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Original Article

Seroprevalence of brucellosis and typing of *Brucella melitensis biovar* 2 in lactating cows in Kuwait

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ABSTRACT

Objective: This study was conducted to determine the seroprevalence and typing of brucellosis in lactating cows in some dairy farms in Kuwait.
Materials and methods: A total of 4671 serum samples were collected from 4671 apparently healthy lactating cows comprising of 486 from Al-Wafra, 348 from Al-Kabed and 3837 from Al-Salebia areas. The sera were tested by Buffered Acidified Plate Antigen Test (BAPAT), Rose Bengal Plate Test (RBPT) and Complement Fixation Test (CFT) for the presence of brucellosis. Besides, Milk Ring Test (MRT) was done with 60 milk samples collected from 60 lactating cows comprising 18 from Al-Wafra, 5 from Al-Kabed and 37 from Al-Salebia areas. The stomach content of aborted feti were tested for typing of *Brucella* organism by using specific antisera.
Results: The results showed that the overall seroprevalence of bovine brucellosis was 339 (7.25%) by BAPAT, 332 (7.1%) by RBPT, and 329 (7.04%) by CFT. The results revealed that, 42 (8.6%), 5 (1.4%) and 292 (7.6%) sera were positive for brucellosis by BAPAT in the cows of Al-Wafra, Al-Kabed and Al-Salebia areas, respectively. Whereas, their respective number and seroreactive cases by RBPT were 39 (8.02%), 5 (1.4%) and 288 (7.4%). Similarly, as confirmatory test by CFT, the number and seroreactive cases in these areas were 39 (8.02%), 5 (1.4%) and 285 (7.46%). MRT revealed that the average positive case was 61.67% (59.46% in Al-Wafra; 60% in Al-Kabed and 66.6% in Al-Salebia). Two *Brucella* isolates could be recovered from the stomach content of the two aborted feti and typed as *Brucella melitensis biovar 2*.
Conclusion: Brucellosis is prevalent among lactating cows in Kuwait. This indicates the potential role of these dairy animals in disseminating and spread of such zoonosis to human. Considering public health significance, appropriate preventive measures are suggestive for combating brucellosis in Kuwait.

KEYWORDS

Brucellosis, Lactating cows, MRT, Seroprevalence

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INTRODUCTION

Brucellosis is still one of the most important bacterial zoonotic diseases, world wide spread, particularly in Middle East, Mediterranean countries, Africa, Asia, Arabian Gulf, and Central and South Americas (OIE, 2012). The disease is responsible for enormous economic losses in affected animals due to abortions, infertility premature birth, reduced reproduction and drop in milk production. It is also represents a great public health problem in endemic areas (Corbel, 2006; Pappas et al., 2006). Getting knowledge about the extent of disease through surveys and data on the incidences in the particular time in certain area will facilitate implementation of the eradication programs, Serological tests still have a major role in the routine diagnosis of brucellosis especially in developing countries due to their ease in handling, high sensitivity and low price (Alton et al., 1988), Serological tests (screening and confirmatory) are corner stone for obtaining reliable data on prevalence of bovine brucellosis (Gall and Nielsen, 2004). The combination of serological tests is desirable to overcome their variation in sensitivity and specificity (Thakur et al., 2002). Serological tests are almost exclusively used in eradication programmes (Morgan, 1967). The milk ring test (MRT) is also available for detecting of anti-*Brucella* antibodies in milk. MRT is simple and cheap, and requires no specialized equipment to perform. It detects anti-Brucella IgM and IgA present in milk, however, the test may be insufficiently sensitive to detect IgM and IgA at low concentrations in milk or when milk that contains colostrum or milk at the end of the lactation period is used (Bercovich and Moerman, 1979).

The MRT is sometimes used for screening the presence of brucellosis in lactating cows. However, the sensitivity of MRT becomes less reliable in screening brucellosis in large herds (>100 lactating cows) because false-positive reactions may occur in cattle that were vaccinated within 4 months, in the cases of mastitis or in samples containing abnormal milk. Therefore, the MRT is not recommended to test brucellosis in farms (OIE, 2012). Although, sheep and goats and their products are the main source of animal and human *Brucella melitensis* infection, *B. melitensis* infection in cattle is an emerging bovine disease of increasingly serious public health problem in some countries like Kuwait, Saudi Arabia, some Southern European countries and Israel. *B. abortus* vaccines do not protect effectively against *B. melitensis* infection and the *B. melitensis* Rev.1. vaccine has not been fully evaluated for use in cattle, thus, *B. melitensis* infection in cattle is problem (Garcia, 1990). *B. melitensis* (biovar 3) is the most dominant biotype of *Brucella* isolated from both animals and human in Egypt (Mohamed and Eisa, 2004; El-Sayed et al., 2011; Abdel Hamid, 2012; Afifi et al., 2015; Mona, 2015). Infections caused by *B. melitensis* are known to cause more sever disease of both clinical and pathological effects and responsible for most world-wide morbidity particularly in developed countries (Nicoletti, 1989).

