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RESEARCH ARTICLE

Cross sectional epidemiological investigations of human brucellosis in pregnant women of Punjab, Pakistan

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ABSTRACT

Human brucellosis is an acute febrile illness responsible of causing serious threats to pregnant women and their developing fetus. It is a neglected disease having zoonotic potential resulting in variety of complications. The present study aimed to detect sero-prevalence of brucellosis in pregnant women and to find out the role of various demographic and potential risk factors associated with this disease during January to July 2024. In this cross-sectional study (n=300) blood samples were collected from pregnant women ranging from 18 to 45 years of age. The information about personal data, demographics and risk factors was gathered via pre-designed structured questionnaire. The anti-*Brucella* antibodies were detected using Rose Bengal Plate test (RBPT) followed by enzyme linked immunosorbent assay (ELISA). The seropositive samples were subjected to qRT-PCR for molecular detection of *Brucella*. The data was subjected to descriptive statistics, Chi square test and Odds ratio using Minitab version 18. The result of study showed the sero-prevalence of brucellosis 9.33% among pregnant women with higher 11.36% in age group (18-28 years). Among the demographic factors, the occupation of farming (P =0.005; OR = 3.51, 95%CI: 1.56–7.85) and absence of education (P = 0.001; OR = 7.20, 95%CI: 2.79-18.59) showed significant association with human brucellosis. Additionally, of the potential risk factors analyzed, keeping animals at home (P =0.001; OR = 4.64, 95%CI: 2.05–10.50), and lack of knowledge about brucellosis (P =0.047; OR = 3.53, 95%CI: 1.03-12.03) were found statistically significant. A comprehensive awareness should be given to females regarding risk factors and spread of brucellosis. Consumption of pasteurized dairy products and adopting personal protection while dealing with animals will prevent pregnant women and their unborn from human brucellosis and its complications.

Keywords: Human brucellosis; pregnant women; risk factors; Pakistan.

INTRODUCTION

Brucellosis is a serious communicable disease of zoonotic origin affecting both humans and animals alike caused by member of genus *Brucella.* It has a feature to multiply inside the host macrophages, dendritic cells and usually restricted to the reproductive organs of its animal host resulting in abortions and infertility (Gonzalez *et al*., 2008). There are four species which are reported pathogenic to humans including *B. melitensis*, *B. abortus*, *B. canis* and *B. suis* that can be transmitted from goats, cattle, dogs and swine respectively. Among these, *B. melitensis* is most pathogenic agent in humans and animals (Zhang *et al*., 2024).

Human brucellosis is transmitted either by contacting the infected animals or by consuming contaminated animal products such as unpasteurized milk, cheese, butter, meat and its products (Coelho *et al*., 2015). The exhales and excreta of infected animals also serve as a vehicle for transmission of this bacterium to human beings (Lapaque *et al*., 2006). The transmission between humans is negligible (Godfroid *et al*., 2005). *Brucella* is shedded in large amount in body fluids including milk, placental fluid, semen and urine (González-Espinoza *et al*., 2021). Brucellosis is a solemn occupational hazard for slaughterer house workers, animal handlers, farmers, veterinarians and laboratory personnel, who experiences close proximity with animals (Pappas *et al*., 2005).

Human brucellosis alternatively known as Undulant fever, Malta fever and Mediterranean fever shows variety of clinical manifestations such as chills, headache, profuse sweating, intermittent fever, splenomegaly, hepatomegaly and arthralgia. Ignored cases lead to osteomyelitis, arthritis, orchitis and epididymitis (Attard *et al*., 2018). Clinical obstacles recorded commonly are intrauterine fetal death, premature delivery and abortions. Infants born to *Brucella* positive mothers may develop meningitis and aspiration pneumonia (Cacace *et al*., 2013; Bosilkovski *et al*., 2020). The adverse obstetric complications accredit disseminated intravascular coagulation (DIC), placentitis and maternal bacteremia followed by release of endotoxins which increase the frequency and intensity of uterine contractions quite similar in the action as Oxytocin do on smooth muscles (Aydın *et al*., 2013).

