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Immunogenetic Markers of Whey Proteins in Bovine Brucellosis

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Abstract: The incidence of brucellosis among examined cows by different diagnostic tests on milk and whey were 11 (22.91%), 10 (20.83%), 8 (16.66%), 7 (14.58%) and 11 (22.91%) for Milk Ring Test (MRT), Whey Rose Bengal Plate Test (WRBPT), Whey Microtiter Agglutination Test (WMAT), Whey Dithiothereitol Agglutination Test (WDAT) and Delayed type Hypersensitivity Test (DHT) respectively. The results of absolute specificity were 26.31, 20.0, 17.07, 29.72 and 29.72% with WRBPT, WMAT, WDAT, MRT and DHT, respectively. The highest incidence of DHT (22.91%) indicated that the sensitivity of this test, which depends upon the cellular immunity response, was independent from specific humoral antibody production. The concordance between reactors cows in the different tests used in this study were 100.0, 97.51, 93.75 and 91.66% for MRT, WRBPT, WMAT and WDAT, respectively. It is noticed that the DHT achieved the highest agreement with MRT (100%). Only one isolate of *Brucella melitensis biovar* 3 could be isolated from milk samples which gave positive with MRT. The results evident that the whey of cows included closed bands in case of infected samples if compared with free and healthy one. Within β-Lg three genotypes were observed: AA, AC and CC. Homozygote CC had the greatest number (5) of these genotypes, but, heterozygote AC had the lowest number (1). A higher frequency for allele C of β-Lg than allele A of β-Lg gene was observed (0.687 and 0.312), respectively. Within α-La the presence of three genotypes were appeared: AA, AB and BB, the highest number being recorded for homozygote BB with higher frequency in $\alpha\text{-La}^B$ gene (0.812) than in all fractions of whey proteins. Concerning the γ-globulin ($S\alpha_2$), the same genotypes of α-La were observed for this fraction, with higher frequency in $S\alpha_2^B$ than $S\alpha_2^A$ (0.687 and 0.312), respectively. The observed homozygous genotype in β-Lg, α-La and γ-globulin variants of whey proteins were higher than those expected heterozygous genotypes. Results revealed that the infected cows suffering from brucella distinguished by high frequency of homozygotic genotypes in all studied loci with predominance of $\alpha\text{-La}^B$ gene marker.

Key words: Genetic markers, cow milk, brucellosis, serological tests, brucellin test, electrophoresis, whey proteins

INTRODUCTION

Brucellosis is a specific contagious disease of animals and humans caused by bacteria of the brucella group. The disease is considered by FAO, WHO and OIE as the most widespread zoonosis in the world (OIE, 2004). Theconsumption of contaminated milk and dairy products made from raw milk, which is a continuing human health risk (Headrick *et al*., 1998; Mohamed, 1999). The infected cows excrete brucella in colostrum, milk and genital secretions such as semen and vaginal fluids. The milk of infected cows may contain large number of viable organisms, which become concentrated in products such as soft cheeses. Throughout the entire lactation period of cow, the counts varied from few organisms up to 15 000 cells mL^{-1} of milk (Rivera *et al*., 2003).

It has been recognized that soft cheese was a major vehicle of infection in the Mediterranean region, the Middle East and Latin America (Headrick *et al*., 1998).

The milk is important in the diagnosis of brucellosis. There are number of specific screening tests for detecting antibodies of brucella in milk (Chand *et al*., 2005). The most commonly used test is the Milk Ring Test (MRT) which could be applied as screening test on the herd level parallel to other serum serological tests (El-Loly and Ghazi, 2002). The MRT depends on the presence of brucella agglutinins in milk (Katz *et al*., 1976).

Although allergic skin tests for diagnosis of brucellosis have not been widely employed through the world, they have reported to be very helpful in some areas (Bercovich *et al*., 1990). The Delayed type Hypersensitivity Test (DHT) is very specific skin allergic test and not all infected animal react. However it has been shown that some animals are negative to be with allergic test at time when they are excreting brucella organisms, although they may show sensitivity later on chronic stage on infection (Alton *et al*., 1988).

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Selection of dairy sires and cows has been based mostly on quantitative traits such as milk, fat or protein yield, which are assumed to be controlled by multiple *loci*. Genetic improvement of quantitative traits is, therefore, relatively slow, as productive traits can only be measured in one sex, are affected by numerous polygenes (each polygene exerting a small effect on the trait) and environmental factors have an important influence on their expression. This undoubtedly lowers the accuracy of genetic evaluation of sires and cows. In addition, productive traits can only be measured in adult animals, thereby increasing the generation interval and lowering the genetic progress per year. For this reason, qualitative characters, such as polymorphisms in blood groups, enzymes, blood serum proteins or milk protein types, are among those being investigated for the possibilities which they provide of improving the accuracy of estimating genetic merit of sires and cows and practicing selection at an earlier age (Lin *et al*., 1992).

