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THE EVALUATION OF *BRUCELLA SPP*. ISOLATION RATES IN RUMINANT ABORTION CASES BY USING DIFFERENT SELECTIVE MEDIA

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ABSTRACT

The aim of this study is to evaluate the success of *Brucella* spp. isolation in ruminant abortion cases by using different selective media. To this end, 58 samples from ruminant abortion cases were utilized. 4 selective media; namely, Farrell Medium (FM), CITA Medium (CM), Modified Thayer Martin (MTM) and Jones & Morgan (JM) were preferred for isolation. In addition to these, one medium with antibiotics was used to extend the range of the results. Suspensions prepared from organ and fetal stomach contents were inoculated to media plates and incubated at 37C° for 5-8 days in 5-10% CO_2 condition. Conventional biotyping method was used to identify *Brucella* isolates within the level of species and biovar. MTM (67.2%) and Farrell (65.5%) outperformed the other media with regards to isolation rate. However, regarding the inhibition ability against contaminant microrganisms, Farrell (86.2%) and CITA (72%) have the highest and second highest percentages respectively. The media's inhibition ability was examined in the samples in which *Brucella* spp. isolation occurred to be able to investigate the correlations between isolation and inhibition. Lower isolation percentage was observed in the samples in which the media displayed the lowest inhibition ability against contaminants. In this context, using two different selective media with high inhibition ability against contaminants may be recommended to enhance the isolation rate. Moreover, the components stimulating the growth of *Brucella* strains might be added to the media to obtain better results.

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

INTRODUCTION

Brucella spp. causes Brucellosis, which is one of the most common zoonotic diseases and which brings about important problems related to health and economy (1, 2, 3). Brucellosis causes economic loss in husbandry; in addition, it poses a risk to public health as it is transmitted to people and causes infections through dairy products. It is possible to trace the roots of this disease in the 5th plague of Egypt around 1600 BC (4, 5). It is defined as a chronic contagious disease causing necrotic inflamatory infections and complications such as abortion, infertility, arthritis, orchitis and mastitis in susceptible hosts (6). According to the World Health Organization (WHO hereafter), there are 500,000 reported Brucellosis cases annually worldwide (7, 8). Due to the transmission of *Brucella* species via aerosol way, it is classified as a potential bioteror agent as well (1, 2). Moreover, Brucella organisms are described as belonging to risk group 3 microorganisms in the manual of WHO laboratory biosecurity (9, 10, 11).

For the diagnosis of Brucellosis, isolation of bacteria is regarded as the gold standard (10). Test-and-slaughter and vaccination are important activities being implemented as part of eradication programs against Brucellosis (12). Furthermore, the investigation of the epidemiological source of the disease is as important as these implementetions (13). Isolation and identification of the etiological agent is necessary for this investigation, which can determine the source and the spread of the

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infection. As the number of contaminant organisms growing fast is big in the diagnostic material, using a selective medium for the isolation of *Brucella* spp. is necessary. (14, 15).

There is a great variety of selective media types including different basal media, antibiotic mixture, and concentration (16). Marin et al. (17) and Vicente et al. (18) contend that every medium has got a specific effect on *Brucella* species, its biovar and contaminants owing to the differences in media. After the first selective medium was created, new species and strains were found out in a variety of hosts and they were included in the *Brucella* genus; and this led to the extension of the ecological range of the *Brucella* genus. (1, 3, 19). For this reason, selective media, which have a significant role in isolation, are undeniably important for bacteriological isolation as a gold standard.

In this context, this study investigates the success of *Brucella* spp. isolation by using different selective media.

MATERIAL AND METHOD

This study was carried out in the Pendik Veterinary Control Institute between 2014 and 2015. 51 organs and 7 fetal stomach content of abortion cases were utilized. The media included in this study involves 4 different selective media and the Tryptic Soy Agar (TSA) as a non-selective medium. The content of Farrell (20), CITA (21), Modified Thayer Martin (14), and Jones and Morgan (22) as selective media is illustrated in Table 1. In addition to these, one medium with antibiotics was used to extend the range of results. This medium is called '*Brucella* medium' and labelled as BM in the following sections of this study.

Amphotericin-B has been preferred instead of natamycin or cycloheximid, which is part of the antimicrobial content of the JM medium. It is considered to be one of the antifungal agents suggested for the selective media for the first isolation of *Mycobacterium* spp. (23), *Campylobacter* spp. (24) and *Brucella* spp. (21).

For this study, the basal medium required for the selective media was prepared and sterilized by autoclaving (121°C ± 3°C, 15 minutes). Antibiotics and sterile new born calf sera were added to the media at about 56°C depending on their contents (25). Sterility controls of media were conducted after they were incubated at 37ºC for 48 hours (26). The organ suspensions were prepared from organ samples diluted 1/10 in a phosphate buffered saline in a biosafety cabinet (18, 27).

