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RESEARCH ARTICLE

Comparative Production of Rapid Slide Agglutination Test (RSAT) Antigen Used in Serological Diagnosis of *Brucella canis* in Different Culture Media in the Fermenter [1]

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Summary

In this study, rapid slide agglutination test (RSAT) antigens were produced by using homologous (*Brucella canis* RM6/66 and a wild *B. canis*) and heterologous brucella strains (*B. abortus* 45/20 and *B. melitensis* B115) for a quick, practical and economic diagnosis of *B. canis* infection in dogs and humans. All the test strains were grown in three different culture media, namely, trypton liquid media (TLM), brucella broth media (BBM) and brain heart infusion broth media (BHIBM) to compare their optimal growths. The best growth rate was obtained by *B. canis* RM6/66 strain. *B. melitensis* B 115 showed the poorest growth in all test media. The culture media that supported the best growth rate in all tested strains was TLM. RSAT antigens from each test strains were produced without any stringy formation and standardised successfully. It was thought that *B. canis* RM6/66 and/or wild *B. canis* might be suitable candidate in commercial RSAT antigen production according to the test results supporting the fact that using homotypic strain increases the diagnostic sensitivity. As a conclusion, it was decided that this antigen will be able to used extensively as a part of routine clinical examination in dogs in Turkey. Furthermore, it was considered that to include RSAT in humans as a part of routine brucellosis diagnosis could help to evaluate the disease more accurately.

Keywords: *Brucella canis, RSAT, Antigen production, Rough Brucella strains*

Brucella canis'in Serolojik Tanısında Kullanılan Çabuk Lam Aglütinasyon Test (ÇLAT) Antijeninin Fermentörde Farklı Besiyerlerinde Karşılaştırmalı Olarak Üretimi

Özet

Bu çalışmada *Brucella canis* infeksiyonunun çabuk, pratik ve ekonomik teşhisi için homolog ve heterolog suşlar ile hazırlanmış çabuk lam aglütinasyon test (ÇLAT) antijenleri üretildi. Antijen üretiminde homolog suşlar (*B. canis* RM6/66 suşu ve saha *B. canis* suşu) ve heterolog suşlar (*B. abortus* 45/20 ve *B. melitensis* B115) kullanıldı. Bu amaçla çalışmada kullanılacak test suşları, tripton sıvı besi yeri (TSB), brusella broth besiyeri (BBB) ve brain heart infüzyon broth besiyeri (BHIB) olmak üzere 3 ayrı besi yerinde optimal üremeyi değerlendirmek üzere karşılaştırmalı olarak üretildiler. Test besiyerlerinde en iyi üremeyi *B. canis* RM6/66 suşu ve en zayıf üremeyi *B. melitensis* B 115 suşu gösterdi. Suşların en iyi üredikleri besi yerleri TSB oldu. Sonuçta tüm test suşlarından hazırlanan ÇLAT antijenleri başarılı bir şekilde üretilip standardize edildiler. Test sonuçlarına göre *B. canis* RM6/66 ve/veya saha *B. canis* suşlarının ticari ÇLAT antijeni üretimi için seçilebileceği düşünüldü. Sonuç olarak üretilen ÇLAT antijeninin Türkiye'de köpeklerde, yaygın olarak rutin klinik muayenenin bir parçası olarak hastalığın serolojik teşhisinde kullanılabileceği ve bu testin insanlardaki klasik bruselloz tanısına dahil edilmesinin, hastalığın insanlardaki durumunun daha sağlıklı olarak değerlendirilmesine olanak sağlayacağı sonucuna varıldı.

Anahtar sözcükler: *Brucella canis, ÇLAT, Antijen üretimi, Rough Brucella suşları*

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INTRODUCTION

Canine brucellosis is an important cause of canine abortion and infertility worldwide. Although the disease is reported from many countries of the world, the exact epidemiologic data regarding to its prevelance in many parts of the world are missing [1-3]. Because of the signs of the disease are relatively mild, diagnosis of the disease is usually based on bacterial isolation which is laborious and lengthy and its sensitivity might be decreased by the intermittent bacteremia [4]. Therefore, serological tests are most commonly used for routine clinical diagnosis. The most widely used serological tests are rapid slide agglutination test, tube agglutination test with and without 2-mercaptoethanol (2ME-RSAT and RSAT; 2ME-TAT and TAT, respectively) and agar gel immunodiffusion test (AGID) [3,5,6].