Also, according to Litwinczuk and Krol (2002) selection dairy cattle on the basis such genetic markers may lead to a 5% quicker genetic improvement than does traditional selection.

Specific proteins in bovine milk include four caseins (α_{s1} , α_{s2} , β- and κ-casein) and two whey proteins, α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), each protein showing at least 2 genetic variants (Eigel *et al*., 1984).

Several studies have reported that some of these bovine protein variants, particularly certain κ-Cn and β-Lg variants, are associated with lactation performance and have a major influence on milk composition and its processing properties, including cheese yield (Aleandri *et al*., 1990).

Reported relationship between genetic variants of the β-Lg and milk yield, milk composition and cheese making ability in cattle (Curik *et al*., 2002) have raised interest for the establishment of the relationship between β-Lg polymorphism and milk production traits in dairy goat and sheep populations.

Genotyping of milk proteins, such as κ-casein, can be performed by electrophoresis, directly from milk samples, as the expression of caseins occurs only during the lactation phase in mammary gland cells. Therefore, the use of electrophoresis for genotyping of milk proteins is strongly limited because it can only be used in cows in the lactation stage (Lara *et al*., 2002).

The objective of this study was to identify the genetic markers (using whey proteins electrophoresis profile), that gave natural resistance to bovine brucellosis, these detection of gene markers could be helpful on selection of brucella resistant cows for breeding purposes and to diagnose bovine brucellosis by using different serological tests.

MATERIALS AND METHODS

Samples: A total number of 48 individual samples of milk were collected from a herd of dairy Holstein-Friesian cows raised in a private farm at Sharkia Governorate. These samples were used for diagnostic examinations. These animals were suffering from reproductive problems and arthritis.

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 min then the whey was filtered through whatman No.2 filter paper and it was kept frozen until used.

Brucella antigens: All of the used antigens, including Rose Bengal, milk ring and standard white antigen were supplied by Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. Bruceller gene OCB (ovine, caprine, bovine) is *Br. melitensis strain* B115 (Rough) protein extract (at least 2000 units) was obtained from Merial, Lyon, France.

Diagnostic test on milk and whey for Brucellosis: Milk Ring Test (MRT), Whey Rose Bengal Plate Test (WRBPT), Whey Microtitre Agglutination Test (WMAT) and Whey Ditheiotheritol Agglutination Test (WDAT) were carried out according to Alton *et al.* (1988) as well as Zaabal and Ghazi (2003).

Delayed type Hypersensitivity Test (DHT), Skin allergic test: The allergic test was carried out according to the method described by Bercovich *et al.* (1990). By inoculating 0.2 mL of the brucellergene OCB intradermally in one side of the caudal fold of the tail and the other in left as control, as well as, in the middle third of one side of the neck in the same animal. The results were recorded after 24, 48 and 72 h. Swelling and congestion at the site of injection was considered as positive reaction.

Bacteriological examination: Isolation and identification of brucella organisms from milk samples were carried out by inoculating the Albimi agar plate containing antibiotics

(Oxide Co., Egypt) with sediment-cream mixture of milk. The plates were incubated at 10 % carbon dioxide tension as described by Alton and Forsyth (1998).

Whey proteins electrophoresis and quantitative measurement by scanning: Analytical SDS-Polacrylamide Slab Gel Electrophoresis (SDS-PAGE) of prepared whey proteins was conducted in polyacrylamide gel containing 0.1% SDS according to the conventional method (Laemmli, 1970) which involved denaturation of proteins by heating for 5 min in 1% SDS in a boiling water bath prior to applying them to the gel. After electrophoresis, proteins were localized in gels using coomasie blue 0.1%. A photograph of stained gel was scanned with an Image densitometry (Biorad G-70) using Gel Pro Analyzer v. 3.0 software. For stained gels, optical transmission method was used at a wavelength of 540 nm. The important parameters in this analysis include molecular weight and the percentage of each separated band.

