Application of Rose Bengal Test and ELISA in Meat Juice for Monitoring of Brucellosis among Cattle Carcasses at Erbil City, Iraq

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Abstract

Brucellosis is a global zoonotic bacterial disease. It is also an ever-increasing public health concern, particularly in endemic regions such as Kurdistan region in Middle East. Samples of meat juice were screened for anti-*Brucella* antibodies via Rose Bengal Test (RBT) and ELISA from January to June 2018. Bacteriological isolation of *Brucella* sp. from meat samples was also performed. The overall prevalence of bovine brucellosis is 7.7%, 6.6%, and 4.9% by RBT, ELISA, and culture-based test respectively. Based on sensitivity and specificity, ELISA outperforms RBT in comparison to culture results as the gold standard test. Nonetheless, both tests showed good efficiency in comparison to culture approach. In terms of temporal changes of brucellosis rate, spring progress is strongly associated (r^2=0.96) with increase in seroprevalence. In conclusion, the prevalence of bovine brucellosis in Erbil city is alarming. Countermeasures should be taken to mitigate the economic losses and transmission to human.

Keywords: *Temporal changes, Brucella abortus, Brucella melitensis, Sensitivity, Specificity*

Introduction

Brucellosisis a highly contagious global zoonotic disease caused by Brucella sp. that have the ability to invade macrophages, dendritic cells, epithelia, and placental trophoblasts (de Figueiredo et al., 2015).This bacterial genus comprises different species that have wide range of animal hosts which facilitates the spread of the disease among humans as well as different domestic and wild animals (Ghaderinia & Shapouri, 2017). *Brucella* infection (Brucellosis) is mainly an infection offood-producinganimals including large and small ruminants. It is recognized by the World Organization for Animal Health (OIE) as a class B animal epidemic (Casalinuovo *et al.*, 2016; Addis and Desalegn, 2018; AL-mashhadany, 2018a).

Different species of *Brucella* tend to be host-specific.Brucellae aregram-negative coccobacilli with straight or slightly curved, facultative intracellular,nonspore forming, non-motile, and non-capsulated cells (Waringa *et al.*, 2016; Patel *et al.*, 2017). Out of twelve species, the most implicated agents of brucellosis are *B.abortus, B. melitensis, B. bovis, B.canis, and B.suis* (Scholz *et al.*, 2016; Sabrina *et al.*, 2018).

Brucellosis has adverse effects on animals and humans as well as economic implications for individuals and communities. Indeed, brucellosis can inflict high economic costs in terms of medical tests, treatment, and worker absence, which makes it also a considerable social problem particularly in developing nations. It also induces fear in the community, which can negatively affect the global trade of meat, milk, and their products (Mufinda *et al.*, 2017; Raghava *et al.*, 2017).

The transmission of brucellosis to humans occurs through different ways. Ingestion of contaminated raw or undercooked meat from infected animals and unpasteurized milk or

milk products are the most common routes of infection acquisition. Transmission also occurs through skin wounds or mucous membranes following direct contact with animal-derived contaminated materials (de Figueiredo *et al.*, 2015). Respiratory, genital, and mother-to-infant transmission is also reported (Najum, 2014;Ali *et al.*, 2015; AL-Shemmari, 2018).

Epidemiologically, World Health Organization (WHO) estimates that 500,000 new cases occur annually, however, this value is expected to represent one-fifth of all cases (Khan *et al.*, 2001; Raghava *et al.*, 2017) with high prevalence in countries of the Mediterranean basin, the Middle East including Iraq, Arabian Peninsula,Africa,Asia, Central and South Americas(CDC, 2017; AL-mashhadany, 2018b).Recently, Jaff (2016) reported that the prevalence of human brucellosis among governorates of Kurdistan region is higher than recorded from adjacent countries.The study also reported occurrence rates of 6.36% in 2011 in Dohuk, 10.7%in Erbil city in 2012, and an annual incidence rate of 976 cases per 100,000 of population were recorded in Sulaymaniyah governorate in 2013.