Brucellosis has been reported to be endemic disease in Kuwait (Ministry of Health, Kuwait, 2006). From this point and from the economic and zoonotic importance of brucellosis, this work was undertaken to study the prevalence of brucellosis in lactating cows in three areas in Kuwait. Also *Brucella* species identification and typing of recovered isolates was performed.

MATERIAL AND METHODS

Animals: A total of 4671 apparently healthy lactating cows (486 from Al-Wafra Area, 348 from Al-Kabed Area and 3837 from Al-Salebia Area) in Kuwait were investigated in this study. The age of cows were ranged from 2-8 years. Most of the cattle were Friesian and Australian breeds. The animals were reared under different managemental system, and were not vaccinated against *Brucella.*

Blood samples: Blood samples were taken from examined animals; about 10 mL. of jugular-vein blood were collected in sterile silicon-coated vacuum tubes 'vacutainers' (catalogue no. 02-683-60, Becton Dickinson, 38241 Meylan, Cedex, France), identified, kept in a slant position in the shade for about 2 h for complete clotting and transferred on ice packs to the laboratory avoiding shaking. Samples were kept overnight at 4°C to allow for separation of serum then centrifuged at 1000 g. for 10 min to obtain amber clear serum. Sera were kept at -20°C each in 2 aliquots in sterile bijou bottles untill examined.

Serological tests: All animal serum samples were subjected to Buffered Acidified Plate Antigen Test (BAPAT), Rose Bengal Plate Test (RBPT) and Complement Fixation test (CFT) according to Alton et al. (1988) and OIE (2012). Antigens for BAPAT and RBPT were obtained from Veterinary Sera and Vaccine Research Institute (VSVRI), Abbassiya, Cairo 11517, Egypt. Antigen for CFT was kindly offered by the National Veterinary Services Laboratories (NVSL), Ames, IA 50010, USA. In CFT, titers of 1/4 were regarded as suspicious, while titers of 1/8+ or above were considered as positive.

Milk samples: Sixty milk samples were taken from 60 lactating cows (18 from Al-Wafra, 5 from Al-Kabed and 37 from Al-Salebia), which were positive abovemen-

tioned three serological tests. Amounts of 20-50 mL. of quarter milk samples were aseptically collected in sterile graduated polystyrene screw-capped conical-bottomed 50 mL. bottles (catalogue no. 05-539-2, Fisher Scientific Company, Springfield, NJ 07081, USA). Firstly, the udder was carefully washed with water and then with 1/1000 solution of potassium permanganate. The first milk streaks were discarded. The udder was dried; the end of each teat was disinfected with a swab of 75% alcohol and was wiped dry beginning with the teats on the far side. Milk samples were taken first from the near teat(s) before those on the off side. Samples were identified and quickly transferred on ice packs to the laboratory. The collected milk samples were subjected to Milk Ring test (MRT).

Milk Ring test (MRT): MRT was carried out individually with 60 samples according to OIE (2012).

Bacteriological examination: Swabs from stomach contents of two aborted feti, also, samples of fetal membranes, uterine discharges of two aborted cows were taken under complete aseptic condition for culture of *Brucella* species. This was performed according to the recommendations of the FAO/WHO Expert Committee on Brucellosis (Alton et al., 1988; OIE, 2012). Using direct culture on *Brucella* Agar Media containing *Brucella* selective antibiotics (Oxoid, England). The plates were examined for *Brucella* colonies. The suspected colonies were identified and typing on the base of colonial morphology, urease, CO_2 requirement, susceptibility to *Brucella* phages, growth in the presence of thionin and basic fuchsin dyes (1:25000, 1:500000, 1:100000, production of H_2S, and antigenic characteristics using specific antisera (A, M, R).