Brucellosis is still among the neglected diseases in Pakistan due to scarcity of surveillance data, lack of awareness about infectious diseases and effective control measures. Majority of the rural population in Pakistan depends on livestock having direct contact with animals. Additionally the low level of literacy rate and very little knowledge about transmission of brucellosis among farmers and staff of livestock farms making brucellosis a public health concern (Saddique *et al*., 2019; Jamil *et al*., 2021).

In Pakistan, nearly 3.8 million abortions occurs during 2023 among the women aged between 15 to 49 years and number of abortions directly attributed to brucellosis is not clear (Sathar *et al*., 2025) and there is very limited surveillance data available on the epidemiology of human brucellosis among women having pregnancy and gynecological complications in Pakistan. Keeping in consideration the public health threat and above stated facts, this study was designed to find out the prevalence of anti-*Brucella* antibodies among pregnant women and to identify risk factors associated with human brucellosis in Punjab, Pakistan. This research will serve to provide important insight into the factors that contribute towards the spread of disease.

MATERIALS AND METHODS

Ethical consideration

The present study was approved on 11 January 2024 under code GCUF/ERC/24/01A by Institutional Ethical Review Committee.

Study design, setting and data collection

This was a cross-sectional epidemiological study conducted on (n=300) pregnant females visiting various hospitals in Faisalabad Division where gynecological care was provided during January to July 2024. The females included in this study were apparently healthy, pregnant, visiting the hospital for their routine antenatal care and willing to participate in the study. No data regarding the females unwilling to participate in the study was included to guarantee their privacy. A questionnaire (closed-ended) was designed and distributed to each woman before sample collection containing dichotomous and multichotomous questions. Data related to demographic factors (age, area, education, occupation, socioeconomic status, pregnancy trimester and previous abortion history) and potential risk factors for human brucellosis (animals at home, consumption of raw milk, knowledge of brucellosis and high risk group) was collected prior to sample collection to find out their association on the transmission of brucellosis. The socioeconomic status is divided into three categories, low (5000-65000PKR/month income), middle (65000-265000 PKR/month income) and high (>265000 PKR/month income). In case of an illiterate woman, an educated and trained medical staff filled the questionnaire on her behalf.

Sample size, procedure and collection

A total of 300 blood samples were collected from pregnant females visiting for their antenatal checkup of Punjab, Pakistan. The sample size estimation was performed by consulting the formula mentioned by (Thrusfield, 2007) keeping in consideration the expected prevalence 23% and desired precision level 5% (Ejaz *et al*., 2024). A non-probability convenience sampling approach was targeted for this study. The blood sample was collected after earning the consent from the female participants which was obtained after clarifying them the study objectives.

About 3-5ml of blood was collected from each woman aseptically from cubital vein by a trained medical laboratory staff in a gel and clot activator tube which was labeled using identification codes, location and date. After blood collection and labeling, tubes were transported to serology section of laboratory and centrifuged at 3000 rpm for 10 mins to separate serum. The serum samples were stored in refrigerator till further processing.

Sample processing

The separated sera samples were subjected to Rose Bengal Plate Test (RBPT) for screening of anti-*Brucella* antibodies and confirmation was done through Enzyme linked immunosorbent assay (ELISA). All the samples showing positive results for both tests were considered as positive because of the discrepancy in the sensitivity and specificity of both tests. The reported sensitivity of RBPT is 89% and false negative results can be observed during the acute phases of the disease. In comparison the sensitivity of ELISA is 98% having highest positive rate in all stages of infection. Therefore, the utilization of both screening and confirmatory tests is more accurate in determining the infection status (Ahmed *et al*., 2023: Xu *et al*., 2023; Barkay *et al*., 2024).

Rose Bengal Plate Test

Firstly all the serum samples, Rose Bengal antigen, and control sera (positive and negative) were equilibrated at room temperature. The positive and negative control serum was procured from national reference laboratory of brucellosis, Friedrich-Loeffler-Institute, Germany. After that equal amounts of serum and antigen (30 µL) were mixed gently with sterile applicator stick on a clean glossy white ceramic tile for 4-5 mins. The serum was considered positive, if visible agglutination appeared (Morgan *et al*., 1969).

