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Comparison of the isolation and inhibition abilities of selective media used for *Brucella* spp. Isolation

Mustafa Sencer KARAGÜL[1,*], Serkan İKİZ[2]
[1]Pendik Veterinary Control Institute, İstanbul, Turkey
[2]Department of Microbiology, Faculty of Veterinary Medicine, İstanbul University, İstanbul, Turkey

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Abstract: The aim of this study was to compare and contrast the isolation ability of selective media developed for *Brucella* spp. isolation and their inhibition ability against contaminant microorganisms. Fifty-one field strains biotyped from abortion case samples and 25 *Brucella* spp. negative organ samples were used. Strain suspensions and organ suspensions were prepared separately. The turbidity of the strain suspensions was measured via spectrophotometer. The bacterial density of the strain suspensions was prepared in such a way that the suspensions would have different turbidity values. Inoculation into Farrell, Agrifood Research and Technology Center of Aragon, Jones & Morgan, and Modified Thayer Martin media was performed simultaneously from a dilution of each strain suspension and organ suspension. It was incubated in a 37 °C, 5%–10% CO_2 condition for 5–8 days. *Brucella* isolates were identified via a conventional biotyping method. The results of this study illustrate that selective media have isolation and cultivation ability for *B. abortus biovar (bv) 3*, *B. abortus bv1*, *B. melitensis bv3*, *B. melitensis bv1*, *Rev1*, and *S-19* strains isolated from the field. There is more variation in the contaminant inhibition ability of the media compared to their isolation sensitivity. Farrell medium has 47 *Brucella* spp. isolations in 51 samples. It showed the highest performance with an isolation sensitivity of 92.1% and an inhibition ability of 80%. In this context, it might be suggested that researchers should initially use Farrell medium and, afterwards, use it simultaneously with another medium to be able to increase the isolation ability.

Key words: Biovar, *Brucella* spp., contaminant inhibition, isolation, selective medium

1. Introduction

Brucellosis is one of the most common zoonotic diseases caused by *Brucella* spp. (1). The World Health Organization (WHO) considers brucellosis a worldwide zoonotic infection that has led to important health and economic problems (2,3). In addition, the WHO laboratory biosecurity manual describes *Brucella* organisms as belonging to risk group 3 microorganisms (4–6).

Isolation of the bacteria is considered as a gold standard for the diagnosis of brucellosis (5). However, no other method that is sensitive enough for all kinds of biological samples instead of classical bacteriology has been suggested so far (7). Using a selective medium is essential for the isolation of *Brucella* spp. because of the high number of fast growing contaminant organisms in the diagnostic material (8,9). Due to the novel species and strains identified in different hosts and added to the genus *Brucella* since the development of the first selective medium, the ecological range of the genus has expanded (2,3,10). Several selective media such as Kuzdas & Morse, Mair, Jones & Morgan (JM), Morgan, Ryan, Farrell, and Ewalt have been developed over the years (9,11). Her et al. (12), De Miguel et al. (13), and Ferreira et al. (14) have introduced some other new media in recent years, as well. As a result of these developments, there are a lot of selective media with different basal media, antibiotic mixtures, and concentrations (15). It was emphasized that each medium has a particular effect on the species and biovar of the genus *Brucella* and contaminants on account of these differences (16,17).

In the current situation regarding the control and eradication program against brucellosis conducted in Turkey, serological diagnosis is not considered to be a valid test except when used for brucellosis-free herds. Therefore, selective media, which have a key role in bacterial isolation, have an undeniable importance for bacteriological culture as a valid test. For this reason, this study aims to evaluate the isolation and inhibition abilities of selective media that could be used in *Brucella* spp. isolation.

* Correspondence: msencerk@hotmail.com

2. Materials and methods

Fifty-one strains biotyped from aborted cases and 25 negative organ samples were used in this study; 15 *B. abortus* bv3 and 15 *B. melitensis* bv3, 5 *B. abortus* bv1, 5 *B. melitensis* bv1, 5 S19, and 6 Rev1 vaccine strains were utilized. Moreover, the media used are four different selective media and tryptic soy agar as a nonselective medium. The content of the Farrell, Agrifood Research and Technology Center of Aragon (CITA), Modified Thayer Martin (MTA) and JM as selective media are listed in Table 1 (8,11,13,18).

