		1		11	5	
4	Introducti on of new animals	Yes	21	19	0.0001	9	0.0001	19	10	0.0001
		No	79	8		1		8	1	
5	Contact with infected animals	Yes	18	16	0.00001	8	0.0002	16	9	0.0002
		No	82	11		2		11	2	
6	Sanitation	Satisfactory	62	5	0.0001	1	0.002	5	1	0.00002
		Unsatisfactory	38	22		9		22	10	
7	Grazing	Yes	60	16	0.92	9	0.04	16	7	0.95
		No	40	11		1		11	4	
8	Condition of animals	Healthy	50	12	0.68	4	0.77	12	7	0.71
		Weak	50	15		6		15	4	

*serum plate agglutination test, **Rose Bengal plate test, ***polymerase chain reaction

Species wise incidence of infection: Of the 100 samples of dairy animals, 8 were found positive by SPAT for *B. melitensis*, while 19 were positive using antigen of *B. abortus*. However, PCR indicated that only 4 were positive for *B. melitensis* and 7 for *B. abortus*. Of the 73 samples from animal handlers, 9 were found positive against *B. melitensis* and *B. abortus* using SPAT, while 6 and 3 were found positive by PCR, respectively.

Interestingly, however, PCR-based positivity of *B. abortus* was found higher (5/50) among veterinary staff compared to *B. melitensis* (4/50) (Fig. 1).

Risk factor influencing incidence of brucellosis in animals and humans: Our results indicated that animals of lower age (3-7 years) were found positive (6/100 and 2/100 by SPAT and PCR, respectively), while animals aged 8-13 years were more infected (21/100 and 9/100 by SPAT and PCR, respectively). Similar relationship of incidence was found in animal handlers and veterinary staff. Of the 73 animal handlers aged 18-30 years old tested, 5 and 1 were found positive by SPAT and PCR, respectively, while of those aged 31-50 years old, 13 and 8 were found positive by SPAT and PCR, respectively.

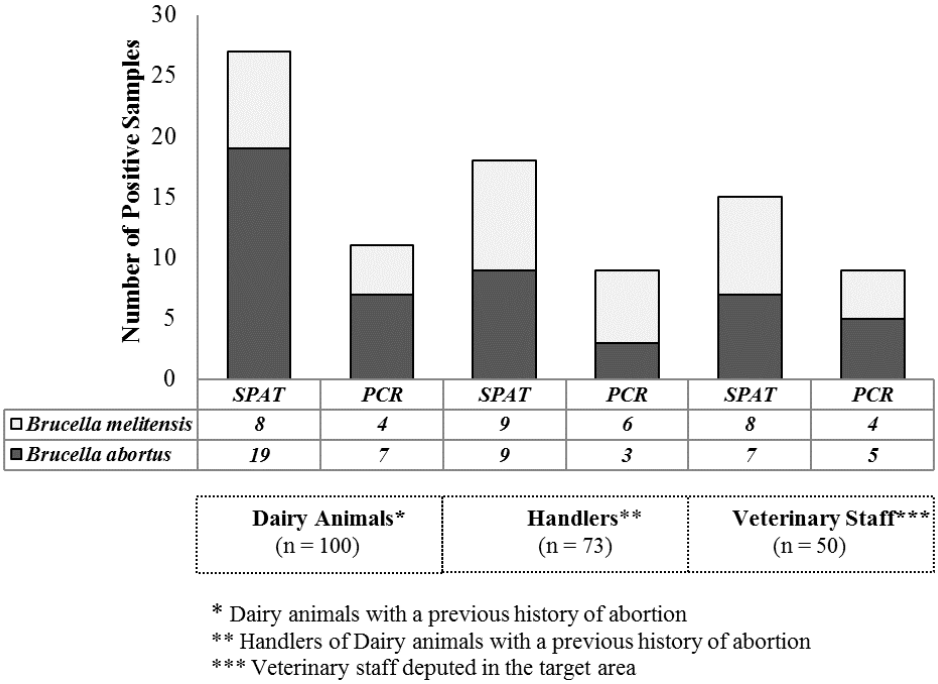

Figure 1 Comparative prevalence of *Brucella abortus* and *Brucella melitensis* in dairy animals, their handlers and veterinary staff by SPAT and PCR