Organ suspensions and fetal stomach content were inoculated to media and incubated in 37°C, 5-10% CO_2 condition for 5-8 days. Biovar identification of isolates was implemented according to CO_2 requirement, H_2S production, growth in media containing thionin (20µg/ml), basic fuchsin (20µg/ml), safranine (100µg/ml), penicillin, streptomycin, and i-erythritol sensitivity, lysis with Tibilisi (TbØ 10^4 RTD) and

Table 1. The contents of the selective media

Content	Farrell	CITA	MTM	JM	BM
Basal medium	BMB-CS	BAB-CS	GC-H	SDA-CS	TSA-CS
Bacitracin (IU/L)	25,000	-	-	25,000	-
Polymyxin (IU/L)	5,000	-	-	6,000	6,000
Nalidixic acid(mg/L)	5	-	-	-	-
Amphotericin-B (mg/L)	-	4	2.5	4	4
Natamycin (mg/L)	50	-	-	-	-
Nitrofurantain(mg/L)	-	10	10	-	10
Vancomycin(mg/L)	20	20	3	-	20
Colistin (mg/L)	-	7.5	7.5	-	-
Nystatin (IU/L)	100,000	100,000	100,000	-	-
Erythritol (g/L)	-	-	-	-	1

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
TSA-CS: Trypton soy agar with calf sera

R/C phages and agglutination with monospecific A and M antisera. Media including streptomisin (2,5 µg/ml), penicillin (5 IU/ml) and i-erythritol (1 mg/ml) were used in the identification of vaccine strains. In addition, we have observed the growth level of contaminant microrganisms.

The classification categories for the inhibition ability of the media against contaminant microorganisms in this study were total inhibition (TI) and partial inhibition (PI). These categories were formed regarding the diffuseness of the contaminant growth through counting the colony forming units (cfu) (26). When the contaminant colony counts were taken into account, the media's inhibition ability was listed by focusing on the range of the contaminant burden. We have classified the ranges into 4 groups; namely, 1 total inhibition group without any contaminant colonies and 3 partial inibition groups with less than 10, ones between 10 and 100, and ones with more than 100 colonies (26, 28, 29). Pearson Chi-Square Test in SPSS18.0 program was used to evaluate the results of this study.

RESULTS

Biyotyping results of the isolates and the media in which isolation was carried out are illustrated in Table 2. The table also shows the inhibition level of the contaminants for each medium in every single sample.

Table 2. The isolation and inhibition results of selective media for each sample

No	Animal	TI*	PI† (<10cfu)	PI‡ (10-100cfu)	PI§ (>100 cfu)	*Brucella* isolation	Biovar
1	Sheep	-	JM	CM, FM	BM, MTM	-	-
2	Goat	-	-	FM	BM, JM, CM, MTM	FM	*B.melitensis* bv3
3	Sheep	FM	CM	JM, BM MTM,	-	-	-
4	Sheep	FM	CM, JM MTM,	BM	-	-	-
5	Sheep	ALL‖	-	-	-	-	-
6	Goat	JM	-	-	CM, FM MTM, BM	MTM, BM, JM	*B.melitensis* bv1
7	Sheep	-	JM, MTM	-	BM, FM, CM	BM, JM MTM	*B.melitensis* bv3
8	Sheep	ALL	-	-	-	ALL	*B.melitensis* bv3
9	Sheep	FM, BM,CM	MTM, JM	-	-	ALL	*B.melitensis* bv1
10	Sheep	FM, BM CM	JM, MTM	-	-	ALL	*B.melitensis* bv3
11	Sheep	FM, JM MTM, CM	BM	-	-	ALL	*B.melitensis* bv3
12	Sheep	JM	MTM	FM, CM, BM	-	ALL	*B.melitensis* bv3
13	Sheep	FM	-	-	CM, JM, BM,MTM	ALL	*B.melitensis* bv3
14	Sheep	FM, BM MTM, CM	JM	-	-	ALL	*B.melitensis* bv3
15	Sheep	ALL	-	-	-	ALL	*B.melitensis* bv3
16	Sheep	FM	-	MTM, CM, BM	JM	FM, BM, CM,MTM	*B.melitensis* bv3
17	Sheep	ALL	-	-	-	ALL	*B.melitensis* bv3
18	Sheep	-	FM	-	MTM, CM, BM, JM	ALL	*B.melitensis* bv1
19	Sheep	-	-	-	ALL	ALL	*B.melitensis* bv3
20	Sheep	-	FM	-	MTM, CM, JM, BM	FM	*B.melitensis* bv3
21	Goat	-	-	-	ALL	MTM, BM, CM	*B.melitensis* bv3