Amphotericin-B, which belongs to the same antifungal agent group as natamycin, has been preferred to natamycin or cycloheximid, which is included in the antimicrobial content of JM medium. Amphotericin-B is regarded as one of the antifungal agents recommended to be added into selective media for the first isolation of *Mycobacterium* spp., (19), *Campylobacter* spp. (20), and *Brucella* spp. (13).

For the preparation of 1 L from each selective medium, basal medium samples from Farrell, CITA, MTM, and JM were weighed in a 2 L flask. Each was rehydrated in 1 L of distilled water and sterilized (121 °C ± 3 °C for 20 min) by autoclaving. After autoclaving, media flasks were placed into a water bath to stabilize the media temperature at around 45 °C. An antibiotic mixture and sterile newborn calf sera were later added to the media based on their contents (8,11,13,18,21). Solidified media were incubated at 37 °C for 48 h for sterility control (22).

For the preparation of organ suspensions of abortion cases, approximately 1 g of *Brucella* spp. negative organ samples taken in a biosafety cabinet was mashed in a sterilized mortar and diluted 10 times with phosphate buffer solution (12,17). Stock field strains kept in storage at a temperature of −80 °C were inoculated to TSA medium. The turbidity of strain suspension was measured via spectrophotometer. Strain suspensions were prepared from bacterial culture grown on a medium plate. The bacterial density of the strain suspensions was prepared in such a way that the suspensions would have different turbidity values. Moreover, inoculation into Farrell, CITA, JM, and MTM media was performed simultaneously from a dilution of each strain suspension and organ suspension.

Field strain suspensions and organ suspensions were inoculated into Farrell, MTM, CITA, JM, and TSA media and incubated in a 37 °C, 5%–10% CO_2 condition for 5–8 days. Previously, all of the strain suspensions were prepared at a specific turbidity value such as the McFarland standard. However, in this study, the suspensions have different turbidity values and a different number of bacteria. With the help of this change, the aim was to approximate the variation of samples containing a different microbial burden and to investigate the effect of the bacteria count on isolation. The reason behind this is that the number of target bacteria included in abort case samples varies according to sample type and sampling time.

Brucella strains isolated at the end of the incubation period were identified by means of a conventional biotyping method. Biovar identification of isolates was carried out according to CO_2 requirements, thionin, basic fuchcine, safranine, penicillin, streptomycin, erythritol

Table 1. Contents of the selective media used.

Content	Farrell	CITA	MTM	JM
Basal medium	BMB-CS	BAB-CS	GC-H	SDA-CS
Bacitracin (IU/L)	25,000	-	-	25,000
Polymyxin (IU/L)	5000	-	-	6000
Nalidixic acid (mg/L)	5	-	-	-
Amphotericin-B (mg/L)	-	4	2.5	4
Natamycin (mg/L)	50	-	-	-
Nitrofurantain (mg/L)	-	10	10	-
Vancomycin (mg/L)	20	20	3	-
Colistin (mg/L)	-	7.5	7.5	-
Nystatin (IU/L)	100,000	100,000	100,000	-

BMB-CS: *Brucella* medium base with calf sera.
BAB-CS: Blood agar base with calf sera.
GC-H: GC agar base with hemoglobin.
SDA-CS: Serum dextrose agar with calf sera.

sensitivity, H_2S production, lysis by Tbilisi and rough/canis (R/C) phages, and agglutination with monospecific A and M antisera (21). In addition, the growth level of contaminant microorganisms was observed.

The media's inhibition ability against contaminant microorganisms was classified as total inhibition (TI) or partial inhibition (PI) with regards to contaminant growth diffuseness by counting colony-forming units (CFUs) (22). According to the contaminant colony counts, the inhibition ability of the media was listed by specifying the range of contaminant burden. The ranges were classified into four groups: one total inhibition group with the identification of no contaminant colonies and three partial inhibition groups, including the ones with less than 10, the ones between 10 and 100, and the ones with more than 100 colonies (22–24). When there was a difference between the media related to *Brucella* growth diffuseness, it was also labeled qualitatively as good growth, weak growth (WG), or zero growth (22).