Tube agglutination test and 2ME-TAT have some technical disadvantages that limit their widespread usage in the field: Inability to detect low level antibody titers in chronically infected and bacteremic dogs, and prozone phenomena. Besides, these tests take at least 48 hours to interpret the results. AGID test has been used but it is a complex test, fails to detect early stages of infection and sometimes precipitin lines are difficult to interpret [3,7]. RSAT is very sensitive, practical and easily interpreted screening test [8-12]. The only disadvantage of RSAT is that many false positive reactions are observed because of the heterospecific reactions between surface antigen of *B. canis* and naturally rough organisms, nonsmooth *Brucella* species, and a variety of other bacterial species [13,14]. Therefore all the RSAT positive samples should be confirmed by more specific tests to alleviate false positives [11].

Production of suitable diagnostic antigen requires considerable attention because of *B. canis* shows great tendency to become ropy and form stringy sediments after long incubation period and in pH below 6.8. These sediments are tried to obviate by adjusting pH of growth media and resuspending buffer to pH level above 7.4 [9,15].

The disease was reported serologically in Turkey and seroprevelance rates in dogs that ranged from 6.3% to 12.7% have been reported in different regions in Turkey [16-18]. There are extremely few data regarding to human infection [19-21]. The impact on public health might be underestimated because of lack of standardized antigens, unawareness of medics to use these specific antigens instead of those for detecting smooth lipo-polysaccharide (sLPS) and poor reporting system in many parts of the world [22,23].

In this study, we present data on the production of four different RSAT antigens prepared by different rough *Brucella* strains and their growth rates in different culture media. The aim of this study was to produce appropriate RSAT antigen that can be used serological diagnosis of canine brucellosis. This antigen also will be able to used in serological diagnosis of brucellosis caused by *B. ovis* infection in sheep and goats and human brucellosis caused by rough species.

MATERIAL and METHODS

Reference Bacterial Strains and Sera

Brucella canis RM6/66 ATCC 23365 strain, *B. canis* positive serum prepared in rabbit (BC4) and negative dog serum, standardized RSAT antigen were kindly provided from Animal Health Veterinary Laboratory Agency (AHVLA), UK. *B.canis* positive serum obtained from experimentally infected specific pathogen free (SPF) beagle by oro-nasal route was kindly supplied from Dr. Carmicheal (Cornell University). Other test strains of *B. abortus* 45/20, *B. melitensis* B115 and *B. canis* wild isolate (10-PBC-87) and other reference materials used in classical biotyping were obtained from culture and reference materials collection from National Reference Laboratory for Brucellosis in Pendik, Istanbul, TURKEY.

Culture Media and Solutions

All the liquid and solid culture media and Tris-maleate buffer (TMB 0.4 M), PBS (0.15 M) and Rose Bengal dye solution (2%) were prepared to the methods described by Alton et al.[24]. *Brucella* Broth Medium (BBM, Becton Dickinson) and Brain Heart Infusion broth media (BHIBM, Himedia) were prepared according to manufacturer's instructions.

Bacteriological Studies

All test strains were identified according to the classical biotyping procedures described in OIE Manuel [25].

Preparation of Seed Cultures of Test Strains

Freeze-dried master culture of test strains were re-hydrated in sterile PBS (pH 6.4) and inoculated on serum dextrose agar slants. The slants were incubated at 37°C for 3 days.

Fermentation

Bioreactor (Biostat A Plus CC, Sartorius, Germany) with a working volume of 1 L was used to produce each test strain for a bulk production. Each of the test media was sterilized by filter and aseptically transferred to previously sterilized bioreactor. The cells from the each seed culture were inoculated to be 5% into bioreactor. The pH of the media was adjusted to 7.4±0.2. Temperature was fixed at 36±1°C. The aeration was maintained by addition sterile compressed air at 4-8 liter/min and agitation speed controlled at 300-600 rpm and the production process was started and after seed inoculation, samples were collected at 24 h interval up to 96 h to determine the viability count,

pH, dissolved oxygen, mucoid appearence, sticky sediment formation, purity and colonial morphology. All viability counts were performed in three separate occasions by three different persons and the results were expressed as the mean value of the viable counts.

