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OCCURRENCE AND EPIDEMIOLOGY OF *BRUCELLA* SPP. IN RAW MILK SAMPLES AT BASRAH PROVINCE, IRAQ

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Summary

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Four hundred and twenty samples of raw cow, buffalo and sheep milk were collected through August 2006 to July 2007 from different sites in the Basrah province of Iraq and tested in the milk ring test to detect *Brucella* antibodies. Positive results were obtained in 24.2% of samples. By means of the enrichment broth technique, 62 *Brucella* isolates, 33 *Brucella abortus* isolates (biotypes 2, 3, 4 and 6); 25 *B. melitensis* isolates (biotypes 2 and 3), and 4 *B. ovis* isolates were recovered. The highest incidence of *Brucella* in raw milk samples was found out in sheep's milk followed by buffalo's raw milk. The prevalence of *Brucella* isolates was high in spring and summer months whereas a lower incidence was found out in cold months. Antibiotic sensitivity tests of the isolates were performed. All *Brucella* isolates showed an ability to grow within the temperature range 18–44 ^{o}C and at pH 4–9.

Key words: *Brucella* spp., buffalo, cow, milk, sheep

INTRODUCTION

Brucellosis is among the most important zoonotic diseases in terms of social and economic impacts. Half a million of new human cases are reported annually worldwide. However, the World Health Organization announced that these numbers greatly underestimate the true incidence of disease and the actual number of cases is estimated to be at least ten times the figures officially announced (WHO, 1997; Seimenis, 2002). Brucellosis has been recognized as a global problem for wild and domestic animals especially cattle, sheep and goats, as it causes decreased reproductive efficiency and abortions (Rijpens *et al.,* 1996). The incidence of human disease is closely tied to the prevalence of infection in animals, as transmission occurs to humans by exposure to infected animals and their infective secreta and excreta during septic abortion, at the time of slaughter and more frequently through consumption of raw animal products, especially milk and dairy products (Wallach *et al.,* 1994; Mater *et al.,* 1996). In Iraq, brucellosis is still endemic among domestic animals and humans in spite of the attempts to control the disease through bilateral projects with some agencies or international organizations (Abed Mohamad*,* 1998). The epidemiology of the disease in the country was studied since the fourth decade of the past century in most animal species like cows, sheep, goats and camels (Al-Zahawi, 1938; Al-Beatti *et al*., 1939). Brucellosis causes considerable economic losses in Iraq and neighbouring countries. The disease might be over-

looked especially in its acute forms (Jamil *et al*., 1989). Therefore, the present study was undertaken to investigate the epidemiological aspects of brucellosis in Basrah province, Iraq.

MATERIALS AND METHODS

Samples

A total of 420 raw milk samples were collected through August 2006 to July 2007 from different sites of Basrah province. Out of these, 120 were cow's, 120 buffaloe's and 180 sheep's milk samples. Milk was collected randomly under sterile conditions. Milk was transported to laboratory in a cooling box (Alton *et al.,* 1988).

Milk ring test

Raw milk samples were stored in a refrigerator at 4 ºC for 12–24 hours. Before the test, samples and the special antigen were removed out of refrigerator and allowed to stand at room temperature for 1 hour. Samples were shaked well and 1 mL was taken in sterilized test tubes, then one drop from the special antigen was added for each tube, shaked and incubated at 37˚C for 1 to 3 hours. The appearance of a blue ring at the upper part of milk pole indicated a positive result (Morgan *et al.* 1978; Alton *et al.,* 1988).

Bacterial isolation and diagnosis

Milk samples were treated according to Brodie & Sinton (1975). A 10 mL sample was centrifuged for 15 min at 4000 rpm. Then, 0.5 mL of each sediment and supernatant were inoculated in two tubes containing 4 mL of brain heart infusion broth (Difco, USA). Tubes were incubated at 37˚C. After 48 h, 0.1 mL of the enriched culture was streaked on *Brucella* selective agar plates (Himedia Labs, India) and incubated at 37˚C for 3–5 days. Plates were observed daily for bacterial growth. Colonies having the characteristics of *Brucella* were subcultured for purification and identification.

Identification and biotyping of Brucella isolates

The bacterial isolates were identified as *Brucella* spp. by means of the following methods: colony morphology and staining; motility test and oxidase test (Paul, 1997); blood haemolysis; lactose fermentation; urease test; catalase test (Cowan & Steel, 1974); gelatin analysis (Merchant & Packer, 1976); indol test (Plazevic & Ederer, 1975).