RESULTS AND DISCUSSION

Brucellosis is one of the most common global zoonoses, especially in the developing nations including Kuwait. Brucellosis was endemic in Kuwait, reported infection rates reached to 68.9 per 100,000 populations in 1985 (Adel et al., 2001). However, the rate decreased to 2.1 per 100,000 populations in 2006 (Ministry of Health, 2006). El Bayoumy and Azmi (2014) carried out a retrospective study considering 220 human brucella cases in Kuwait Sabah Medical Area Hospitals, and concluded that the magnitude of human brucella infection in Kuwait may serve as an indicator that the disease is persistently prevalent in domestic animals of the area. Eradication of brucellosis in animals is the key to prevent infection in humans. Attempts to eliminate brucellosis had been successful in many developed countries where they maintain their brucellosis-free herds by continuous serologic testing, quarantine, and other precautionary measures (Hafez, 1986). Accurate and rapid diagnosis of brucellosis is urgent for any seroprevalence survey, monitoring and eradication programs. Serological investigation still has played a dominant role in diagnosis of the disease (Konstantinidis et al., 2007). Despite its potential impact on public health, the epidemiologic situation of Brucella infection in Kuwait is need further investigations. So, it was utmost to investigate the Seroprevalence of brucellosis in some dairy cattle farms in Kuwait and try to identify and further typing the recovered isolates for obtaining clear picture about present status of the disease.

Most surveys performed to reveal the prevalence of bovine brucellosis have been depend upon the agglutination test because it is easy and economic application, In the present study, BAPAT, RBPT and CFT were used for as screening and confirmatory tests for diagnosis of bovine brucellosis and detection naturally infected cases in a total of 4671 apparently healthy lactating cows (486 from Al-Wafra Area, 348 from Al-Kabed Area and 3837 from Al-Salebia Area) in state of Kuwait. In the present study the overall prevalence of bovine brucellosis were 339 (7.25%), 332 (7.1%) and 329 (7.04%) as determined by BAPAT, RBPT and CFT, respectively (**Table 1**). The results revealed that, 42 (8.6%), 5 (1.4%) and 292 (7.6%) were seropositive for brucellosis in BAPAT in cattle of Al-Wafra, Al-Kabed Area and Al-Salebia, respectively. Whereas, their respective number and present of sero-reactive by RBPT were 39 (8.02%), 5 (1.4%) and 288 (7.4%). By using confirmatory test as CFT, the number and present of seroreactive in three areas were 39 (8.02%), 5 (1.4%) and 285 (7.46%) (**Table 1**). In one study conducted in Sharkia Governorate, Egypt, the occurrence of Brucella infection was 6.72% in cows (El-Sayed et al., 2011). In another study carried out on a total of 608 selected apparently healthy from 2.830 Holstein-Friesian dairy cattle aged between 2 to 5 years, from farms located at Dakahlia, Damietta, and Port Said Governorates, Egypt, the overall prevalence in samples collected from Dakahlia, and Port Said were 53.7%, 67.98% and 59.1% by RPAT, ELISA and Fluorescent Polarization Assay, respectively where all samples from Damietta were negative (Gwida et al., 2015). Moreover, in Egypt, a total of 520 Holstein cows tested for Brucella antibodies using four serological tests (BAPAT, RBPT, CFT and I- ELISA) at Gamasa districts in Dakahlia Governorate of different ages and production status, revealed, 84 (16.15%) serum samples were positive for BAPAT, 75 (14.42%) for the RBPT, 55 (10.57%) by IELISA and 36 (6.9%) serum samples were positive for CFT as confirmatory test (Mona, 2015). The sensitivities

Table 1: Seroprevalence of brucellosis in examined lactating cows at different dairy cattle farms in Kuwait as determined by BAPAT, RBPT and CFT.

Locality	No. of examined cows	BAPAT		RBPT		CFT	
		No. of +ve (%)	No. of -ve (%)	No. of +ve (%)	No. of -ve (%)	No. of +ve (%)	No. of -ve (%)
AL-Wafra	486	42 (8.6)	444 (91.4)	39 (8.02)	447 (91.9)	39 (8.02)	447 (91.9)
Al-Kabed	348	5 (1.4)	343 (98.6)	5 (1.4)	343 (98.6)	5 (1.4)	343 (98.6)
Al-Salebia	3837	292 (7.6)	3546 (92.4)	288 (7.5)	3549 (92.5)	285 (7.4)	3552 (92.6)
Total	4671	339 (7.25)	4333 (92.76)	332 (7.1)	4339 (92.9)	329 (7.04)	4342 (92.95)

BAPAT, Buffered Acidified plat antigen test. RBPT, Rose Bengal plat test. CFT, Complement Fixation test. + Ve, positive result. -Ve, negative result.