Enzyme Linked Immunosorbent Assay

The serum samples detected positive by RBPT were subjected to ELISA for confirmation of anti-*Brucella* IgM antibodies (NovaLisa, GmbH, Germany). Firstly the serum samples were diluted followed by addition of test serum, positive and negative controls in pre-coated allocated wells of ELISA plates. After one hour of incubation at 37°C, washing of wells was performed. The wells were then dispensed with rabbit anti-human IgM antibodies coupled with horseradish peroxidase enzyme and incubated for another one hour. After this 2nd washing of wells, a substrate (Tetramethylbenzidine) was added followed by a dark incubation of 15-20 minutes. At the end, stop solution was dispensed in the wells and values were recorded with the help ELISA reader (BioRad, USA) at 450nm (Alrodhan, 2017).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Assay

The DNA of all the seropositive blood samples was extracted using FavorPrep blood DNA extraction mini kit according to the introductions and methodology described by manufacturer. The quantification of extracted DNA was performed using a Nanodrop UV spectrophotometer (Thermo, USA) by measuring absorbance at 260 nm (Ejaz *et al*., 2024). The quantitative real-time PCR (qRT-PCR) was done for the detection of genus-specific *BCSP-31* gene of *Brucella.* The targeted gene, primer sequence and PCR thermal conditions was adopted as previously demonstrated by (Hassan *et al*., 2022).

Statistical Analysis

The data was gathered from questionnaires in Microsoft Excel sheet. A Chi-square test and odds ratio with 95% confidence interval were also determined by univariate logistic regression analysis. The value $p< 0.05$ was considered as significant (García-Valle *et al*., 2024).

RESULTS

A total of 300 pregnant females participated in this study and tested for anti-*Brucella* antibodies among which 28 were detected positive making an overall sero-prevalence of 9.33%. The samples of seropositive (n=28) women were subjected to qRT-PCR and *Brucella* DNA was detected among 19 (67.85%) females. It was found that females ranging between 18-28 years showed higher sero-prevalence of brucellosis (11.36%) than females ranging between 29-38 years (7.69%) while none of the female of age above 38 found seropositive for brucellosis.

The sero-prevalence of human brucellosis among pregnant females based on their educational status showed that the education (Secondary or above) was inversely linked with brucellosis. It was recorded highest among illiterate/uneducated females (25.80%) while lowest among those who were educated (4.60%) [OR= 7.20, 95% CI= 2.79-18.59]. On the basis of occupation, farmers women were found more prone to *Brucella* infection 21.62% in comparison to employed 7.05% and house wives 0% [OR= 3.51, 95% CI= 1.56-7.85]. These results were statistically significant (P<0.05) as shown in (Table 1).

This study revealed that regarding the residential location, the prevalence of anti-*Brucella* antibodies in serum was detected 10.52% in rural and 8.10% in urban pregnant residents. A non-significant relation of brucellosis was found with residency (P=0.512) as well as socioeconomic status (P=0.145). In the current study the sero-prevalence of human brucellosis in pregnant women was found highest in third trimester 10.17% followed by second trimester 9.83% and first trimester 6.66%. Similarly the females having history of miscarriages showed raised sero-prevalence 11.59% in contrast to those who did not have previous miscarriages 7.40%. Both of these results were found non-significant (P> 0.05) as shown in (Table 1).

The association of potential risk factors with human brucellosis was also detected in this study and it was found that females having animals in their homes showed higher (19.14%) sero-prevalence of brucellosis in contrast to those who did not have animals in their homes 4.85% [OR= 4.64, 95% CI= 2.05-10.50]. The knowledge of brucellosis is also considered a prominent factor in *Brucella* infection and it was observed that females having knowledge of brucellosis showed much lower sero-prevalence of brucellosis 3.57% in comparison to those who don't have any knowledge about the disease [OR= 3.53, 95% CI= 1.03-12.03]. These findings were found statistically significant (P< 0.05). A non-significant result was observed between human brucellosis and consumption of raw milk and belonging to risk group (P> 0.05) as shown in (Table 2).

