DIAGNOSTIC VALIDATION OF OVINE AND CAPRINE BRUCELLOSIS USING SERUM- AND MILK-ELISA

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A b s t r a c t : *Aim:* To introduce and validate the new method of diagnosing ovine and caprine brucellosis in a rapid, accurate and inexpensive manner by using i-ELISA (serum/milk) technique.

Methods: Serum and milk samples from brucella RB and CFT negative (n = 881) and positive (n = 755) animals were used. Standardization of tests was through the Bommeli ELISA-BESW (*Brucella Bang*) standard and our Institute's (MKD) working standards (positive serum and milk based on *B. melitensis* antigen).

Results: Validation of serum/ milk ELISA for detecting ovine and caprine brucellosis was completed. The specificity obtained for the serum ELISA was 99.0% for the Bommeli system (at cut-off of 30% of positivity – PP) and 99.4% for the MKD system (at cut-off 15% PP). The sensitivities of serum ELISAs at the same cut-off were 98.5% for the Bommeli and 96.6% for the MKD test. Parallel milk samples from the same animals showed a specificity of 99.5% in the Bommeli system (at cut-of 30% PP) and 99.8% in the MKD system (at cut-off 25% PP). The sensitivity of the milk ELISAs were 94.6% for the Bommeli test and 95.6% for the MKD test.

Conclusion: The Bommeli ELISA and MKD ELISA were successfully standardized and validated as confirmatory tests for the diagnosis of *B. melitensis* in sheep and goat samples (milk/sera). Using our Institute's milk standard, we confirmed successful screening of brucellosis in pooled milk samples from 100 sheep and 100 goats.

Key words: sheep, goats, *Brucella melitensis*, ELISA-serum, ELISA-milk, validation.

Introduction

B.melitensis infection in small ruminants continues to be a problem in many countries, especially in the Mediterranean region and Eastern Europe [1–6].

In Macedonia, brucellosis caused by *B. melitensis* is one of the most important diseases in small ruminants. The impact of its effects on sheep and goat production and reproduction (abortions), and especially its human health hazard, implies that this disease should be controlled and eradicated [4, 7, 8]. The disease has persisted for about 30 years and efforts of government programmes to effectively control and eradicate the disease have been not successful. From 2008 to the present, a new strategy has been introduced, based on "diagnose and remove" for infected animals and different types of vaccination have been performed, depending on the infection level in the epidemiological units [2, 3, 9].

The Republic of Macedonia has a domestic animal populations of about 215,000 cattle, 800,000 sheep, 115,000 goats and 235,000 pigs. The prevalence of brucellosis in sheep and goats *(B. melitensis,* biovar 2) for a period of 30 years was less than 2%. Brucellosis in cattle is limited to sporadic cases, especially where cattle cohabit with sheep and goats. Approximately 400-500 human cases have been reported annually in the last 20 years [7]. Diagnosing and control/eradication of the disease using the classical serological methods has been: the Rose Bengal plate agglutination test (RBT) used for screening flocks and individual animals and the Complement Fixation Test (CFT) and the Indirect i-ELISA serum/milk (used as a confirmatory test for small ruminants and for cattle). The above-mentioned tests and temporary serum agglutination test (SAT) and milk ring test (MRT) are used for bovine brucellosis. For pooled milk samples (screening test) there are useful tests, such as MRT (only for cattle) and i-ELISA (for cattle, sheep and goats).

The intention of this work is to report the efforts to find a suitable ELISA test for individual serum/milk samples, like confirmatory and pooled (bulk) milk samples like a screening test, especially for small ruminants. Preliminary data in this field have previously been presented in 1991, 1994 and 1997 [8, 10–13]. It is known that *B. melitensis* infections in small ruminants are not as well confirmed by using serological tests such as those for *B. abortus* in cattle [1–3, 14, 15]. Using the RBPT and CFT and slaughtering infected animals, it seems impossible to eradicate the disease among the small ruminant populations. Our validation has shown that the ELISA serum/milk tests are applicable for routine diagnosis of brucellosis infection in small ruminants. These tests are more accurate, rapid and inexpensive, and may be automated for detecting brucellosis, especially in infected flocks [16, 17].