Preparation of Stock Antigens for Slide Agglutination Test

Antigens prepared from each test strains were produced as described with some modifications [9,10]. Briefly, all the test strains cultured in bioreactors were harvested 96 hours later of their incubation and inactivated by heat at 60°C for 2 h. Inactivated cultures were checked for mucoidness, purity and viability. Then all test cultures were centrifuged at 3500 g for 30 min. After centrifugation, the supernatant fluid was decanted and resulted pellet washed twice in PBS (0.15 M, pH 7.4). The washed cells were resuspended to approximately 10% packed cell volume (PCV) in PBS and labeled as "stock RSAT antigen" for future use of standardized RSAT antigens production. The stock cell suspensions then were stained by the stock solution of 2% Rose Bengal (Sigma) dye previously filtered through Whatman no 1 filter paper. Stained suspensions were agitated at slow speed with magnetic stirrer overnight at 4°C to avoid foaming and then centrifuged at 3500 g for 30 min to sediment stained bacteria. Stained pellets were suspended with vigorous shaking in 0.4 M TMB buffer (pH 9.0) using a magnetic stirrer and filtered to exclude possible large bacterial clumps. The suspensions were diluted to PCV of 6% and filtered through sterile glass wool. Finally, sodium azide was added to each suspension for a final concentration of 0.1%.

Standardisation of RSAT Antigens

The prepared four separate RSAT antigens were standardised by using positive BC4 serum dilutions of 1/10, 1/40 and 1/80 made in TMB buffer. Equal amount of serum and each test antigens and standard antigen were mixed on a glass plate with a wooden stick and the plate was rocked gently for 4 min. Positive and negative control sera were included in each test. If agglutination appears within 15 sec, it was recorded as a "++" reaction and any agglutination occured later was scored as a "+" reaction. No agglutination was recorded as negative. Each test antigens was evaluated as standardised when it produced positive agglutination at a 1/10 and 1/40 dilution of BC4 serum and no agglutination at a 1/80 dilution of control and negative serum. Each test was carried out in three separate trials. Final products were checked for sterility.

Each test RSAT antigens were also tested with serially diluted *B. canis* positive serum from experimentally infected SPF beagle. This serum (AS 1048) which was kindly supplied from Dr. Carmicheal was obtained from a beagle experimentaly infected with *B. canis* by oro nasal route 9 months post infection and had TAT test titer of 1/500. The degree of agglutination was scored from +1 to +4 of agglutination reaction.

Statistical Analysis

For statistical comparisons of the test culture media and test strains, one-way analysis of variance (ANOVA) was done (with 95% confidence level) using SPSS (IBM, SPSS Inc.).

RESULTS

All test strains were identified and results were consisted with described reference strain characteristics [25]. Test results were shown in *Table 1*. All test strains were lysed by R/C phage while none of them was lysed by Tbilisi phage *(Fig. 1)*.

Table 1. *Cultural characteristics of reference and test strains*
Tablo 1. *Referans ve test suşlarının kültürel özellikleri*

Cultural Characteristics	*B. canis RM/66*	*B. abortus 45/20*	*B. melitensis B115*	*B. canis 10-PBC-87*
Agglutination with acriflavine	+	+	+	+
CO_2 requirement	-	-	-	-
H_2S production	-	+	-	-
Growth on thionine 20 µg/ml	+	-	+	+
Growth on basic fuchsin 20 µg/ml	-	+	+	+
Agglutination with monospecific A serum	-	-	-	-
Agglutination with monospecific M serum	-	-	-	-
Agglutination with monospecific R serum	+	+	+	+
Serum requirement	-	-	-	-
Urease production	+	+	+	+
Oxidase production	+	+	+	+
Lysis by Tbilisi phage	-	-	-	-
Lysis by R/C phage	+	+	+	+

Fig 1. Lysis evaluation of test strains by R/C and Tbilisi phage
Şekil 1. Test suşlarının R/C ve Tbilisi fajı ile lizis değerlendirmesi

All four strains were grown in the three different culture media, namely, TLM, BHIBM and BBM in a bioreactor. Samples were taken daily intervals for a viability count. Best growth (CFU/ml) was obtained after 72 h of incubation for each test strain and in each test culture media *(Table 2)*. Each test culture was terminated after 96 h of incubation and viability counts for each test strain in test media were summarized in *Table 3*. During growth of the test strains, bacterial cultures did not become stringy and no sticky sediments formed. But bacterial growth in BHIBM produced more foam compared to others and to compensate the bioreactor pumped antifoam solution into the reactor at more frequent intervals. The maximum viable count expressed as CFU per ml for all test culture media was obtained by *B. canis* RM6/66, *B. canis* 10-PBC-87, *B. abortus* 45/20 and *B. melitensis* B 115 strains, respectively. The culture medium yielded the best growth for each test strain was TLM, BBM, and BHIBM, respectively.