For the classification of *Brucella* species and biotypes, the following tests were performed: carbon dioxide requirement for growth; hydrogen sulfide production; dye sensitivity test (viz. basic fuchsin 1:50,000 and 1:100,000; thionin 1:25,000; 1:50,000 and 1:100,000). Agglutination with monospecific antisera of *Brucella abortus* (A) and *B. melitensis* (M) were done by the slide agglutination test (Alton *et al.,* 1975; 1988; Shang 1990).

Antibiotic sensitivity tests

Bacterial suspensions were spreaded on nutrient agar using a L-shaped glass and lifted for 5 min. Then the antibiotic discs (Himedia Labs, India) were fixed on the agar plate with a sterilized forceps and incubated at 37 ºC for 24 hours. The zones of inhibition were measured using a caliper.

Effect of pH and temperature on bacterial growth

Bacterial strains were streaked on *Brucella* agar medium with different pH values – 2, 4, 6, 8, 9 and 10 (Phillips *et al.,* 1997). Plates were incubated at 37˚C for 24–48 hour and the growth were observed

daily. The pH was adjusted using NaOH and HCl.

Each strain was streaked on *Brucella* agar medium and incubated for 24–48 hours at different temperatures: 4, 10, 15, 18, 20, 30, 37, 40, 42, 43, 44, 45 and 50 °C. (Alton, 1985). Growth was observed daily and the results were recorded.

RESULTS AND DISCUSSION

The results showed that 24.2% of all 420 collected milk samples were seropositive in the milk ring test (Table 1), with high rates in spring and summer months of the year. This may be attributed to the feeding type in this season which depends mainly on grazing of green stuff as well as to higher calving rates, that create predisposition for pasture contamination by discharges and foetal membranes from infected cow, which is the commonest method of spreading (Radostits *et al*., 2000). Previous studies in different regions of Iraq and other countries revealed that 24.4% of 61 tested goat milk samples and 21.2% from 53 sheep milk samples were positive (Salman, 1997). Shanshal (1999) reported 22.8% positive milk samples from a total number of 250 (obtained from 125 cattle and 125 sheep). Althwyni *et al.* (1995) found out 24.61% seropositive samples of buffaloe's milk, 18.8% – of cow milk and 15.6% – of sheep milk. Al-Rodhan (2005) showed that from 98 milk samples tested in the milk ring test, 10.20% gave positive results .

The result of bacterial isolation from 420 raw milk samples revealed that 62 isolates (14.7%) were obtained, out of these 10% from cow's, 15.8% from buffaloe's and 17.2% from sheep's milk. Among these, 33 isolates (7.8%) were identified as *B. abortus* biotype 2, 3, 4 and 6, 25 isolates (5.9%) – as *B. melitensis* biotype 2 and 3, while 4 isolates (0.9%) were identified as *B. ovis* (Tables 2 and 3). The prevalence of *B. abortus* biotype 3, 4 and *B. melitensis* biotype 2, 3 was higher compared to the other isolates. These results were similar to those of Al-Izzi *et al.* (1985) who obtained 6 isolates of *B. melitensis* biotype 3 from 36 sheep's milk samples and to data reported by Shanshal (1999) who obtained 16 isolates from 600 milk and milk products samples:

Table 1. Samples from cow's (n=120), buffalo's (n=120) and sheep's (n=180) raw milk, positive in the milk ring test and of *Brucella* isolates recovered from milk samples by culture isolation and their seasonal distribution

Sample source	Number	Seasonal distribution			
		spring	summer	autumn	winter
Milk ring test positive samples					
Cow's milk	31 (25.8%)	8	11	5	7
Buffaloe's milk	42 (35.0%)	14	13	7	8
Sheep's milk	29 (16.1%)	9	7	2	11
Total	102 (24.2%)	31	31	14	26
Brucella isolates					
Cow's milk	12 (10.0%)	0	2	6	4
Buffaloe's milk	19 (15.8%)	1	2	8	8
Sheep's milk	31 (17.2%)	9	1	9	12
Total	62 (14.7%)	10	5	23	24

Table 2. Distribution of isolated *Brucella* biotypes in studied milk samples.

Brucella spp.	Biotypes						Total number (%)*
	1	2	3	4	5	6	
Brucella abortus	–	5	11	16	0	1	33 (7.8%)
Brucella melitensis	–	17	8	–	–	–	25 (5.9%)
Brucella ovis	–	–	–	–	–	–	4 (0.9%)
Total number	0	22	19	16	0	1	62 (14.7%)

* percentages are calculated vs the total number of studied milk samples.