Nationwide studies of bovine brucellosis in developing countries are scarce. In fact, prevalence of brucellosis in livestock in many developing countries is alarming as reflected by the high numbers documented in epidemiology reports of the past decade. Cattle sero prevalence estimates have been observed to range between 3% and 15% (Godfroid *et al.*, 2011 and references therein). For instance, in Bangladesh, the overall bovine prevalence is 3.7% (2.1%-66% confidence interval) (Islam *et al.*, 2013), while in Egypt, according to the General Organization of Veterinary Service (GOVS), the prevalence of bovine brucellosis dropped from 1.27% in 2002 to 0.35% in 2011 (Wareth *et al.*, 2014).

Since the early appreciation of *Brucella* as the causative agent of brucellosis, a number of serological diagnostic tests were developed. Serological tests are generally divided into three categories: (i) the classical or conventional tests [such as RBT, Complement Fixation (CF), Milk Ring Test (MRT)], (ii) primary binding assays [such as Radioimmunoassay and ELISA] and (iii) developing technology [PCR assays] (best reviewed in Godfoid *et al.*, 2010; Poester *et al.*, 2010).Little is known about the role of meat and its products in transmission of *Brucella* to human. Therefore, the objectives of this work are to determine the occurrence of *Brucella* antibodies and *Brucella* speciesin red meat samplesfrom markets of Erbil City. The sensitivity, specificity and total efficacy of RBT and ELISA in comparison to traditional culture-based approach was also investigated.The relationship between the prevalence of anti-*Brucella* antibodies in red meat with months during the period of study was also addressed.

Materials and Methods

1- Study Design and Sampling

Three hundred and fifty (350) meat samples were collected from cattle carcasses during the period from January to June 2018.Sample units comprise 115 thigh muscles, 125 lumber lymph nodes (L.Ns), and 110 shoulder musclesfrom different retail butchers in Erbil city.Each sample weighted about 50-100 grams separately collected in sterile polyethylene bags and rapidly transported to Laboratory of Microbiology, Pathological Analysis Department, Knowledge University, Erbil City in ice box with a minimum delay. Upon arrival, samples were immediately deep-frozen and stored at -20°C up to one week.

2- Preparation of Meat Juice

According to Wallander *et al.*, (2015), after one week of deep freezing, samples were thawed at 20-25ºC. About 2-5 ml of meat juices were collected in an Eppendorf tube and separated by centrifugation into two parts; for RBT and ELISA assays.

3- Detection of *Brucella* antibodies

A- Rose Bengal Test (RBT)

Equal quantities of antigen and meat juice sample were mixed on a clean slide by a stirring stick. The slide was manually rotated for 3-4 minutes and inspected for any level of agglutination. When observed, the RBT was considered positive.

B - ELISA

The ELISA tests were performed according to a published protocol.

4- Isolation of *Brucella*

The isolation of *Brucella* from meat samples was done under sterile conditions at the Microbiology Laboratory, Pathological Analysis Department, following standard procedures

Table1. Occurrence of *Brucella* antibodies among meat juice according to RBT and ELISA tests.

Type of meat	No. Examined	Positive samples n (%)	
		RBT	ELISA
Thigh muscle	115	9 (7.8)	7 (6.1)
Lumbar L.Ns[1]	125	12 (9.6)	11 (8.8)
Shoulder muscle	110	6 (5.5)	5 (4.5)
Total	**350**	**27 (7.7)**	**23 (6.6)**

[1]L. Ns; Lymph nodes.

Table 2. Isolation of *Brucella* species from different types of meat.