There was no significant difference (P>0.05) in growth between *B. canis* RM6/66 and *B. canis* 10-PBC-87 in different test media at 95% confidence level but the viability counts of these strains were significantly different (P<0.05) from the rest of the strains. The viability counts were significantly higher (P<0.05) in TLM than those of BBM and BHIB. The number of bacteria found after 48 h of incubation were not significantly different (P>0.05) than those of 72 h of incubation.

RSAT antigens prepared from each test strains were standardized against reference antigens and known positive and negative sera. All of them agglutinated 1/10 and 1/40 serum dilution of BC4 serum while there was no agglutination occurred with 1/80 dilution of the control serum. No bacterial and fungal contamination was detected in any RSAT antigens *(Table 4)*.

RSAT antigens were also tested by various serum dilutions of positive dog antiserum (AS 1048). Agglutination degree was scored from +1 (minimum degree of agglutination) to +4 (maximum degree of agglutination) reaction. The weakest reaction was observed in the

Table 2. *Viability counts (CFU/ml) in test media after 72 hours of incubation in the bioreactor*
Tablo 2. *Biyoreaktörde 72 saatlik inkübasyon sonrasında test besiyerlerindeki canlılık sayımları (KOB/ml)*

Test Strain	Test Culture Media		
	TLM	BHIBM	BBM
B. canis RM6/66	42.5X10^9	32.5 X10^9	35.8 X10^9
*B. canis*10-PBC-87	31.3 X10^9	24.9 X10^9	30.3 X10^9
B. abortus 45/20	18.9 X10^9	12.7 X10^9	15.9 X10^9
B. melitensis B115	11.9 X10^9	8.9 X10^9	10.3 X10^9

Table 3. *Viability counts (CFU/ml) in test media after 96 hours of incubation in the bioreactor*
Tablo 3. *Biyoreaktörde 96 saatlik inkübasyon sonrasında test besiyerlerindeki canlılık sayımları (KOB/ml)*

Test Strain	Test Culture Media		
	TLM	BHIBM	BBM
B. canis RM6/66	39.1X10^9	30.5 X10^9	31.2 X10^9
*B. canis*10-PBC-87	22.3 X10^9	18.3 X10^9	20.3 X10^9
B. abortus 45/20	17.6 X10^9	10.6 X10^9	12.6 X10^9
B. melitensis B115	9.1 X10^9	7.1 X10^9	8.8 X10^9

Table 4. *Results of standardization of RSAT antigens*
Tablo 4. *ÇLAT antijenlerinin standardizasyon sonuçları*

Test Strains of RSAT Antigens	BC4 Positive Serum Dilutions				Sterility
	1/10	1/40	1/80	Negative Serum	
B. canis RM6/66	++, ++,++	+, +,+	-, -,-	-, -,-	Sterile
B. canis PBC-10-87	++, ++,++	+, +,+	-, -,-	-, -,-	Sterile
B. abortus 45/20	++, ++,++	+, +,+	-, -,-	-, -,-	Sterile
B. melitensis B115	++, ++,++	+, +,+	-, -,-	-, -,-	Sterile
Standard antigen	++, ++,++	+, +,+	-, -,-	-, -,-	Sterile

Table 5. *Results of agglutination reactions of RSAT antigens with positive dog serum dilutions*
Tablo 5. *ÇLAT antijenlerinin pozitif köpek serumunun dilüsyonları ile aglütinasyon sonuçları*

Test Strains of RSAT Antigens	Positive Dog Serum (AS 1048) Dilutions					
	1/5	1/10	1/15	1/20	1/25	1/30
B. melitensis B115	++ ++	++ ++	+ +	- -	- -	- -
B. canis PBC-10-87	++++ ++++	+++ +++	++ ++	++ +	+ -	- -
B. abortus 45/20	++++ ++++	+++ +++	++ ++	+ +	- -	- -
B. canis RM6/66	++++ ++++	+++ +++	++ ++	+ +	- -	- -
Standard antigen	++++ ++++	+++ +++	++ ++	+ +	- -	- -

agglutination caused by *B. melitensis* B115. The sensitivity of RSAT antigen prepared from *B. canis* PBC-10-87 seemed to be highest since only this antigen gave positive reaction with the 1/25 dilution of positive dog serum. Results were shown in *Table 5*.