Brucellosis is highly zoonotic, but requires close physical contact or consumption of raw animal materials contaminated with *Brucella* to get infected. The present study investigated whether close contact was associated with higher infection rate in animal handlers and veterinary staff. Our results indicated that animal handlers with close contact with infected animals were found more positive [7 of 9 (77%) by SPAT and 3/9 (33%) by PCR] than people who had no contact with infected animals [11/64 (17%) by SPAT and 6/64 (9%) by PCR]. Other risk factors such as sanitation, grazing/deeding behaviors and condition of animals were investigated for animals while the use of mask, gloves, etc. during handling of animals was investigated for animal handlers; the results obtained are presented in Tables 1-3.

Altogether, statistical analysis revealed significant ($p < 0.05$) association between the incidence of brucellosis in dairy animals and breed, breeding method, age, introduction of new animals, contact with infected animals and farm hygienic conditions. Similarly, clinical signs and contact with infected animals also showed strongly significant association. Surprisingly, however, non-significant association was observed between the occurrence of brucellosis and grazing, physical health of subject and history of aborted animals ($p > 0.05$) (Tables 2-3). Similarly, no significant association was observed between the occurrence of brucellosis and handling of retained placenta with or without gloves or mask.

Discussion

Brucellosis, due to its obvious potential of zoonosis, holds importance in addition to adversely affecting production capabilities of livestock animals. In this report, the incidence of brucellosis in dairy animals, animal handlers and veterinary professionals in Bannu District of Khyber Pakhunkhwa, Pakistan was investigated. Overall, our results indicated high seroprevalence in animals including humans who were at high risk of getting infection. A strong association of the occurrence of disease and breed, breeding method, age, introduction of new animals, contact with infected animals and farm hygienic conditions was observed.

Brucella species can infect a range of types of animals including human beings. This current report mainly focused on dairy animals (both farm and domestic) and people who handled these animals during treatment or feeding such as veterinarians and farmers. Our results indicated a higher occurrence of 27% and 11% (based on SPAT and PCR, respectively). The recorded observations are quite higher compared to the results of other studies conducted in Pakistan; one study reported the seroprevalence in cattle and buffaloes of 5.05% and 5.45%, respectively [10], and our previous study reported the findings of 4.67 (Abdul Qadeer Khan et al., 2017 accepted article). A previous study conducted in Balochistan, Pakistan indicated overall prevalence of 3% and 8.5% in cattle and

buffaloes using milk ring test and i-ELISA, respectively, by screening a total of 200 samples (Shafee M t al., 2011). Similar lower prevalence rate was also reported in cows and buffaloes in Punjab, Pakistan by screening 1,473 cattle and 481 buffaloes by Rose Bengal test (RBT), indicating 14.7% and 17.4% in cattle and buffaloes, respectively (Nasir A t al., 2004). The discrepancy between the 27% seropositivity of brucellosis by SPAT in non-vaccinated animals and 11% PCR-based positivity may not be intriguing in this case, as SPAT is considerably non-specific and false positive results might be expected. Moreover, previous exposure to brucellosis that has been cleared out may be the reason for positive SPAT. However, PCR detects specific genomic part of brucellosis, and healthy animals with previous exposure will be declared negative by PCR. The incidence of brucellosis in the neighboring countries of Pakistan and around the world has also been reported, however it occurs at different frequencies. For example, in Sarab city of Iran, 4.06% of 1,500 livestock animals were found positive for brucellosis using RBPT (Akbarmehr J and Ghiyamirad M, 2011). Similarly, in India 3-5% of animals were found infected with brucellosis using blood samples (Renukaradhya G et al., 2002), however, milk ELISA screening indicated significantly higher (18-20%) prevalence in the province of Punjab, India (Aulakh H et al., 2008). In China, the prevalence of brucellosis in animals was recorded at an average rate of 0.06-0.09 in 1991-98, while 0.74% was recorded for human. However, since 1994 onward human cases of brucellosis infection gradually increased from less than 500 cases to 1,500-3,000 case per year (Deqiu S et al., 2002).