Type of meat	No. examined	Positive isolates n (%)	Isolates of *Brucella* species n (%)	
			B. abortus	*B. melitensis*
Thigh muscle	115	5 (4.3)	3 (60.0)	2 (40.0)
Lumbar L.Ns	125	9 (7.2)	6 (66.7)	3 (33.3)
Shoulder muscle	110	3(2.7)	2 (66.7)	1 (33.3)
Total	**350**	**17(4.9)**	**11 (64.7)**	**6 (35.3)**

(Corbel *et al.*, 2006).The plates of Brucella agar (HiMedia, India) were incubated aerobically and in the presence of 5%–10% carbon dioxide at 37°C up to 10 days with daily inspection for the presence of bacterial growth.

5- Identification of *Brucella*

The identification of *B. abortus* and *B. melitensis* were confirmed by traditional biochemical tests (Tille, 2018).

6- Sensitivity and Specificity of RBT and ELISA

The sensitivity and specificity of RBT and ELISA were calculated using the bacterial isolation diagnostic method as the gold standard.

7- Statistical analysis

Data were analyzed using the SPSS software version 21.Confidence intervals of prevalence were estimated using normal distribution approximation at alpha level of 0.05. Confidence intervals for sensitivity, specificity and accuracy are "exact" Clopper-Pearson confidence intervals.

Results

Seroprevalence of Brucellosis

Of the 350 meat samples, 27 (7.7%) were positive by RBT assay. The result shows that the highest rate of *Brucella* antibodies was found in lumber lymph nodes (9.6%), while the lowest occurrence rate of *Brucella* antibodies was in shoulder muscles (5.5%). On the other hand, the overall prevalence of *Brucella* antibodies in different types of meat samples according to ELISA is 6.6%. The highest rate of occurrence of *Brucella* antibodies was found in lumbar L.Ns. 8.8% (Table1).

Detected *Brucella* species

In terms of culture-based diagnosis, it was found that only 4.9% of the samples were culture-positive for *Brucella* isolates. It's obvious that *B. abortus* is more prevalent than *B. melitensis* among the evaluated meat samples. The distribution of the isolated *Brucella* spp.in various meat samples is summarized in Table 2. It should be noted that approximately 53% (9/17) of *Brucella* isolates were recovered from lymph nodes samples.

Assessment of RBT and ELISA Performance

Compared to traditional culture approach, RBT has a good specificity (97%) but low sensitivity (62.9%). On the contrary, the ELISA test has higher specificity and sensitivity (Table 3). Despite low sensitivities of both RBT and ELISA, their accuracy (efficiency) is high (94% and 97.6%) in comparison to the results of culture method.

Temporal changes in *Brucella* seroprevalence

For a period of six months, the highest rate of seropositive samples was found in June 7/60(11.7%), while the lowest rates were found in March3/58(5.2%) and January 3/ 60 (5.0%). A

Table 3. Comparison between RBT and ELISA capabilities in detecting brucellosis in meat juice samples

Test	Rose Bengal test (95% CI)	ELISA (95% CI)
Sensitivity	62.9%(42.4-80.6)	71.4% (29-96.3)
Specificity	97% (94.7-98.6)	98.2% (96.2-99.3)
PVP	62.9% (46.4-76.9)	45.45%(24.9-67.6)
PVN	97.1%(95.3-98.2)	99.4%(98.1-99.8)
Efficiency	94.6%(91.8-96.7)	97.6% (95.4-98.9)

Bacteriological culture approach is the gold standard for evaluation.

Table 4. Relationship between Months and Prevalence of *Brucella* antibodies (RBT) during period of study.