DISCUSSION

Serological diagnosis of the canine brucellosis constitutes a vital first stage in control and eradication of the disease. In this context, a sensitive, practical and easily interpreted screening serological test that allows to diagnose early stage of infection is highly required for routine diagnosis [3,4]. The production of RSAT antigens that employs M+ virulent strains requires a great care because of the tendency of the agent to become stringy and to form sticky sediments especially for longer incubation period. These M+ strains are always mucoid in nature and autoagglutinates in relatively acidic pH, which is something that is feared to be happened in agglutination test antigens, and might be responsible for lack of complete agglutinations [4,10,13]. Because of the difficulties reported in antigen production using *B. canis*, some heterologous strains were also chosen in antigen production to evaluate their potential usage in this test.

Today, commercial RSAT kits for *B. canis* employ *B. ovis* as antigen. However, being more fastidious and having strict requirements for CO_2 and serum for growth [24], *B. ovis* might not be the best alternative for RSAT antigens production. It was reported that the usage of *B. canis* instead of *B. ovis* in RSAT production reduced the false positives from 50% to 12% and they concluded that the specificity of RSAT might be increased using homologous strains in antigen production [15]. There are some other researchers who supported the same conclusions [12,26,27].

The growth rate of *B. canis* RM6/66 and *B. canis* 10-PBC-87 was found as similar statistically in all tested media ($P>0.05$). But heterologous strains grew poorly compared to the *B. canis* strains ($P<0.05$). The highest viability rate was obtained by using TLM, BBM, and BHIBM, respectively. TLM is the medium of choice for growth of brucella vaccine strains (*B. melitensis* Rev.1 and *B. abortus* S19) [25] and we have been using this medium for vaccine production in our laboratory. BHIBM supported the poorest growth rate among others. This might be due to dilution of growth media by antifoam solution pumped into bioreactor because of foamy growth of test strains in this medium. Since the aim of this study was to produce cost effective reagent for commercial bulk production in fermenter, we decided to determine the growth media that stimulated the highest number of bacteria and test strain that grew optimally in this test media for antigen production. The viability counts made from the samples taken after 48 h of incubation were not significantly different ($P>0.05$) than those of 72 h of incubation. But it was significantly different from those observed in 96 h of incubation. The commercial biological production should not only consider the highest yield but also consider to reach same results by using minimum sources. In this context, to harvest cultures after 48 h of incubation instead of 96 h of incubation will significantly decrease the operational costs by bioreactor and will serve to more economical production.

Longer incubation periods in growth media especially in those of having pH below 6.8 were reported to cause sticky sediments in culture media [24]. In our study, no such sediments were observed. Stained pellet was suspended with vigorous shaking in 0.4 M TMB buffer (pH 9.0). It was reported that nonspecific agglutination was reduced by increasing the molarity of TMB to 0.2 M or greater [10]. The disease was reported serologically in Turkey in both dogs [16-18] and in humans [19-21]. But there are no enough data to clearly identify the disease situation in Turkey. The lack of commercially available standardized antigens that show no cross reactivity between antibodies to *B. canis* and smooth *brucella* species in serologic diagnosis of the disease might be responsible for few studies conducted with relatively low number of serum samples.

Human infections are probably more common than indicated in published reports because routine brucellosis diagnosis does not include serological test with *B. canis* antigen. Therefore, infection with this species or any rough species of brucella might be undetected [23,28,29]. Therefore, a RSAT that employs a suitable rough strain should be a part of a routine serologic brucellosis scheme in humans in order to evaluate the disease situation more precisely.

It has been reported that *B. canis* strain has the advantage of making a satisfactory antigen by replacing AGID as a screeining test for diagnosis of *B. ovis* infection which causes a genital disease in sheep [26].

According to the results of the study, *B. canis* RM6/66 and *B. canis* PBC-10-87 strains might be used in commercial large scale antigen production by using TLM. However, future studies that determine sensitivity and specificity of each test antigen comparatively in the field with large number of serum samples in carnivores, sheep and goats and in humans would be necessary to decide the best antigen for serologic diagnosis of infection caused by rough *brucella* strains.

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