Table 2 Prevalence of brucellosis in serum samples of animal handlers using SPAT, RBPT and PCR and association of different factors with the disease

Variables	Category	SPAT*			RBPT**		PCR***		
		N	+ ive	P value	+ ive	P value	N	+ ive	P value
Gender	Male	50	11	0.43	3	0.67	11	4	0.21
	Female	23	7		2		7	5	
	Total	73	18 (24.6%)		5 (6.84%)		18	9 (12.3%)	
Age	18-30 (years)	31	5	0.042	1	0.95	5	1	0.41
	31-50 (years)	42	13		4		13	8	
Clinical signs	+	33	16	0.0001	5	0.011	16	9	0.0001
	–	40	2		0		2	0	
Contact with infected animals	Yes	9	7	0.05	4	0.0001	7	3	0.006
	No	64	11		1		11	6	
Abortion history in family	Yes	9	4	0.14	1	0.58	4	2	0.57
	No	64	14		4		14	7	

*serum plate agglutination test, **Rose Bengal plate test, ***polymerase chain reaction

Table 3 Prevalence of brucellosis in serum samples of veterinary staff using SPAT, RBPT and PCR and association of different factors with the disease

Variables	Category	SPAT			RBPT		PCR		
		N	+ ive	P value	+ ive	P value	N	+ ive	P value
Designation	Vet Dr[1]	10	3	0.20	0	0.08	3	2	0.41
	Vet Asst[2]	25	5		0		5	4	
	AI Tech[3]	15	7		2		7	3	
	Total	50	15 (30%)		2 (4%)		15	9 (18%)	
Handling of aborted animal	Yes	35	11	0.73	2	0.34	11	7	0.63
	No	15	4		0		4	2	
Handling of retained placenta	Yes	19	5	0.65	0	0.25	5	3	0.51
	No	31	10		2		10	6	
Use of mask	Yes	23	6	0.57	0	0.18	6	3	0.51
	No	27	9		2		9	6	
Use of gloves	Yes	21	7	0.66	1	0.81	7	4	0.83
	No	29	8		1		8	5	

*serum plate agglutination test, **Rose Bengal plate test, ***polymerase chain reaction
[1]veterinary doctor, [2]veterinary assistant, [3]AI technician

Of note, our results of statically significant association of the incidence of brucellosis and increasing age is quite alarming since it indicates increased chances of exposure to infected animals or materials. Conversely, it indicates that most of the infection is more likely acquired due to contamination in the environment. Thus, the circulation of *Brucella* species in prevailing environment is highly dangerous and attention should be given for proper control and elimination of the disease. Additionally, the higher level of seroprevalence of brucellosis among older animals may be attributed to the sexual development of the animals (Amin KM et al., 2005). The higher level of seroprevalence in adult stock has been endorsed in other studies as well (Abubakar M et al., 2012; Rahman M et al., 2006). Besides, other risk factors such as abortion and handling of aborted fetus or placenta and infected animals may increase the chances of getting *Brucella* infection. Our results also elaborated that animals artificially inseminated were more infected than those bred naturally. This could be due to unhygienic handling by veterinary professionals of

materials like AI rods or semen contents being infected. Therefore, special awareness programmes are needed for professionals to ensure hygienic handling of animals. Furthermore, semen screening should be screened and tightly regulated.

Our results indicated a higher incidence of zoonotic brucellosis in veterinary professionals (30% by SPAT and 18% by PCR) and animal handlers (24% by SPAT and 12.3% by PCR). In contrast, our previous report from Kuram Agency of Pakistan, which is located quite close (~100 miles) to Bannu District, documented the incidence of brucellosis in human at 4.5% (Abdul Qadeer et al., 2017). Similarly, a significantly lower (3-5%) level of incidence was reported by other authors (Apan TZ et al., 2007; Ahmad R and Naz N, 1999). Taken together, our results indicate the alarmingly high seroprevalence of brucellosis and simultaneous higher occurrence of brucellosis in Bannu District, Pakistan.