22	Cattle	JM	FM	CM, BM, MTM	-	JM, FM, MTM, CM BM(WG¶)	*B.abortus* S-19
23	Sheep	-	-	ALL	-	ALL	*B.melitensis* bv3
24	Goat	FM,CM, BM, MTM	JM	-	-	-	-
25	Sheep	CM, FM, BM	JM, MTM	-	-	ALL	*B.melitensis* bv1
26	Sheep	-	-	FM	MTM, CM, BM, JM	FM	*B.melitensis* bv3
27	Sheep	-	-	FM, CM	MTM, JM,BM	ALL	*B.melitensis* bv3
28	Goat	-	-	FM	MTM, CM, JM, BM	-	-
29	Sheep	FM, CM, BM,MTM,	JM	-	-	-	-
30	Sheep	ALL	-	-	-	-	-
31	Sheep	-	FM	-	MTM, CM, JM, BM	ALL	*B.melitensis* bv1
32	Sheep	-	JM	BM, CM, MTM,FM	-	ALL	*B.melitensis* bv3
33	Sheep	-	FM	CM,BM MTM	JM	-	-
34	Sheep	FM,CM, MTM	JM, BM	-	-	-	-
35	Sheep	-	-	-	ALL	-	-
36	Sheep	-	FM	-	CM, BM, MTM, JM	-	-
37	Sheep	-	-	-	ALL	-	-
38	Sheep	ALL	-	-	-	-	-
39	Sheep	-	-	-	ALL	-	-
40	Sheep	-	-	ALL	-	ALL	*B.melitensis* bv3
41	Sheep	-	-	JM, CM, FM	BM, MTM	JM, CM, FM, BM	*B.melitensis* Rev1
42	Sheep	ALL	-	-	-	ALL	*B.melitensis* bv3
43	Cattle	-	JM	CM,MTM	FM, BM	ALL	*B.abortus* bv3
44	Sheep	FM, CM	JM, BM	MTM	-	MTM	*B.abortus* bv3
45	Cattle	-	-	MTM, CM BM, FM,	JM	ALL	*B.abortus* bv3
46	Sheep	CM,JM, MTM,	BM	FM	-	ALL	*B.melitensis* bv1
47	Sheep	MTM	CM, JM	FM, BM	-	ALL	*B.melitensis* bv3
48	Sheep	ALL	-	-	-	ALL	*B.melitensis* bv3
49	Sheep	ALL	-	-	-	ALL	*B.melitensis* bv3
50	Cattle	-	FM, JM MTM,	CM, BM	-	ALL	*B.abortus* bv3
51	Cattle	CM	MTM, JM	FM, BM	-	ALL	*B.abortus* bv3
52	Cattle	ALL				ALL	*B.abortus* bv3
53	Cattle	ALL				ALL	*B.abortus* bv3
54	Cattle	ALL				ALL	*B.abortus* bv3
55	Cattle	-	-	FM	CM, MTM, JM, BM	ALL	*B.abortus* bv3
56	Cattle	FM, JM, CM, BM	MTM	-	-	ALL	*B.abortus* bv3
57	Cattle	ALL	-	-	-	ALL	*B.abortus* bv3
58	Cattle	ALL	-	-	-	MTM	*B.abortus* bv3

∗.TI (Total Inhibition): The media listed in this column inhibited all the contaminants. No contaminant colony was observed
†. PI (Partial Inhibition)<10cfu: The media listed in this column partially inhibited the contaminants. Less than 10 contaminant colonies were observed
‡. PI (Partial Inhibition)10-100cfu: The media listed in this column partially inhibited the contaminants. Between 10 and 100 contaminant colonies were observed
§. PI (Partial Inhibition) >100 cfu: The media listed in this column partially inhibited the contaminants. More than 100 contaminant colonies were observed
ǁ. ALL It represents all the media
¶. WG: (Weak Growth) It shows that growth diffuseness in this medium is less than the other media

Table 3. The number of *Brucella* spp. isolations and the distribution of inhibition ability

Media	TI	PI (<10 cfu)	PI (10-100cfu)	PI (>100cfu)	*Brucella* spp. isolation
Farrel	28	7	15	8	38
CITA	26	3	13	16	36
MTM	21	9	10	18	39
JM	20	16	4	18	36
BM	21	4	13	20	37+ 1 (S-19)

Table 4. The isolation and the inhibition ability of the media in percentages

Media	Isolation%	Inhibition%
Farrel	65.5	86.2
CITA	62	72.4
MTM	67.2	68.9
JM	62	68.9
BM	65.5	65.5

According to the aforementioned results, isolations could not be carried out in every medium. Moreover, selective media had different performance levels in terms of inhibition ability against contaminants. In addition to these, even though *B. abortus* S19 was isolated in *Brucella* medium after inoculation of sample No. 22, the growth of strain was at weak growth (WG) level and the growth diffuseness was clearly lower than the other media. The detailed results in Table 2 are summarized in Table 3 and Table 4. The numbers of *Brucella* isolation and the distribution of inhibition abilities are listed in Table 3.