Month	Total Examined	Positiven (%)
January	60	3(5.0)
February	57	5 (8.8)
March	58	3(5.2)
April	60	4 (6.7)
May	55	5 (9.1)
June	60	7 (11.7)
Total	**350**	**27 (7.7)**

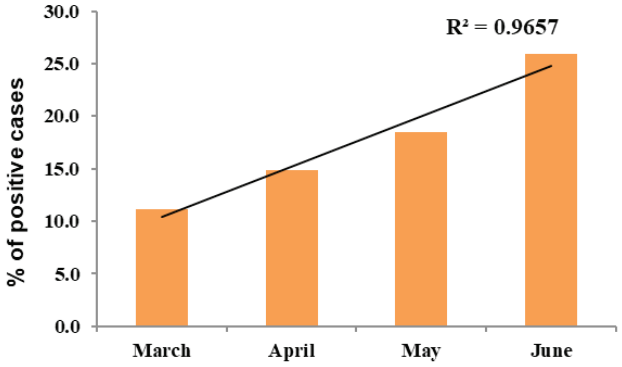

Figure 1. Correlation between spring and seroprevalence of bovine brucellosis

strong correlation (r^2= 0.96) was found between spring progress (March to June) and increased seropositivity (Fig.1). Based on this sample, 4 - 5.2% of meat samples in Erbil markets will be positive for RBT (95% CI).

Discussion

Brucellosis is mainly a disease of food producing animals such as cattle, buffalo, camels, sheep, goats, swine, but transmission to humans occurs in several ways most commonly by foodborne route(AL-mashhadany, 2018a; Islam *et al.*, 2018;Zheng *et al.*, 2018).Accurate and rapid detection of brucellosis is crucial for both health and economic purposes. Bacteriological isolation of brucellae from tissues and milk specimens is the only mean of definitive diagnosis of bovine brucellosis. However, culture-based diagnosis is not always feasible especially in field or in slaughterhouses. Alternatively, other serological tests were developed and put in practice. Each test has its own advantages and disadvantages which render the test more or less adopted (Poester *et al.*, 2010).Rose Bengal Test (RBT) is commonly used to detect anti-*Brucella* antibodies in the blood due to its low cost and simplicity. Meanwhile, meat juice can be used in serological assays to monitor the presence of infectious diseases, particularly brucellosis within the food chain (Hammed,1996;Szulowski *et al.*, 2000; Wallander *et al.*, 2015).

According to the available literature, this study seems to be the first in Kurdistan region, Iraq, to address the screening of *Brucella* sp. in cattle meat. The study has documented a high prevalence of brucellosis in various types of cattle meat samples via RBT and ELISA tests (Table 1).The overall prevalence of *Brucella* antibodies in juice from all collected meat samples according to RBT was 7.7%(27/350), which is slightly lower than the rate foundin Italy (9.3%) and in Egypt (11.1%) according to RBT (Casalinuovo *et al.*, 2018; Salem *et al.*, 2014). However, according to ELISA, *Brucella* seroprevalence documented in the present study is 6.6% (23/350).Yet, a lower prevalence of brucellosis in Pakistan was reported to be 3% by RBT and 3.2% by i-ELISA (Shafee *et al.*, 2012). In contrast, in sub-Saharan region of Africa, a higher rate of *Brucella* seroprevalence in cattle was estimated to be 16.2% (10.2 - 25%,95% CI) according to RBT (Mangen *et al.*,

2002). Furthermore, a higher prevalence rate was reported in an Indian serological study (Trangadia *et al.*, 2009).They also showed that ELISA detected higher rates of brucellosis in cattle than RBT. The prevalence in different regions ranged from 6.2% -56.4% according to ELISA. On the other hand, the prevalence according to RBT ranged from 42.3% - 2.7%.

In terms of evaluation of employed serological tests, ELISA assay outperforms RBT (Table 3). This finding is in good agreement with previous reports (Konstantinidis *et al.*, 2007; Weiner *et al.*, 2010). Indeed, RBT has been reported to produce false positive results in response to *Yersinia enterocolitica* or *Leptospira* infections (Mainar-Jaime *et al.*, 2005; Munoz *et al.*, 2005). Nonetheless, another study claims that serum agglutination, Coombs, competitive ELISA, Brucellacapt, lateral flow immunochromatography, immunoprecipitation, and ELISA did not outperform RBT in screening of brucellosis (Díaz *et al.*, 2011).However, a recent study of brucellosis in cattle found RBT and ELISA to be the most suitable diagnostic sero tests due to their high sensitivity and specificity (Matope *et al.*, 2011).

