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DIAGNOSIS OF BOVINE BRUCELLOSIS USING MILK RING AND SEROLOGICAL TESTS WITH SPECIAL REFERENCE TO COLOSTRUM AND MILK IMMUNOGLOBULINS.

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ABSTRACT

Blood and whey serological tests (RBPT, TAT, Riv.T) as well as milk ring test were preformed on individual samples obtained from 84 dairy cows for diagnosis of bovine brucellosis. Also, samples were taken from colostrum and milk to evaluate the effect of brucellosis on immunoglobulin concentrations using ELISA. In addition, milk samples were used for isolation and identification of brucella organisms.

Results revealed that the incidence of brucellosis using blood serum were 21.42, 25.0 and 20.23% with RBPT, TAT and Riv.T, respectively. Meanwhile, in whey the corresponding figures were 11.90, 10.71 and 13.09%. The milk ring test revealed that the incidence of brucellosis was 13.09 %.

Brucellosis caused significant increases in all immunoglobulin fractions in mammary secretions, especially IgA in colostrum as well as IgG and IgA and total Igs in milk of infected cows, as compared to that of the normal control cows. While, IgM was obviously lower in milk of brucella positive cows.

Only two isolates of brucella *melitensis biovar* 3 were isolated from 21 positive TAT reactors milk samples.

It was concluded that for proper rapid screening of brucellosis, MRT should not be performed alone.

Keywords: Cow, colostrum, milk, blood, brucellosis, serological tests, ELISA, immunoglobulins.

INTRODUCTION

Brucellosis is a widespread economic disease, particularly among dairy cattle (Radostits *et al.,* 1994 and OIE, 2001). Also, it is an important zoonotic disease that could be transmitted from infected animals, through drinking of raw milk to man causing undulant fever (Chamberlain, 2000). In cattle, brucellosis leads to great losses in calves through abortion, reduction in milk yields and reduced fertility (Amin *et al.,* 1995).

Infected cows excrete brucella organisms in their milk sporadically throughout the entire lactation period in counts varied from a few up to 15 000 cells / ml milk (El-Gibaly *et al.*, 1991) depending upon the stage of lactation with the largest number of organisms found in the milk at the onset of lactation, but both the occurrence and numbers of brucella excreted at any time can vary (Mohamed, 1999). Udder from infected animals appears clinically normal, but is an important source of infection for consumers (Vanzini *et al.,* 1998).

Currently, the diagnosis of brucellosis is based on bacteriological and serological techniques. Culture techniques are time consuming to grow and identify the organisms (Lulu *et al.,* 1988). Neither single milk or blood serological tests was sufficient to give conclusive diagnosis (El-Gibaly *et al.,*

1990). There are number of specific screening tests for detecting antibodies of brucella in milk, the most commonly used test is the milk ring test (MRT), which depends on the presence of brucella agglutinins in the milk (Katz *et al.*, 1976). The potency and duration of reaction depend on the amount of agglutinins and fat content of milk, the higher fat content, the more strongly and rapidly the reaction would take place. In some cases agglutinins of brucella may present in milk before blood (Sutra *et al.*, 1986). Immunoglobulin A (IgA) mainly produced in the mammary gland and occurs in the milk earlier after infection and it is the most active immunoglobulin in coloured ring formation in MRT, (Sutra and Dubray, 1987). Ruminants are born virtually without antibodies, and their immune system depends on the ingested maternal colostrum. The newborn ruminant's gut allows unselective transfer of immunoglobulins and other macro molecules into circulation only during the first 12-36 hours after birth (Lilius and Marnila, 2001).

Cow's colostrum and milk contain virtually all compounds of bovine cellular and humoral immune defense, including antibodies and complement proteins (Korhonen *et al.*, 2000).

The immunoglobulins in mammary secretions are both humoral, arising from the blood stream (IgG_1 and IgG_2) and local, produced by plasmacytes (IgM and IgA) in the mammary gland (Butler, 1974 and Larson, 1992).