Immunogenetic markers: Genotyping distribution and gene frequencies of some whey proteins of brucella positive cows were done according to Mercoreva (1977) as follows:

$$p^2 + 2pq + q^2 = 2$$

where:

p : Gene frequency of allele A
P^2 : Frequency of genotype AA
q : Gene frequency of allele B
q^2 : Frequency of genotype BB

RESULTS AND DISCUSSION

The incidence of brucellosis among examined cows by different diagnostic tests on milk and whey were 11 (22.91%), 10 (20.83%), 8 (16.66%), 7 (14.58%) and 11 (22.91%) for Milk Ring Test (MRT), Whey Rose Bengal Plate Test (WRBPT), Whey Microtitre Agglutination Test (WMAT), Whey Dithiothereitol Agglutination Test (WDAT) and Brucellin skin Test (DHT), respectively. From Table 1, it is shown that the MRT gave the highest positive reactors (22.91%), this might be due to defating process before the performance of whey tests (El-Loly and Ghazi, 2002). Also, MRT depends upon the presence of IgA produced in the mammary gland, which is the most active immunoglobulins in the coloured ring formation of MRT (Sutra *et al*., 1986; El-Gibaly *et al*., 1990).

The evaluation of the diagnostic tests on milk and whey as well as Brucellin test were estimated on the basis of concordance, absolute sensitivity and absolute specificity. The results of absolute sensitivity were 26.31, 20.0, 17.07, 29.72 and 29.72% with WRBPT, WMAT, WDAT, MRT and DHT, respectively. While, the results of absolute specificity were 79.16, 83.33, 85.41, 77.08 and 77.08% with the same tests (Table 2).

Table 1: Incidence of brucellosis among dairy cows examined by different diagnostic tests on milk and whey

	Samples (n = 48)			
	Positive		Negative	
Tests	No.	(%)	No.	(%)
MRT	11	22.91	37	77.08
WRBPT	10	20.83	38	79.17
WMAT	8	16.66	40	83.33
WDAT	7	14.58	41	85.42
DHT	11	22.91	37	77.08

Table 2: Absolute sensitivity and specificity of different tests for diagnosis of bovine brucellosis

Items	WRBPT	WMAT	WDAT	MRT	DHT
No. samples test	48	48	48	48	48
No of reactors	10	8	7	11	11
No of negative	38	40	41	37	37
Sensitivity %	26.31	20.00	17.07	29.72	29.72
Specificity %	79.16	83.33	85.41	77.08	77.08

The results of these estimations revealed the highest sensitivity of both MRT and DHT. This was contrast with the specificity of WDAT which was highest (85.41%) followed by WMAT (83.33%). Generally finding of this study cleared higher sensitivity of MRT and DHT as compared with the other diagnostic tests on whey, indicating their ability to detect the higher number of positive reactors to brucellosis. These results come in coordination with those of El-Loly and Ghazi (2002) who concluded that MRT could be applied as screening test for detecting of brucella infection in dairy farms.

Concerning to DHT, which was used for diagnosis of brucellosis (Ghazi *et al*., 2004). It was the highest incidence of sensitivity (22.91%), which depends upon the cellular immunity response and was independent from specific humoral antibody production. These results were forced by Bercovich *et al*. (1990) who found that DHT test gave positive results even through serologic test were negative. Meanwhile, Bercovich and Leak (1990) showed that the sensitivity of DHT test was 100%, while the specificity was 83%, it was corrected well with those of the Complement Fixation Test (CFT).

Regarding, the concordance, relative sensitivity and relative specifity of DHT, they were estimated and evaluated with other diagnostic and MRT tests (Table 3).

The concordance (Agreement) between reactors cows in the different tests used in this study were 100.0, 97.51, 93.75 and 91.66% for MRT, WRBPT, WMAT and WDAT, respectively. From these results, it is noticed that the DHT achieved the highest agreement with MRT (100%).

Table 3: Comparison between Brucellin skin test reactors and the other diagnostic tests on milk and whey

Items	DHT Vs MRT	DHT Vs WRBPT	DHT Vs WMAT	DHT Vs VDAT
No. sample test	48	48	48	48
Both test positive	11	10	8	7
Both test negative	37	37	37	37
Concordance %	100.00	97.91	93.75	91.66
R. sensitivity %	100.00	90.90	72.70	63.60
R. specificity %	100.00	97.36	92.50	90.24

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

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

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

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

Concerning with estimation of relative senstivity and specifity on the basis of DHT of all applied tests used showed high values with MRT and WRBPT. This explained that the used methods are highly valuable in diagnosis of bovine brucellosis in the present study.

From 48 milk samples obtained, out of 11 samples gave positive with MRT, only one isolate 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; Salem and Hosein, 1990).

Figure 1 shows the whey proteins electrophoresis of brucella positive cow with control samples (negative). The results evident that the whey of cows included closed bands in case of infected animals if compared with free and healthy one, with predomonomeric of homozygotic genotypes in most genetic loci, it maybe due to the responsibility of these genes to natural resistance of animals against brucella.