Table 3. Distribution of *Brucella* species according to samples sources

Sample source	Total isolates	*B. abortus,* number (%)	*B. melitensis* number (%)	*B. ovis,* number (%)
Cow's milk	12	9 (75.0%)	3 (25.0%)	–
Buffaloe's milk	19	14 (73.6%)	5 (26.4%)	–
Sheep's milk	31	10 (32.2%)	17 (54.9%)	4 (12.9)

seven isolates of *B. abortus* biotype 4, three isolates of *B. abortus* biotype 3, four isolates of *B. melitensis* biotype 2 and one isolate of *B. melitensis* biotype 3. Hadad *et al.* (1997) however, found 8 *B. abortus* isolates in 80 buffaloe's milk and 80 cream samples, 5 *B. abortus* biotype 3 and 3 *B. abortus* biotype 4 isolates.

All *Brucella* isolates were sensitive to streptomycin, gentamicin, rifampin, trimethoprim, trimethoprim with sulfamethoxazole and kanamycin, whereas 98.5% were sensitive to tetracycline. It was also established that some of our isolates were sensitive to doxycline (85.9%), cephalexin (61.9%), and less sensitive to cefotaxim (40.8%), ampicillin (30.9%) and erythromycin (29.5%). *Brucella* spp. is able to survive in macrophages and because of this resistance to phagocytosis, the organisms are not destroyed. Therefore the use of antibiotics is aimed at facilitating macrophages' resistance and at destroying the pathogens (Rafie-Kolpin *et al.,* 1996). Our results are similar to those of Shanshal (1999) who reported a 100% sensitivity of these microorganisms to streptomycin, tetracycline, gentamicin, rifampin, trimethoprim, trimethoprim with sulfamethoxazole and kanamycin but also a resistance to cefotaxim (58.9%), erythromycin (9.2%) and amoxicillin. Korji (1991) found out 100% sensitivity to streptomycin, tetracycline, gentamicin, kanamycin, tobramycin and lesser sensitivity (54%) to neomycin, chloramphenicol, cephalexin and erythromycin.

The investigation of the effect of pH on *Brucella* growth showed that these bacteria tolerated acidic medium up to pH 4 and thus, the bacterial cells could survive in acidic milk (Table 4). The tolerance for *Brucella abortus* was previously suggested. The mechanism is related to acidic shock adaptation and is induced by exposure to reduced pH (Phillips *et al*., 1997). The contribution of acid tolerance to virulence has been studied and a strong correlation was found to exist (Portillo *et al*.,1993; Wilmes–Riesenberg *et al*., 1996).

Table 4. Growth of *Brucella* spp. at different pH values and temperatures

	B. abortus	*B. melitensis*	*B. ovis*
Temperature °C			
4	–	–	–
10	–	–	–
15	–	–	–
18	+	+	+
20	+	+	+
30	+	+	+
37	+	+	+
40	+	+	+
42	+	+	+
43	+	+	+
44	+	+	+
45	–	–	–
pH			
2	+/–	+/–	+/–
4			
6	+	+	+
8	+	+	+
9	+	+	+
10	–	–	–

+ presence of growth; +/– weak growth; – no growth.

The present study established that *Brucella* strains grew within a temperature range of 18–44 °C (Table 4). Therefore, they are able to survive in milk for a long time if it is not pasteurized (Nicoletti, 1980; Plomeet *et al*., 1988; Nicoletti, 1989).

The survival of *Brucella* in milk and dairy products is related to a variety of factors including pH, humidity level, presence of bacterial species and temperature. The optimum growth temperature is 36–38˚C, but most *Brucella* strains can grow between 20 ˚C and 40 ˚C. *Brucella* is inactivated by pasteurization or by prolonged boiling for 10 min (Alton, 1985; FAO, 1994).

In conclusion, the overall prevalence of *Brucella* in milk produced in the Basrah province of Iraq, was 14.7% as per culture isolation results. The highest percentage of *Brucella* isolates originated from sheep's milk followed by buffalo's raw milk. The prevalence of *Brucella* isolates was higher in spring and summer months. *Brucella abortus* was the most dominant among recovered *Brucella* species. All *Brucella* isolates showed an ability to grow within the temperature range of 18–44 °C and at pH 4–9.

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