In a project supported by FAO/AEA and the World Bank (2003), from 1995 to 2003 the Veterinary Institute in Skopje in carried out the largest and longest experiments in the field of introducing serum/milk – ELISA technology for diagnosing *B. melitensis* in small ruminants [8, 10, 11]. Additional studies were performed (2003–05) to introduce the Polymerase Chain Reaction (PCR) by extracting DNA of *B. melltensis* directly from the raw milk of small rumi-

nants, but without success. According to a review of literature and the opinion of the independent experts (6 missions) of the projects, they were apparently the first scientific studies of diagnosing of *B. melitensis* in sheep and goats by individual serum/milk ELISA, especially using bulk (pooled) milk. In view of the lack of international standards for diagnosing *B. melitensis* in small ruminants, these methods were for the first time prepared as standards for national and international use. The results of this work were utilized in two directions; first, the method was used as a prevalidation test [7, 10, 11, 16] and second, it was successfully performed as a validation test for diagnosing *B. melitensis* in sheep and goats by serum / milk ELISA [12, 17].

Methods

The best method for validation of a test for diagnosing *B. melitensis* in small ruminants for the serum/milk ELISA is the use of the "gold standard" from truly positive animals where *B. melitensis* is isolated from each animal. But in the absence of such animals (according to Jacobson), we used a different "gold standard", animals serologically positive and negative to the RBPT and CFT, and compared results with iELISA [18, 19, 20].

Serum and milk specimens from *Brucella* negative (n = 881) and positive (n = 755) animals were used (material for investigation). The samples were selected and taken from selected regions of R. Macedonia. The negative serum/milk samples originated from a region where no brucellosis had been recorded, either clinically or serologically, during the last 40 years. The individual animals were sampled simultaneously for both serum / milk (from each one). The positive serum/milk samples were obtained from permanently infected flocks, where clinical symptoms (abortions) occurred and family members of the famers were heavily infected.

For preparing standards for diagnosing *B. melitensis*, in one epidemiological unit, we indentified one heavily infected flock of sheep and goats. We identified 9 sheep and 6 goats with high serological titers. These animals were separated for one month, milk was collected every day and serum every 7 days. After that period, these animals were purchased for culling in the abattoir. Serum and milk samples were frozen at -70°C. Each serum and milk sample was titrated by the i-ELISA method, using positive control serum samples from Weybridge (bovine origin), Bulgarian national reference serum at CFT diagnosis (rabbit) and from the Bommeli ELISA kit (2nd International anti brucella abortus serum, diluted 1: 64). For the determination of a positive cut-off for RBPT minimum positivity of (+), CFT (1: 5 dilution equivalent to 35 CFU) was used, and for serum/milk ELISA a percent positivity (PP) of strong (++) positive standard was used [1, 21–24]. The weak positive standard (+) was obtained with the dilution of the strong positive standard.

     The reagents for performing the RBT and CFT tests were obtained from Rhone- Merieux and the ELISA kits from Dr. Bommeli, Switzerland. Testing of samples (serum/milk) was performed in duplicate and controls in triplicate. The average value of optical density (OD) and validation of standards and samples were according to manufacturer's recommendations (Dr. Bommeli).

     The serum/milk standards used in the Macedonian (MKD) ELISA were obtained from sheep and goats, previously tested negative/positive on RBT, CFT and commercial ELISA tests for both serum and milk. The titrations of different positive and negative serum/milk domestic standards were performed [16, 17, 25–27]. For measuring the optical density, a Multiskan Plus-Finland spectrophotometer was used [12, 16, 17]. Simultaneously, other international cattle standards, such as Weybridge, Bommeli and Bulgarian, were compared with our sheep and goat standards.

     The Bommeli (Chekit) ELISA-Brucellotest kit was used according to the protocol of the producer. The antigen used in the MKD ELISA was *Brucella* Bang LPS (FAO/IAEA), determined by chess titration with positive standard [21]. The titration of the anti-ruminant monoclonal conjugate (Bommeli AG, Switzerland) was also performed [21, 23]. The MKD positive standard was used in a final dilution of 1: 3000 and the negative in a dilution of 1: 200. The procedure of MKD ELISA (Box 1) started with serum samples at a dilution of 1: 200 and the milk samples 1:2 in diluent buffer (PBS, pH 7.4 with 0.05% Tween 20).