The calculation of the isolation sensitivity of each medium was done via isolation results and by using a TP (true positive)/(TP + WN, wrong negative) × 100 formula (25,26). The results were evaluated with Pearson's chi-square test in SPSS 18.0.

3. Results

The results of the study illustrate that selective media have isolation and cultivation ability for *B. abortus* bv3, *B. abortus* bv1, *B. melitensis* bv3, *B. melitensis* bv1, Rev1, and S-19 strains isolated from the field. The number of *Brucella* isolations and the distribution of inhibition abilities are listed in Table 2.

As illustrated in Table 2, Farrell medium has the highest isolation rate, with 47 *Brucella* spp. isolations in 51 samples. In addition, Farrell medium outperforms the other media with regards to inhibition ability. The aforementioned results are listed in percentages in Table 3 to show the media's isolation sensitivity and inhibition ability. The value of the inhibition ability in Table 3 was calculated by taking the sum of TI and PI inhibition values (except when the PI >100 CFU) into consideration. Even if two of the values (PI <10 CFU, PI = 10–100 CFU) represent partial inhibition ability, they are regarded as sufficient inhibition ability because they provide an opportunity for the isolation of *Brucella* spp.

As can be seen in Table 3, there are not big differences between the isolation sensitivity of the media. When the results of Farrell medium, which has the highest percentage, and JM medium, which has the lowest percentage, have been analyzed, the chi-square value (X^2 = 2.468 P = 0.338) is not statistically significant. As there are bigger differences between the inhibition abilities of the media, the results of the statistical analyses are shown in Table 4. Although the P-value of the inhibition abilities is lower than that of the isolation sensitivity, the values obtained are not statistically significant as they are higher than 0.05.

What is more, when two different inoculations, including different counts of *Brucella* bacteria with the same negative organ suspensions, were compared and contrasted, the inoculation including more *Brucella* bacteria brought about *Brucella* spp. isolation at the level of WG in Farrell medium. However, the inoculation including fewer *Brucella* bacteria (approximately 5 times less) led to no isolation in Farrell medium. At the end of the incubation period of these two inoculations, Farrell medium had the lowest level of inhibition ability (PI > 100 CFU), which is considered to be inadequate.

Not only the inoculation of fewer bacteria but also the medium's inadequate inhibition ability should be considered as the reason for not observing isolation in the medium. In other inoculations, where the media

Table 2. The number of *Brucella* spp. isolation and the distribution of inhibition ability.

Media	TI[a]	PI (<10) CFU)[b]	PI (10–100) CFU)[c]	PI (>100) CFU)[d]	*Brucella* spp. (+)
Farrell	23	7	11	10	47
CITA	8	12	15	16	45
MTM	7	16	14	14	45
JM	8	9	17	17	44

a. Contaminants were totally inhibited
b. Less than 10 CFU contaminants were observed
c. Between 10 and 100 CFU contaminants
d. More than 100 CFU contaminants were observed

Table 3. Isolation sensitivity and inhibition ability of the media.

Media	Isolation %	Inhibition %
Farrell	92.1	80.4
CITA	88.2	68.6
MTM	88.2	72.5
JM	86.2	66.7

Table 4. Statistical analysis of inhibition abilities.

Chi-square tests	Inhibition ability	
Pearson's chi-square	X^2 value	P-value
Farrell & JM	2.468	0.116
Farrell & CITA	1.858	0.173
Farrell & MTM	0.872	0.350

showed adequate inhibition ability, there was no change in the growth level and isolation of *Brucella* bacteria, even though much fewer *Brucella* bacteria were inoculated.

4. Discussion

According to the growth results of the samples, Farrell medium had the highest isolation sensitivity and inhibition ability. MTM and CITA media, on the other hand, had the second best isolation sensitivity. In addition, they had lower inhibition abilities than Farrell medium. In this context, the media's similar order of success in terms of isolation sensitivity and inhibition ability illustrates a positive correlation between the contaminant inhibition and *Brucella* spp. isolation.