Table 1. Sero-prevalence of brucellosis in pregnant women on the basis of demographic factors

Variables	Total Sampled	Total Positive (%)	Chi Square Test (P Value)	Odds Ratio	95% CI
Age					
18-28 Years	176	20 (11.36)			
29-38 Years	104	08 (7.69)	0.368	1.53	0.65–3.63
>38 Years	20	00 (00)			
Residency					
Rural	152	16 (10.52)	0.512	1.33	0.60–2.92
Urban	148	12 (8.10)			
Education					
Uneducated	62	16 (25.80)			
Primary	86	05 (5.81)	**0.001**	**5.63**	**1.93–16.38**
Secondary & Above	152	07 (4.60)		**7.20**	**2.79–18.59**
Occupation					
Farmer	76	16 (21.05)			
Employed	170	12 (7.05)	**0.005**	**3.51**	**1.56–7.85**
House wife	54	00 (00)			
Socioeconomic status					
Low	168	12 (7.14)			
Middle	126	16 (12.69)	0.145	1.89	0.86–4.15
High	06	00 (00)			
Trimester					
First	60	04 (6.66)			
Second	122	12 (9.83)	0.763	1.58	0.48–5.14
Third	118	12 (10.16)		1.03	0.44–2.41
Miscarriage history					
Yes	138	16 (11.59)			
No	162	12 (7.40)	0.258	1.63	0.74–3.59

Table 2. Sero-prevalence of brucellosis in pregnant women on the basis of risk factors

Risk Factors	Total Sampled	Total Positive (%)	Chi Square Test (P Value)	Odds Ratio	95% CI
Animals at home					
Yes	94	18 (19.14)	**0.001**	**4.64**	**2.05–10.50**
No	206	10 (4.85)			
Consuming raw milk					
Yes	92	10 (10.87)	0.581	1.28	0.56–2.90
No	208	18 (8.65)			
Knowledge of Brucellosis					
Yes	84	03 (3.57)	**0.047**	**3.53**	**1.03–12.03**
No	216	25 (11.57)			
Risk Group					
Yes	38	04 (10.52)	0.806	1.16	0.38–3.56
No	262	24 (9.16)			

DISCUSSION

Brucellosis is a neglected infectious disease which infects both human and animals. In Pakistan it is endemic in ruminants having a zoonotic potential (Nawaz *et al*., 2021). As being neglected disease, very few studies have been conducted on brucellosis particularly in pregnant females who are considered to be at exceptional risk of abortion.

In the present study, 9.33% of pregnant Pakistani women were found to be seropositive for brucellosis which is elevated than the results of (5.8%) in Pakistan (Ali *et al*., 2016), and lower than the findings of (10.9%) in Tanzania (Makala *et al*., 2020), (11.25%) in Nepal (Thapa & Maharjan, 2018) and (17.4%) in Nigeria (Folagbade *et al*., 2017). This variation in the sero-prevalence of brucellosis in pregnant females is due to the difference in their habits including contact with animals, consumption of unprocessed animal food, level of knowledge regarding infectious zoonotic diseases and livestock population of that area. The age group found most vulnerable for human brucellosis in pregnant women was 18-28 years with 11.36% which coincides with the finding of (Abdullah *et al*., 2018) from Yemen, (Makala *et al*., 2020) from Tanzania and (Thapa & Maharjan, 2018) from Nepal. The findings of (Ahmadi *et al*., 2017) in Iran and (Kledmanee *et al*., 2019) in Thailand also support the fact that middle aged pregnant females are at more risk of acquiring brucellosis. The reason behind is that females of age 20-40 years are sexually active as well as prominently participating in livestock related activities including milking, washing and feeding of animals.

According to the results of this study, the pregnant women residing in rural areas and serving as farmer or livestock associated jobs showed higher sero-prevalence of brucellosis in contrast to urban residents and house wives or students which is in accordance with previous findings of (Ejaz *et al*., 2024), (Nawaz *et al*., 2021) and (Ali *et al*., 2018) from different localities of Pakistan and (Tumwine *et al*., 2015) in Uganda. This increased prevalence in both groups is due to routine practices such as herding of livestock, birthing of calves, close contact with animals and consumption of raw milk which put them at high risk of getting infected by *Brucella.* The sero-prevalence of brucellosis was also inspected based on the education of females and found inversely associated with education. As the education level increases among the pregnant women, the prevalence declines. This association is in line with the results of (Ali *et al*., 2018), (Madut *et al*., 2018) and (Nawaz *et al*., 2021). These results might be observed because the educated individuals preferably consume pasteurized milk, use proper protection while dealing with animals and have a concept of zoonotic disease transmission.