Box 1. Procedure for the MKD ELISA
1. Dilute samples and controls to the appropriate wells   (serum samples are diluted 1:200, and milk samples 1:2 in diluent buffer)
2. Add samples and controls in a volume of 100 µL/well
3. Incubate in a shaker for 1 hour at 37°C
4. Wash plates 3 times with 300 µL wash buffer
5. Add conjugate diluted 1:200 in diluent buffer in a volume of 100 µL/well
6. Incubate in a shaker for 1 hour at 37°C
7. Wash plates 3 times with wash buffer with 300 µL wash buffer
8. Add chromogen in a volume of 100 µL/well
9. Incubate 60 minutes at room temperature
10. Add the stopping solution in a volume of 100 µl/well

     The controls and samples were added in an amount of 100µL/well and incubated 1 hour in a shaker at 37°C and washed 3 times with a buffer before adding the conjugate. The conjugate was used in a dilution of 1: 200 in diluent buffer and added in an amount of 100µL/well. After incubation of 1 hour in a shaker at 37°C the plates were washed 3 times and the chromogen added. The

chromogen was created by diluting 1 tablet of ABTS (Sigma) in 6 ml of phosphate/citrate buffer (1 tablet in 100 ml dd water), (Sigma).

The stopping solution was prepared as a 4% dilution of sodium dodecyl sulphate (SDS) double distilled water. The optical densities were measured using a filter of 405 nm.

For the interpretation of results ordinary and special statistical methods were used, as well as our own approach for interpretation [18–20, 22, 23, 26, 27].

Results and discussion

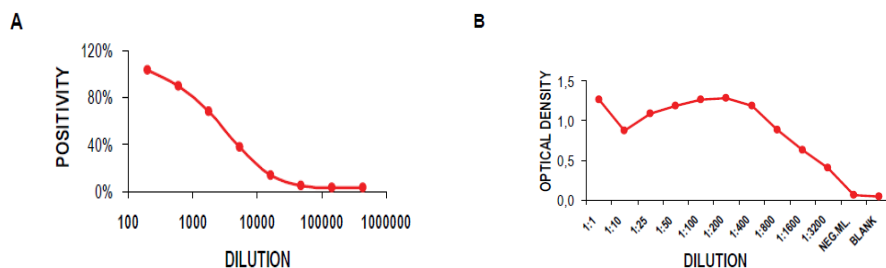The results of the whole study are shown in the following figures and tables:

Figure 1 – Titration of the standards. A) Titration of the serum positive standard. The sheep MKD serum standard gave a similar curve to international bovine standards (BESW); B) Titration of the milk positive standard in negative milk. The dilution curve shows that it is possible to use the ELISA milk standard for determination of brucellosis in pooled milk samples of at least 100 animals
Слика 1 – Титрација на стандардите. А) Титрација на серум позитивните стандарди. МКД серум стандардот кај овци даде слична крива како интернационалниот говедски стандард (BESW); Б) Титрација на позитивен стандард за млеко во негативно млеко. Кривата на разредување покажува дека е можна употреба на ЕЛИСА стандард за млеко за утврдување на бруцелоза во примероци на молзено млеко од најмалку 100 животни

A mixture of all sheep positive standard serum samples and a mixture of all positive standard sheep milk samples was also included in the titration curve showing for the first time (2003) a successful standardization of sheep serum and milk samples for validation experiments. These standards, achieved from these experiments aimed at identification of anti-brucella antibodies in sheep and goat serum and milk samples, have been established as national reference materials for standardization of the ELISA techniques.
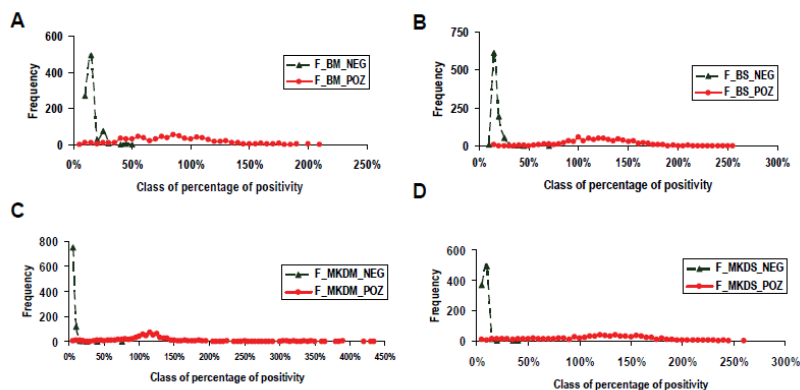
Figure 2 – Frequency distribution of the positive and negative samples: A) in milk samples analyzed by Bommeli ELISA system (755 positive and 881 negative samples); B) serum samples by Bommeli ELISA system; C) in milk samples by MKD ELISA system; D) in serum samples by MKD ELISA system