The immunological function mediated by the immunoglobulins depends on the immunoglobulin class. IgG antibodies have a multitude of functions, the most important of which is the activation of complement mediated bacteriolytic reactions. Another vital function of Igs is their ability to augment the recognition and phagocytosis of bacteria by leukocytes (opsonization). Immunoglobulins are also capable of preventing adhesion of bacteria and viruses to surfaces, inhibit bacterial metabolism, agglutinating bacteria, and neutralizing toxins (Korhonen *et al.*, 2000).

This study was undertaken to determine the correlation between brucella agglutinins in milk and serum from reactor cows by using different diagnostic tests. Also, the effect of brucella infection on cow's colostrum and milk immunoglobulin fractions was evaluated by using Enzyme Linked Immunosorbent Assay (ELISA).

MATERIALS AND METHODS

Samples:

A total number of 84 individual samples of blood and milk were collected from a herd of pluriparous dairy Holstein-Friesian cows raised in a private farm at Sharkia Governorate. These samples were used for serological examinations. This herd is suffering from reproductive problems such as abortions, stills birth and retained placenta.

The milk samples were aseptically collected and examined using Schalm test (A.P.H.A., 1985) to exclude subclinical mastitis and to avoid other factors that may affect the results of milk ring test (MRT).

The milk was defatted by centrifugation, whey was obtained by adjusting the pH of skim milk to 4.6 (25°C) using 0.1 *N* HCL. Casein was separated by centrifugation at 4000 r.p.m. for 30 minutes, then the whey was filtered through whatman No.2 filter paper, and it was kept frozen until used.

Brucella antigens:

All of the examined antigens, including Rose Bengal plate test (RBPT), standard Tube agglutination test (TAT) and Rivanol test (Riv.T) were supplied by Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.

Serological tests:

Rose Bengal plate test (RBPT), Tube agglutination test (TAT) and Rivanol test (Riv.T) were done as described by Alton, *et al.* (1988).

Diagnostic test on milk and whey for Brucellosis:

Milk ring test (MRT), whey rose bengal plate test (WRBPT), whey tube agglutination testy (WTAT) and whey rivanol test (WRriv.T) were carried out according to N.V.S.L. (1985) and Alton *et al.* (1988).

Bacteriological examination:

Isolation and identification of brucella organisms from milk samples were carried out by inoculating the Albimi agar plate containing antibiotics (Oxide) with sediment-cream mixture of milk. The plates were incubated at 10 % carbon dioxide tension as described by Alton and Forsyth (1998).

Determination of immunoglobulin concentrations in colostrum and milk:

Three colostrum and 8 milk samples, which were positively reacted with MRT as well as 3 colostrum and 4 milk samples. Which were collected from normal healthy cows (as control), were used to determine the immunoglobulin concentrations by using Enzyme Linked Immunosorbent Assay (ELISA) technique as described by Erhard *et al.* (1992).

Data obtained from colostrum and milk samples were statistically analysed as designed by Snedecor and Cochran 1980.

RESULTS AND DISCUSSION

In the present study, three diagnostic tools i.e. blood, milk and whey were used for diagnosis of bovine brucellosis. Table (1) represents the serological tests used in blood serum and whey. The results revealed that the incidence of brucellosis in blood serum were 21.42, 25.0 and 20.23% with RBPT, TAT and Riv.T, respectively. Meanwhile, in whey the respective incidence of 11.90, 10.71 and 13.09% was reported. These results indicated that the serum samples showed higher incidence of reactors than whey test with serological examination. This might be attributed to defateing process before the performance of whey tests (Sutra *et al.,* 1986 and El-Gibaly *et al.,* 1990).

The milk ring test gave an incidence of brucella positive reactors was 13.09 %. This might be due to the fact that MRT depends upon the presence

of IgA, produced in the mammary gland, which is the most active Igs in the coloured ring formation of MRT. Meanwhile, the other serum serological tests depend on the presence of IgG and IgM in serum of infected animals (Collin 1976 and Sutra *et al.*, 1986).

Table (2) correlates the results of MRT and other serological tests of brucellosis in whey. The percentage of positive reactors were 13.09, 11.9, 10.71 and 13.09 % with MRT, WRBPT, WTAT and WRiv.T, respectively. A complete correlation was found between MRT and WRiv.T.