The detected level of antibodies against brucellosis was higher in women in their 3rd trimester 10.16% in contrast to 2nd 9.83% and 1st trimester 6.66% which is agreement with the records of (Vilchez *et al*., 2015), (Abdullah *et al*., 2018) and (Thapa & Maharjan, 2018) in Peru, Yemen and Nepal respectively. It was also observed that the frequency of *Brucella* seropositivity was more in females with history of miscarriage. Similar results were also recorded by Ali *et al*. (2021), Hassan *et al*. (2022) and Ejaz *et al*. (2024) in Pakistan. Due to limited number of samples and positive cases in all the studies reported from Pakistan, we can only recommend further studies to evaluate these findings because brucellosis can pose a serious risk to newborns.

In the present study, association of certain risk factors with human brucellosis in pregnant women was also evaluated. Keeping animals within home is thought to be one of the major factor linked with human brucellosis and the results revealed that the risk of brucellosis is four times (OR=4.64) more in females having animals at their homes. This fact is supported by some recent findings of (Hassan *et al*., 2022) in Pakistan, (Mwatondo *et al*., 2023) in Kenya and (Ejaz *et al*., 2024) in Pakistan. Similar results were also reported previously by Ali *et al*. (2016, 2018) and Nawaz *et al*. (2021) in pregnant women and general population of Pakistan. The family members and females face a high risk of possible domestic exposure due to the vicinity of animals in residential spaces making them prone to brucellosis because infected animals act as reservoir and shed *Brucella* species in aerosol, saliva, urine and aborted placenta.

Consuming raw milk is also considered a prominent risk factor for human brucellosis. In this study the frequency of brucellosis was recorded higher (10.87%) in females who consumed raw milk and this factor is found non-significantly associated with the prevalence of brucellosis in pregnant women. The higher sero-prevalence of brucellosis among raw milk consumers was profoundly reported by Tumwine *et al*. (2015) in Uganda, Folagbade *et al*. (2017) in Nigeria, Ali *et al*. (2021) in Pakistan and Mwatondo *et al*. (2023) in Kenya. *Brucella* can excreted in urine, semen, saliva and milk of infected animals and consuming unpasteurized milk is direct threat for pregnant women.

A statistically significant association was detected between *Brucella* infection and knowledge of human brucellosis. The sero-prevalence of brucellosis was found 3.5 times more in females who lacks the knowledge of *Brucella* infection (OR= 3.53). These findings are in agreement with results of Tumwine *et al*. (2015) in Uganda, Ali *et al*. (2018) and Nawaz *et al*. (2021) in Pakistan. The knowledge about risk factors and transmission of zoonotic diseases is very much important to curtail the threats of brucellosis and other infectious diseases.

The results of this study showed that 67.85% seropositive females detected positive for *Brucella* DNA by real-time PCR. The qRT-PCR is a reliable, less time consuming, highly sensitive and specific technique for molecular detection but it depends on the availability of specific DNA in the serum sample. So it is preferred to use combination of serological method along with qRT-PCR for accurate, precise and acute phase diagnosis of brucellosis (Yousaf *et al*., 2021).

CONCLUSION

It was concluded from the results of this study that brucellosis is a neglected zoonotic disease and its sero-prevalence is high among pregnant women of Pakistan (9.33%). It is a serious threat and public health concern for pregnant women, their unborn and newborns. The study highlights that pregnant women having animals in their homes and direct contact with animals (milking, washing and feeding) are at significantly higher risk of acquiring brucellosis. Lack of education, consuming unpasteurized dairy products and absence of knowledge about brucellosis are the important risk factors associated with brucellosis in pregnant women. Awareness regarding vital risk factors, pasteurization of dairy products, personal care while dealing with animals and vaccination of animals is recommended for effective control of this disease.

Conflict of interest

The authors declare that they have no conflict of interest.

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