Слика 2 – Дистрибуција на фреквенциите на позитивните и негативните примероци: А) во примероци од млеко анализирани со Бомели ЕЛИСА систем (755 позитивни и 881 негативни примероци); Б) во примероци од серум анализирани со Бомели ЕЛИСА систем; В) во примероци од млеко анализирани со МКД ЕЛИСА систем; Г) во примероци од серум анализирани со МКД ЕЛИСА систем
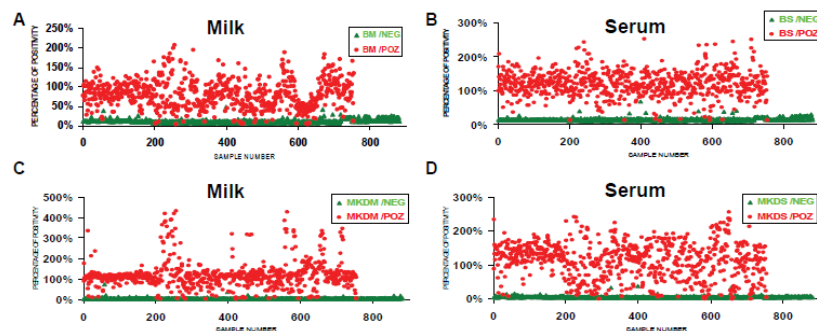

Figure 3 – Dispersion of the percentages of positivity of positive and negative samples: A) in milk analyzed by Bommeli ELISA system; B) in serum samples by Bommeli ELISA system; C) in milk samples by MKD ELISA system; D) in serum samples by MKD ELISA system

Слика 3 – Дисперзија на процентите на позитивност на позитивните и негативните примероци: А) во примероци од млеко анализирани со Бомели ЕЛИСА систем; Б) во примероци од серум анализирани со Бомели ЕЛИСА систем; В) во примероци од млеко анализирани со МКД ЕЛИСА систем; Г) во примероци од серум анализирани со МКД ЕЛИСА систем
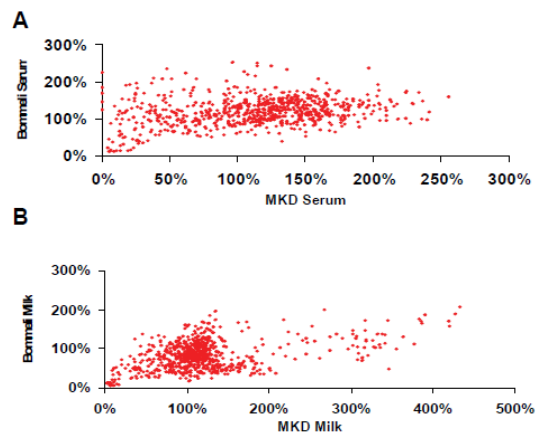
Figure 4 – Correlation between the percentages of positivity of the Bommeli and MKD ELISAs: A) in serum samples; B) in milk samples