Estimation of the concordance, relative sensitivity and relative specificity percentages between MRT and other serological tests (Table 3), revealed the highest concordance between MRT and Riv.T (92.85%), followed by RBPT (91.66%) then TAT (88.09%). The relative sensitivity of MRT was 61.11, 52.38 and 64.70 % versus RBPT, TAT and Riv.T, respectively. However, relative specificity percentages of MRT were 90.41, 86.30 and 91.78% versus RBPT, TAT and Riv.T, respectively.

Generally, the present results indicated higher relative specificity and lower relative sensitivity between MRT and other serum serological tests. These findings indicated the presence of false negative results of MRT, as compared with other serum serological tests. These data came in coordination with those of Martin (1977) who reported that the lack of specificity leads to false negative results. Therefore, it was recommend that MRT should be applied only as a preliminary screening test (Sutra *et al.*, 1986 and Hosein *et al.*, 1991). On the contrary, El-Gibaly *et al.*, (1990), reported that MRT is an efficient complementary test and has close correlation with results of blood serological tests for detecting of bovine Brucellosis.

From 21 milk samples obtained from serological positive cows (TAT) and 11 of them were also positive for MRT, only two isolates of brucella *melitensis biovar* 3 could be isolated. This is mainly attributed to the fact that brucella organisms are discharged intermittently in milk (Blood *et al.*, 1983), and also due to fastidious properties of brucella organisms in their nutritional requirements as proved by Robertson *et al.* (1977); Hosein (1987) as well as Salem and Hosein (1990).

Table (1): Incidence of positive reactors by serological tests used for diagnosis of brucellosis in blood serum, whey and milk.

Tests	Samples (n = 84)					
	Serum		Whey		Milk	
	No.*	%	No.	%	No.	%
RBPT	18	21.43	10	11.90	-	-
TAT	21	25.00	9	10.71	-	-
Riv.T	17	20.23	11	13.09	-	-
MRT	-	-	-	-	11	13.09

* : Positive reactors.

Table (2): Correlation between MRT and other serological tests used for diagnosis of brucellosis in whey.

Tests / Reaction	Positive		Negative	
	No.	%	No.	%
MRT	11	13.09	73	86.90
WRBPT	10	11.90	74	88.09
WTAT	9	10.71	75	89.28
WRiv.T	11	13.09	73	86.90

Table (3): Comparison between MRT reactors and other serum serological tests used for diagnosis of cattle brucellosis.

Items	MRT Vs RBPT	MRT Vs TAT	MRT Vs Riv.T
No. sample test	84	84	84
Both test positive	11	11	11
Both test negative	66	63	67
Concordance %	91.66	88.09	92.85
R. sensitivity %	61.11	52.38	64.70
R. specificity %	90.41	86.30	91.78

$$\text{Concordance \%} = \frac{\text{Both test reactor + Both test negative}}{\text{Total cases examined}} \times 100$$

$$\text{Relative sensitivity \%} = \frac{\text{True positive}}{\text{True positive + False positive}} \times 100$$

$$\text{Relative specificity \%} = \frac{\text{True negative}}{\text{True negative + False negative}} \times 100$$

True positive : Reactor sera in the two comparable tests.
False negative : Reactor sera in one test but proved negative by another tests.
True negative : Negative sera in the two comparable tests.

Table (4) represents the changes in colostrum and milk immunoglobulin concentrations in relation to brucellosis. It was noticed that all immunoglobulin concentrations significantly increased, especially IgA in colostrum as well as IgG, IgA and total Igs ($p<0.001$) in milk of the infected cows, as compared with the normal control cows. While in milk, IgM was significantly lower ($p<0.01$) in infected cows as compared to the normal control cows.

Kholif and El-Loly (2001) reported lower values of colostrum immunoglobulin in goats and ewes as measured by ELISA, meanwhile, higher values were detected in buffaloes colostrum as measured by Single Redial Immunodiffusion (El-Loly 1996). In addition, our data are in agreement with those noted by (El-Loly 1996) in buffaloes milk and it was lower than those found by (Kholif and El-Loly 2001) in goat and ewe milks. Furthermore, Wernicki, (1984) could detect lower values of IgG and slightly higher values of IgM and IgA in cow milk, as compared with the results obtained in the present work. Also, it was clear that the IgG was the major immunoglobulins in cow's colostrum and milk, the same observation was reported by Butler, (1969).