Table 2: Results of Milk Ring test done on milk samples of lactating cows sero-reactors of three serological tests.

Locality	BAPAT +ve (n)	RBPT +ve (n)	CFT +ve (n)	MRT +ve (%)
Al-Wafra	18	18	18	12 (66.6)
Al-Kabed	5	5	5	3 (60)
Al-Salebia	37	37	37	22 (59.46)
Total	60	60	60	37 (61.67)

BAPAT, Buffered Acidified plat antigen test. RBPT, Rose Bengal plat test. CFT, Complement Fixation test. MRT, Milk Ring test. +ve, positive result. -ve, negative result.

Table 3: Results of isolation and typing of recovered *Brucella* isolates. Two *Brucella* isolates could be recovered, from the stomach content of the two aborted feti.

Strain source		**CO_2 need**	**H_2S production**	**Urease**	**Growth on dyes**					**Lysis by phage**				**MS**			**Conclusion**
					Thionin			BF		Tb		Iz_1	R/C	A	M	R	
					a	b	c	b	c	RTD	RTD 10^4	RTD	RTD				
Field strain	2 stomach content	-	-	+ (20 h)	-	+	+	+	+	-	-	+	-	+	-	-	*B. melitensis* biovar 2
Reference strains	*B. melitensis* Ether	-	-	+ (18-24 h)	-	+	+	+	+	-	-	+	-	+	+	-	*B. melitensis* biovar 3
	B. abortus 544	-	+	+ (2 h)	-	-	-	+	+	+	+	+	-	+	-	-	*B. abortus* 1
	B. suis 1330	-	+++	++ (<15 min)	+	+	+	-	-	-	+	+	-	+	-	-	*B. suis* 1

All isolates were typed as *Brucella melitensis biovar* 2.

RTD: routine test dilution a: 1:25000 b: 1:500000 c: 1:100000

Tb: Tbilisi IZ1: Izatnagar R/C: Rough Brucella. B: Brucella, MS=Monospecific sera.

of BAPAT, RBPT, IELiSA and CFT were 90.6, 84.4, 96.9 and 93.7%, respectively whereas; their respective specificities were 84.6, 61.2, 84.6 and 100%. The author added that the BAPAT and RBPT positive samples should be confirmed by I-ELISA or CFT and 11 (5%) of 220 examined lactating cows were seroreactors.

Variations in infection in different examined areas in Kuwait (**Table 1**) may be attributed to environmental factors, number of examined cows in each area and stress, which may modulate susceptibility to infection, together with number of examined cows in each area.

The BAPAT and RBPT are well-known buffered Brucella acidified antigen tests introduced in many countries as the standard screening tests, because they simple, quickly and present sensitive results for detection low titer of antibodies which may present in chronic brucellosis. Acidification of the antigen in Rose Bengal Test (RBT) (pH 3.3) and BAPAT (pH 4.02) limits reactions with IgM, which persists in vaccinated animals, and non-specific agglutination due to IgM so, it prioritizes reactions with IgG, which is predominates in infected animals (Alton et al., 1988). Montasser et al. (2011) reported higher sensitivities of BAPAT (97.4%) and RBPT (94.9%) in detection of infection in tested animals and attributed the higher sensitivity rates due to its abilities to detect both IgG and IgM molecules. BAPAT so that these tests are applied as suitable screening tests for brucellosis and must be confirmed by CFT which was recommended by OIE as CFT which can detect little amount of IgG1 which specific for the infection (OIE, 2009).

Radostitis et al. (2000) reported that brucellosis remains of particular concern causing economic losses in animal production due to abortion, reduced milk production sterility, and the costs of animals' replacement in developing countries, beside it is the second most important zoonotic disease in the world after rabies (Cutler and Whatmore, 2003). El-Diasty (2004) found the prevalence of brucellosis in dairy cattle in Egypt during the period between 2002 and 2003 was 7.6%, 7.05% and 6.5% as determined by three serological tests (BAPAT, RBPT and Riv.T). However, Samaha et al. (2008) found that percentage of brucellosis in Egypt among cattle was 5.44%. Al-Habaty et al. (2015) studied the prevalence of brucellosis in Assuit Governorate during the period from January 2013 to July 2014 and found that prevalence of brucellosis using screening tests (BAPAT and RBPT) were 10.23% and 9.76% in cattle.