Слика 4 – Крелација йомеѓу йроценшише на йозишивносш на Бомели и МКД ЕЛИСА: А) во йримероци од серум; Б) во йримероци од млеко
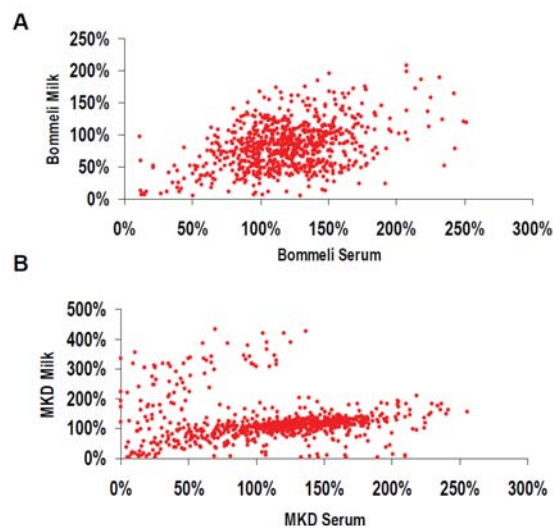

Figure 5 – Correlation between the percentages of positivity in serum and milk samples: A) analyzed by Bommeli system; and B) by MKD system
Слика 5 − Корелација йомеѓу йроценшише на йозишивносш во йримероци од серум и од млеко: А) анализирани со Бомели сисшем; и Б) со МКД сисшем

Table 1 – Табела 1

Optimisation chart for determination of the cut offs in different types of ELISAs
(A total of 881 negative and 755 positive samples have been analyzed)
Опͭͭимизациски ͖риказ за оͨределување на границиͭͭе во различни ͭͭиͨови
на ЕЛИСА (вкуͨно 881 неͱаͭͭивни и 755 ͨозиͭͭивни анализирани ͨримероци)

CUT_OFF	BOMMELI MILK		BOMMELI SERUM		MKD MILK		MKD SERUM	
	SPEC*	SENS**	SPEC*	SENS**	SPEC*	SENS**	SPEC*	SENS**
5%		99.9		100.0	84.7	99.1	41.8	98.9
10%	30.6	98.7	1.0	100.0	98.3	97.7	97.8	98.1
15%	86.8	97.5	70.1	99.1	99.1	96.7	99.4	96.6
20%	90.0	97.0	91.8	98.9	99.5	96.2	99.7	95.1
25%	98.6	95.8	98.0	98.7	99.8	95.9	99.7	93.4
30%	99.5	94.6	99.0	98.5	99.8	95.6	99.7	91.7
35%	99.5	93.1	99.4	98.3	99.8	95.0	99.9	90.6
40%	99.7	88.7	99.8	97.6	99.9	93.8	100.0	88.9

Green represents the determined optimal cut-offs in each of the ELISAs.
SPEC* = specificity; SENS** = sensitivity

With this data, it is possible to evaluate the diagnostic specificity (DSp) and diagnostic sensitivity (DSe) of the Chekit-Brucellotest ELISA (Dr. Bommeli) and our Institute (MKD) ELISA. A total of 881 negative and 755 positive samples of both sera and milk were included in the analyses. Samples used in the preliminary validation were previously confirmed with the "gold standard" methods, RBT and CFT. The specificity obtained for the serum ELISA was 99.0% for the Bommeli system (at a cut-off of 30% PP) and 99.4% for the MKD system (at a cut-off 15% PP). The sensitivity of serum ELISAs at the same cut-offs were 98.5% for the Bommeli and 96.6% for the MKD test. Parallel milk samples from the same animals showed a specificity of 99.5% in the Bommeli system (at a cut-of 30% PP) and 99.8% in the MKD system (at a cut-off 25% PP). The sensitivity of the milk ELISAs was 94.6% for the Bommeli test and 95.6% for the MKD test.

Taken together, we successfully standardized and validated the Bommeli ELISA and MKD ELISA. It is possible to diagnose brucellosis in a quick, safe, exact and cheap manner by using the i-ELISA (serum/milk) technique. Also, by introducing ELISA analyses of pooled milk samples, it is possible to maintain a "free status" of flocks, certain regions, epidemiological units or an entire country. It is very important for farmers and countries to certify brucellosis "free status" for numerous reasons. Maybe, this is one of the easiest and cheapest

ways for the protection and fast screening of large flocks for brucellosis. However, following identification of infected farms, what then would be the ideal policy that should be taken to control and further eradicate the disease?

It is possible to examine both individual serum/milk samples (confirmatory test) and pooled milk samples (screening test) with the i-ELISA method. ELISA on pooled milk samples is useful for examination of flocks of 100 sheep and 100 goats (*B. melitensis*).