Conclusion

Bannu District is located in the eastern side of Khyber Pakhtunkhwa, Pakistan with people highly dependent on dairy animals like cows. Brucellosis is highly contagious and is a zoonotic disease of public health and veterinary health importance. The status of brucellosis is unknown. The present study reports the seroprevalence and incidence of brucellosis among dairy animals, their handlers and veterinary professionals. The higher level of seroprevalence and incidence is a matter of concern. Comprehensive surveillance is imperative and an immediate urgent action should be taken to control and eradicate the disease in parallel with mass education campaign.

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***Conflict of interests*:** Nothing to declare

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บทคัดย่อ

อุบัติการณ์ของโรค บรูเซลโลซีส ในสัตว์แท้งและสัตวแพทย์ ในเขตแบนนู แคว้นไคเบอร์ปัคตูนควา ประเทศปากีสถาน

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โรคบรูเซลโลซีสในสัตว์เป็นปัญหาด้านสุขภาพทั่วโลก เนื่องจากเป็นโรคสัตว์สู่คน และส่งผลต่อความสูญเสียทางเศรษฐกิจ โดยมีรายงานของโรคบรูเซลโลซีส ในหลายภาคของประเทศปากีสถาน อย่างไรก็ตามในเขตแบนนู ประเทศปากีสถาน ยังขาดข้อมูลอุบัติการณ์และระบาดวิทยาของโรคในคนและสัตว์ การศึกษาครั้งนี้ ได้หาอุบัติการณ์ของโรคบรูเซลโลซีส ในสัตว์เลี้ยงและการติดเชื้อข้ามมายังคนที่มีอาชีพกลุ่มเสี่ยง ในพื้นที่เขต แบนนู แคว้นไคเบอร์ปัคตูนควา ประเทศปากีสถาน โดยเก็บตัวอย่างซีรัม จำนวน 223 ตัวอย่าง (สัตว์ = 100 คนสัตว์เลี้ยง = 73 สัตวแพทย์ = 50 คน) และตรวจตัวอย่างด้วยวิธี Serum plate agglutination test (SPAT), Rose Bengal plate test (RBPT) และ polymerase chain reaction (PCR) ที่จำเพาะต่อเชื้อ *Brucella melitensis* และ *B. bovis*. ผลการศึกษา พบอุบัติการณ์ของเชื้อ 27% (27/100) โดยวิธี SPAT, 10% (10/100) โดยวิธี RBPT และ 11% (11/100) โดยวิธี PCR และแบ่งเป็นอุบัติการณ์ของโรคในสัตว์ 24.6% (18/73) โดยวิธี SPAT, 6.84% (5/73) โดยวิธี RBPT และ 12.3% (12.3% (9/73) โดยวิธี PCR ในขณะที่อุบัติการณ์การติดเชื้อในคนคิดเป็น 30% (15/50) โดยวิธี SPAT, 4% (2/50) โดยวิธี RBPT และ 18% (9/50) โดยวิธี PCR และพบว่าปัจจัยที่เกี่ยวข้อง เช่น อายุ เพศ การนำสัตว์ใหม่เข้าฝูง การสัมผัสกับสัตว์ที่ติดเชื้อ วิธีการผสมพันธุ์ และสุขอนามัย มีความเกี่ยวข้องกับอุบัติการณ์การเกิดโรค บรูเซลโลซีสอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) โดยสรุปโรคบรูเซลโลซีลเป็นภัยคุกคาม ต่อสุขภาพของประชาชนในเขตแบนนู ประเทศปากีสถาน ดังนั้นการจัดการที่เหมาะสมและ ให้ความรู้เป็นสิ่งจำเป็น

คำสำคัญ: บรูเซลโลซีส PCR ปัจจัยเสี่ยง ผู้เลี้ยงสัตว์ อุบัติการณ์

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