The MRT is an agglutination test applied on fresh milk, but it does not work on pasteurized or homogenized milk used, it detects IgM and IgA antibodies bound to fat globules, wide acceptable used as a routine periodic test for brucellosis due to it is cost effective, easy to perform and can finishing many samples in a short time (Cadmus et al., 2008). In the present study, MRT was performed on 60 lactating cows (18 from Al-Wafra, 5 from Al-Kabed and 37 from Al-Salebia), which were positive abovementioned three serological tests. The results of MRT shown in **Table 2** revealed that the percentages of positive were 61.67 (59.46 in Al-Wafra; 60 in Al-Kabed and 66.6 in Al-Salebia).

MRT is considered as an ideal method for detecting infected herds of brucellosis in individual animals (Noriello, 2004), but, false positive reactions may occur in colostrum or milk at the end of the lactation period and milk from cows suffering from a hormonal disorder or mastitis (Morgan, 1967). Cadmus et al. (2008) tested milk and blood samples collected from 532 trade cows to be slaughtered at Bodija abattoir, Ibadan (southwestern, Nigeria) for antibodies to *Brucella* using the MRT and the Rose Bengal Test (RBT). They found that ninety-nine (18.61%) of the milk samples were positive for MRT, while 52 (9.77%) of the serum samples were positive for RBT. Thirty-two (6.02%) of the samples were positive in both tests. They recommended other confirmatory tests e.g. ELISA, CFT; SAT must be applied in conjunction with MRT and RBT.

Salman and El-Nasri (2012) detected the prevalence of bovine brucellosis in Khartoum State, Sudan through examination of milk samples using MRT and ELISA and serum samples by RBPT and serum-ELISA samples. They found that prevalence of brucellosis was 34.7% and 32.5% using ELISA and milk ring test, respectively. However, in serum samples the prevalence was 27% and 24.4% using RBPT and serum-ELISA, respectively. They concluded that sensitivity of MRT and RBPT was 85% and 92% and specificity of MRT and RBPT were 95% and 94%, respectively. On the other hand, Salman et al. (2014) estimate the prevalence of bovine brucellosis in Khartoum state using the Milk Ring Test (MRT) and Milk Elisa (M Elisa) for the milk samples and the Rose Bengal Plate Test (RBPT) and the Serum Elisa (S Elisa) for the serum and they found that, the overall prevalence of bovine brucellosis within the milking cows was 38.2% and 40.8% for the milk samples using MRT and M ELisa respectively and 32 % and 38.8% of the serum samples were positive to the RBPT and S Elisa, respectively. Moreover, Mohamand et al. (2014) reported that 18.35% of the milk samples from 109 dairy cows were positive by

MRT. Furthermore, Najum (2014) found that 11(9.16%) goat milk samples collected from Al-Samawa city were positive for MRT.

In the present study, two *Brucella* isolates could be recovered from the stomach content of the two aborted foeti by culture on artificial media, followed by isolates identification by its morphology and growth characteristics of the colonies and biochemical tests. Two isolates were typed as *B. melitensis biovar 2* based on as it does not required CO_2 for growth, negative for H_2S production, grow in the presence of thionin and basic fuchsin dye (1:250000 and 1:500000), urease positive after 20 h, phage (Izatnagar) lyses and agglutinated only with A monospecific antisera (**Table 3**). Soliman (2006) reported that *B. melitensis* was the prevalent *Brucella* strain in Egypt. Moreover, *B. melitensis* (biovar 3) is the most dominant biotype of *Brucella* isolated from both animals and human in Egypt as reported by many authors (Mohamed and Eisa, 2004; El-Diasty, 2009; El-Sayed et al., 2011; Abdel Hamid, 2012; Afifi et al., 2015).

CONCLUSION

It is concluded that brucellosis is present at a level of 7% (as determined by CFT) among the examined lactating cows in Kuwait. A combination of several serological tests such as BAPAT and RBPT, followed by a confirmatory test of high specificity such as CFT can be used for diagnosis of brucellosis. Two isolates of *Brucella* are typed as *B. melitensis biovar* 2. This is represented a zoonotic threat to the public health. Routine screening of animals for brucellosis is crucial that may help to detect positive cases and reduce the risk of transmission of the disease. Effective implementation of control measures including test and culling of the infected animals, quarantine and movement controls may prevent the spread of infection. Application of hygienic measures which help in the control of brucellosis in the dairy farms. Further studies concerning molecular typing and sequencing of the recovered strain and tracing the source of infection in other animal hosts in Kuwait are necessary.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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