However, there is more variation in the contaminant inhibition ability of the media compared to their isolation ability. For this reason, the increases in the microbial burden of the sample might negatively affect inhibition ability, and it might cause a decrease in the isolation rate. In this sense, it is stated that fungal contaminant growth could inhibit bacterial growth, and this could lead to a decline in the sensitivity of the bacteriological diagnosis (17). Farrell and Robertson (27) stated that an equal isolation rate between media can be obtained by choosing media with high selectivity.

As the results gained from this study have indicated, enhancing the inhibition ability of selective media has a key role in increasing their isolation sensitivity. In this context, isolation sensitivity can be increased by means of following and developing the inhibition ability of selective media, in particular microflora of different samples.

Even in the inoculation of the strains in higher dilutions, the isolation result in the samples, where the contaminants have been adequately inhibited, has not changed. Therefore, these findings bring about the idea that the success in isolation can be primarily reached through the adequate inhibition of contaminants. These findings indicate the significance of collecting appropriate samples at the right time. Otherwise, when the target bacteria count decreases but the number of contaminant organisms increases in the samples, the decline of isolation sensitivity is a possible result.

Even though the difference between the media in terms of the isolation and inhibition abilities is not statistically significant, Farrell medium has the highest inhibition and isolation percentages. In a similar study, *Brucella* agar, Farrell, and CITA media were compared and contrasted and, in spite of the similarities between the isolation numbers of the media, Farrell medium was found to be the best in terms of the inhibition of the contaminants, and it is considered the best selective medium for microbiological diagnosis (28). In this sense, it might be suggested that researchers should initially use Farrell medium and, afterwards, use it with another medium simultaneously to be able to increase the isolation ability. In cattle brucellosis, as well, using two media simultaneously in order to increase isolation sensitivity is recommended (6,16). In this study, the isolation rate could reach 96% by simultaneously using Farrell medium with MTM or CITA. This rise in isolation sensitivity indicates the positive effect of using different

media simultaneously for higher diagnostic performance. Furthermore, it was pointed out that using CITA with Farrell or MTM simultaneously brought about the best diagnostic performance in the isolation of *B. melitensis*, *B. suis*, and *B. ovis*, respectively (13).

Büyükcangaz et al. used different media in the isolation of *B. melitensis* bv3 that caused epididymitis and orchitis in a Merino ram. During the incubation period, they could not observe any colony growth on the other agar plates except for *Brucella* on Columbia Agar (29). In another study, Büyükcangaz et al. investigated small ruminant abortion cases in northwestern Turkey within the scope of *Brucella* spp. by using two media. They identified an atypical variant of a *Brucella melitensis* strain among the other isolations (30). In this sense, the use of media with better inhibition ability and different content would be more plausible in order not to miss the isolation of these atypical strains normally identified only by biotyping.

The members of the genus *Brucella* are fastidious microorganisms and need a longer incubation period than the contaminants in the samples that grow quickly (8,9,21). In addition, it was also indicated that observing colonies on selective media may take a few more days than the usual incubation period on the nonselective media (21). In their study, Stack et al. (9) could not recognize the growth of *Brucella* bacteria in some of the infected milk samples with *Brucella* spp. In their opinion, it was because of the fact that *Brucella* colonies were masked by contaminants present in the milk samples. In light of the results obtained, maximizing the inhibition ability of the media may ensure easier *Brucella* spp. isolation among contaminants, and this ease may increase isolation sensitivity.

In this sense, in order to be able to passage *Brucella* suspect colonies before the contaminants cover the surface of the media, examining *Brucella* suspect colonies on the third day of incubation is recommended (21). In this study, too, it was observed that if the passage of the *Brucella* suspect colonies was delayed one more day, identifying the *Brucella* colonies became impossible because of the contaminants masking the *Brucella* colonies.

The findings of this study illustrate the benefits of using media that have better inhibition ability to increase isolation performance. Moreover, the use of two different media simultaneously and prioritizing other media that are as effective as Farrell medium could be recommended. The results obtained present an opinion about the isolation and inhibition ability of the selected media against strains circulated in the field and the microflora of abortion samples. Nevertheless, the aforementioned results have provided a basis for prospective studies that will evaluate antimicrobial agents that may be added to media against uninhibited contaminants and the addition of supporting components to increase the isolation rate.

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