The values of immunoglobulin concentrations in infected cows were 32, 57, 113 and 38 % higher than that of the values in normal colostrum and

53, 90, 75 and 59 % higher than that of the values in normal milk for IgG, IgM, IgA and total Igs, respectively. The effect of brucella infection on colostrum and milk immunoglobulin concentrations was not traced in the available literature, so, the present data may nearly throw light on this new information.

Table (4): Effect of brucllosis on immunoglobulin concentrations in colostrum and milk (Mean ± SE).

Items	Obtained from	No. samples	Immunoglobulin concentrations (mg/ml)			
			G	M	A	Total
Colostrum*	Normal cow	3	23.48^{E} ± 0.26	3.56^{E} ± 0.23	1.15^{A} ± 0.05	28.19^{E} ± 0.16
	Infected cow	3	30.90^{F} ± 0.31	5.58^{F} ± 0.18	2.45^{B} ± 0.13	38.94^{F} ± 0.38
Milk	Normal cow	4	12.40^{A} ± 0.21	1.89^{C} ± 0.08	0.70^{A} ± 0.09	15.00^{A} ± 0.20
	Infected cow	8	18.94^{B} ± 0.22	3.60^{D} ± 0.21	1.23^{B} ± 0.06	23.77^{B} ± 0.24

*** Samples obtained within 22-36 hrs. after calving.**
Means with different supscripits significantly differ at $p<0.001$ for A & B, at $p<0.01$ for C & D and at $p<0.05$ for E & F.

In conclusion, one can expect that brucellosis caused significant increases in all colostrum and milk immunoglobulin concentrations, especially IgA in colostrum ($p<0.01$). Therefore, colostrum is of great value in the diagnosis of bovine brucellosis because of the colostrum shows very high significant change in IgA fraction between infected and non-infected cows. Meanwhile, the milk is important in the diagnosis of the disease due to the highly significant increase of total immunoglobulins, especially IgG and IgA. These results supported our results of MRT, where the positive MRT depend mainly upon IgA.

However, in general, milk ring test could be applied as screening test parallel to the other serum serological tests but not alone for detecting of brucella infection in dairy farms.

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تشخيص البروسيلا فى الأبقار بإستخدام إختبار اللبن الحلقى وبعض الإختبارات السيرولوجية مع التركيز على مستوى الجلوبيولينات المناعية فى السرسوب واللبن

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تم إستخدام عينات اللبن لإجراء إختبار اللبن الحلقى (MRT) ، وكذلك عينات الدم والشرش لإجراء بعض الإختبارات السيرولوجية (RBPT, TAT, Riv.T) وذلك لتشخيص مرض البروسيلا فى ٨٤ بقرة حلابة. كما تم إستخدام بعض عينات من السرسوب واللبن لقياس تأثير الإصابة على مستوى الجلوبيولينات المناعية بإستخدام الـ ELISA ، وأيضا تم زراعة بعض عينات اللبن بكتريولوجيا لعزل الميكروب المسبب للمرض.

أشارت النتائج إلى أن النسبة المئوية للإصابة فى عينات الدم كانت ٢١,٤٢ ، ٢٥,٠ ، ٢٠,٢٣ %، بينما كانت فى عينات الشرش ١١,٩٠ ، ١٠,٧١، ١٣,٠٩ % على الترتيب. بينما كانت نسبة الإصابة ١٣,٠٩ % فى اللبن بإستخدام الإختبار الحلقى. وتم عزل عترتين من الميكروب المسبب لمرض البروسيلا من ٢١ عينة ايجابية لإختبار TAT.

إزداد تركيز الجلوبيولينات المناعية معنوياً فى كل من سرسوب ولبن الحيوانات المصابة عنها فى الحيوانات السليمة خاصة المشتق IgA فى السرسوب ، والـ IgG ، IgA ، total Igs فى اللبن . خلص البحث إلى أنه لا يمكن الإعتماد على إختبار اللبن الحلقى فقط فى التشخيص السريع لمرض البروسيلا بل يجب إجراء على الأقل إختبار آخر سيرلوجى بجانبه.