Conclusions

According to the results of the preliminary validation of individual serum/milk ELISA and pooled milk ELISA, it is possible to conclude the following:

1. To examine individual serum/milk samples (confirmatory test) and pooled milk samples (screening test) is possible by using the ELISA. An ELISA for pooled milk samples is useful for examination of flocks of 100 sheep or 100 goats.

2. The results of MKD ELISA highly correlated to those of classical methods (RBT and CFT) in determination of *Brucella* infected sheep and goats;

3. The specificity of the serum ELISA Bommeli system was 99.0% (at a cut-off of 30% PP) and in the MKD system it was 99.8% (at a cut-off 25% PP);

4. The sensitivity of serum ELISA (at a cut-off of 30% PP and 15% PP for Bommeli and MKD ELISA, respectively) was 98.5% for Bommeli and 96.6% for the MKD test;

5. The specificity of the milk ELISA was 99.5% in the Bommeli system (at a cut-off 30 % PP), and 99.8% in the MKD system (at a cut-off 25% PP).

6. The sensitivity of the milk ELISAs were 94.6% (at a cut-off 30%) for the Bommeli test and 95.6% (at a cu-off 25%) for the MKD test;

7. The diagnostic performance of MKD ELISA was very similar to that of recognized, commercial producer Bommeli, but at different cut-offs as determined in our laboratory;

8. Application of milk ELISA in the routine diagnosis of infected sheep or goats will significantly decrease the costs of the control/eradication programmes, without losses in the diagnostic performances compared to the serum ELISA;

9. The results of the validation encourage the full OIE validation of the serum/milk ELISA for small ruminants, using *B. melitensis* antigen;

10. The results of this validation test need to be further confirmed with the direct detection of *Brucella melitensis* in milk samples by PCR, through additional investigations.

11. Recently, OIE Reference Laboratories for Brucellosis have established an OIE International Reference Serum for *B. melitensis* in small ruminants (ISaBms). It is now possible to compare the MKD national serum and national milk standards to the international ISaMbs serum.

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Р е з и м е

ДИЈАГНОСТИЧКА ВАЛИДАЦИЈА НА БРУЦЕЛОЗАТА КАЈ ОВЦИТЕ И КОЗИТЕ СО УПОТРЕБА НА СЕРУМ- И МЛЕКО-ELISA

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Цел: Да се воведе и валидира нова метода за дијагностицирање на бруцелозата кај овците и козите на брз, прецизен и евтин начин со помош на i-ELISA (серум/млеко) техниката.

Меѿоди: Користени се примероци на серум и млеко кои на бруцелоза со RB и реакција на врзување на комплементи (РВК) се негативни (n = 881) и позитивни (n = 755). Стандардизација не тестовите беше направена преку Bommeli ELISA-BESW *(Brucella Bang)* стандардот и работните стандарди (МКД) на нашиот институт (позитивен серум и млеко врз основа на *B. melitensis* антигенот).

Резулѿаѿи: Валидирање на серум/млеко ELISA за откривање на бруцелозата кај овците и козите беше извршено. Специфичноста добиена за серум ELISA беше 99.0% според Bommeli системот (со гранична вредност – cut-off – од 30% на позитивност – ПП) и 99.4% според МКД системот (со

гранична вредност – cut-off – од 15% на позитивност – ПП). Чувствителноста на серум ELISA за истата гранична вредност беше 98.5% според Bommeli и 96.6% според МКД тестот. Паралелните примероци на млеко од истите животни покажаа специфичност од 99.5% според Bommeli системот (со гранична вредност од 30% ПП) и 99.8% според МКД системот (со гранична вредност од 25% ПП). Чувствителноста на млеко ELISA беше 94.6% според Bommeli и 95.6% според МКД тестот.

Заклучок: Bommeli ELISA и МКД ELISA беа успешно стандардизи-рани и валидирани како тестови за потврдување на дијагнозата на *B. melitensis* со примероци од овци и кози (млеко/серум). Користејќи го стандардот за млеко од нашиот институт, ние го потврдивме успешниот скрининг на бруцелозата од збирни примероци на млеко од 100 овци и 100 кози.

Клучни зборови: овци, кози, *Brucella melitensis*, ELISA-серум, ELISA-млеко, валидација.

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