Electrophoretic pattern of milk proteins has been reported by Ilahi *et al*. (2000), Marletta *et al*. (2000), Zaabal *et al*. (2002). They reported that the milk protein loci could be used as genetic marker for genetic analysis and linkage analysis with economically important production traits.

These immunogenetic characteristics are shown in Table 4 and Fig. 1. Results illustrated that within β-Lg three genotypes were observed: AA, AC and C C. Homozygote CC had the greatest number (5) of these genotypes, but, heterozygote AC had the lowest number (1). A higher frequency for allele C of β-Lg than allele A of β-Lg gene was observed (0.687 and 0.312), respectively. The same

Table 4: Genotyping distribution and gene frequencies of some whey proteins loci of brucella positive cows

	Genotypes			
Protein loci	Gene	Observed	Expected	Gene frequency
β-lactoglobulin	AA	2	0.8	$\beta\text{-Lg}^A$: 0.312
(β-Lg)	AC	1	4.4	
	CC	5	3.7	$\beta\text{-Lg}^C$: 0.687
α-lactalbumin	AA	1	0.3	$\alpha\text{-La}^A$: 0.187
(α-La)	AB	1	2.4	
	BB	6	5.3	$\alpha\text{-La}^B$: 0.812
γ-globulin	AA	1	0.9	$S\alpha_2^A$: 0.312
($S\alpha_2$)	AB	3	4.4	
	BB	4	3.7	$S\alpha_2^B$: 0.687

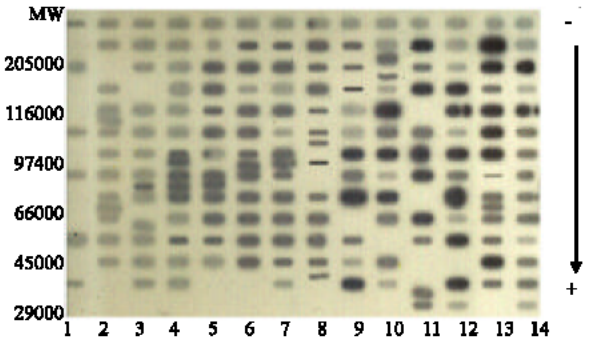

Fig. 1: Electrophoretic pattern in whey proteins of bovine brucellosis Lane 1: Molecular weight standard Lane from 2 to 11: Brucella positive samples Lane from 12 to 14: Brucella negative samples

observation was reported by Litwinczuk *et al*. (1999, 2001) and Litwinczuk and Krol (2002).

Twelve polymorphic variants of β-Lg are known in cattle, but the 2 most frequent, A and B, were shown to be associated with differences in milk protein yield and composition. Allele B of β-lactoglobulin is associated with high casein and fat contents in cow's milk, while, milk of Holstein cows with the β-Lg AA genotype were shown to contain more whey proteins and total protein than those of the other genotypes (Lunden *et al*., 1997).

Within α-La the presence of three genotypes were appeared: AA, AB and BB, the highest number being recorded for homozygot BB with higher frequency in $\alpha\text{-La}^B$ gene (0.812) than in all fractions of whey proteins._α-La is considered a valuable genetic marker for milk production traits in cattle.

Moreover, cows with allele A of the gene have higher milk yield, protein yield and fat yield the allele B of α-La is associated with a higher percentage of protein and fat (Martin *et al*., 2002).

Concerning the γ-globulin ($S\alpha_2$), the same genotypes of α-La were observed of this fraction, with higher frequency in allele $S\alpha_2^B$than allele $S\alpha_2^A$, namely, 0.687 and 0.312, respectively.

The high frequency of genotypes in brucella positive cases gives evidence to correlation between these genes and susceptibility to infection by brucella.

One of the main objectives of this study was to use whey proteins loci as genetic markers to clarify the expected relationships between specific alleles and susceptibility to infection by brucella.

All whey proteins, in the present study were polymorphic and these results confirm the fact that loci were defined as polymorpheric when the frequency of the most common allele was less than 0.95 (Kantanen *et al*., 1999).

In this study, the most genotypes were heterozygous, these results were similar to those reported by (Leberg, 1992) who mentioned that average expected heterozygous genotypes has been increased due to more equal distribution of allele frequencies.

The observed homozygous genotype in β-Lg, α-La and γ-globulin variants of whey proteins were higher than those expected heterozygous genotypes.

Results revealed that the infected cows suffering from brucella distinguished by high frequency of homozygotic genotypes in all studied loci with predominance of α-La^{B} gene marker.

CONCLUSION

We can conclude that the use of whey proteins loci as genetic markers is useful to clarify the expected relationships between specific alleles and susceptibility to infection with Brucella organisms. This could be helpful on selection of brucella resistant cows for breeding purposes.

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