Meat juice samples were found equivalent to sera as a material for ELISA-based diagnosis but such high correlation in results was obtained for samples from hares rather than cattle (Szulowski *et al.*, 2000), consequently, a cautious view should be held in generalizing the findings.

The overall isolation rate for brucellae from different meats is4.9% (17/350) (Table 2). The finding of about 53% (9/17) of *Brucella* isolates were from lymph nodes sample is consistent with *Brucella* tendency to invade macrophages which are abundant in lymph nodes as a part of the immune response to the infection (de Figueiredo *et al.*, 2015).The abundance of brucellae in samples of lymph nodes was also reported in Egypt (Ali and Mahdey, 2010) and in Iran (Faham *et al.*, 2014). The same study also showed that *B. melitensis* was isolated from edible offal of serologically positive slaughtered cattle at Beni- Suef slaughterhouse constituting 40%, 50%, 15%, 20%, 15% and 50% for Liver, Spleen, Lung, Kidney, Heart and lymph nodes respectively. Moreover, nearly analogous findings were reported by El Nesser *et al.*, 2007. Differences in frequency of Brucellae isolation from various organs and from several species may be attributed to the alterations in stage of infection as well as the efficiency of microbiological methods used for isolation of the organism.

Szulowski and colleagues (2013) in Poland, studied the bacteriological investigations of cattle slaughtered during period 2002 – 2011. Serum and tissues samples from 176 cows were examined. All sera were RBT and SAT positive, while in bacteriological examination, *B. abortus* was not isolated, *Brucella suis* biovar 2 were was isolated from 5 cows. Furthermore, Faham and associates (2014) in Iran reported that 11.38% and 13.01% of blood and Lymph nodes samples were positive for *Brucella* species. The study also indicated that 3.2% and1.6% of camel lymph nodes samples were positive for *B. abortus*and *B. melitensis* respectively.

The temporal distribution of seropositive meat samples from cattle carcasses (Table 4) shows strong association with spring progress (Figure 1).The possible cause of gradual increase in the seropositive rates of brucellosis in meat samples is still difficult to draw. First, the documented rates during study period are based on RBT test that could give false positive results. However, if the infection occurred previously in latewinter, subsequent gradual increase of antibodies during spring may account for the increase of serpositivity rate despite external environmental factors. Indeed, the real cause(s) is not currently known. Another point worth mentioning is that these findings contradict very recent observations of increase in seropositive cases during spring (AL-mashhadany, unpublished data).These observations are likely due to the gradual decrease in temperature, humidity, and rain level in Kurdistan region during months of spring. For instance, wet season was found to be a risk factor for seropositive brucellosis in cattle carcasses (Megersa *et al.*, 2012).Indeed, the decrease in *Brucella* seroprevalence in Erbil among cattle and buffalo populations during spring has been recently foundin tested milk samples(AL-mashhadany, unpublished data).

Conclusion

The prevalence of bovine brucellosis in Erbil city is still high which may pose a serious threat to human health an economic growth. Indeed, transmission to farmers, butchery personnel and consumers of meat is highly expected; consequently, meat should be analyzed before

marketing. Despite being specific, RBT showed a low sensitivity in detecting brucellosis in meat juice samples which may hinder its usefulness as a rapid, straightforward, and affordable screening test. As a result, a search for an equivalent, affordable, and sensitive agglutination test form eat juice is recommended. Lymph nodes are the most samples harbored *B. abortus* and *B. melitensis*. Therefore, special care in proper cooking should be mounted among consumers to prevent transmission to humans. Spring is associated with gradual increase in *Brucella* seroprevalence. Environmental factors (temperature, humidity, etc) that may contribute to the epidemiology of bovine brucellosis are recommended to be investigated.

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