Table 3 shows that MTM medium's isolation percentage is the highest one with 39 *Brucella* spp. isolates when we consider all 58 samples. Moreover, Farrell medium's performance is a lot better than the other media regarding the inhibition ability.

We have listed the inhibition and isolation ability of the media as percentages in Table 4 so as to clarify the numbers in Table 3. The percentages of the inhibition ability in Table 4 was found by taking the sum of TI and PI inhibition abilities without including the PI (>100 cfu). Even if two of them (PI <10 cfu, PI=10-100 cfu) show partial inhibition ability, they are considered to be sufficient inhibition ability as they make the isolation of *Brucella* spp possible.

The media's isolation and inhibition ability illustrated in Table 4 was analyzed statistically. The media's isolation percentages are similar and they are not statistically significant. There are far more differences in the media's inhibition ability against contaminants than their isolation ability. The statistical analysis of these differences is outlined in Table 5.

In Table 4, all the media except the Farrell medium have similar inhibition ability percentages. Therefore, in Table 5 statistical analyses were carried out only between the results of Farrell, which has the highest inhibition percentage, and the other media. The *p* value between Farrell and the

Table 5. The results of the statistical analysis related to the media's inhibition ability

Chi-SquareTest	Inhibition ability	
Pearson Chi-Square	**X^2 value**	**P value**
Farrell & BM	6.778	0.009
Farrell & MTM	4.957	0.026
Farrell & JM	4.957	0.026
Farrell & CITA	3.362	0.067

Table 6. The distribution of the media's inhibition ability in the samples where *Brucella* spp. was isolated

Medium	*Brucella* spp. isolation	TI	PI <10cfu	PI 10-100cfu	PI >100 cfu
Farrell	38	19	5	13	1 (1/8: 12.5%)
CITA	36	18	1 (1/3:33.3%)	11	6 (6/16: 37.5%)
MTM	39	15	8	8	8 (8/18: 44.4%)
JM	36	16	10	3	7 (7/18: 38.8%)
BM	38	15	2 (2/4: 50%)	10	11 (11/20: 55%)

other media except CITA medium is smaller than 0.05; therefore, the difference between Farrell and CITA is statistically insignificant but the difference between Farrell and the other media is statistically significant.

In order to investigate the correlations between isolation and inhibition, the inhibition ability of the media was examined in the samples in which *Brucella* spp. isolation occurred. The distribution of the media's inhibition ability for these samples is listed in Table 6.

Based on the distribution results, it can be stated that the highest *Brucella* spp. isolation for each medium was obtained in the samples in which all the contaminants were totally inhibited (TI). In addition, in Table 3, the distribution of the media's inhibition ability was made based on 58 samples regardless of *Brucella* spp. isolation. In this table, on the other hand, the media's inhibition ability was calculated for the samples in which *Brucella* spp. was isolated. When the values of these two tables were evaluated, the isolation percentage of the samples in which the contaminants were partially inhibited at the level of PI >100 was lower than the other levels (TI, PI <10, and PI 10-100), except for two results shown in italics. These low percentages of isolation were given in the last column of Table 6.

DISCUSSION

Regarding the number of isolations, MTM has the highest and Farrell medium has the second highest isolation percentage. Marin et. al. (17) obtained a higher isolation sensitivity for *Brucella melitensis* in MTM than they did in Farrell, which was actually developed for *Brucella abortus* isolation. In this study, it was found out that using these two media simultaneously could increase the isolation percentage up to 74.1% with 43 successful isolations (15). In OIE Cattle Brucellosis Chapter, too, using two media simultaneously to be able to augment the isolation sensitivity is recommended (17, 11). Similarly, Ferreira et al. (30) suggested using more than one selective medium to enhance the isolation sensitivity.

When the inhibition ability is taken into consideration, the Farrell medium has the highest percentage while CITA has the second highest percentage. Even though the difference between the Farrell medium and the CITA medium is statistically insignificant, the difference between the Farrell medium and the other media is statistically significant. It is pointed out that the Farrell medium is able to inhibit most of the contaminants; thus, it is the most common selective medium for the bacteriological diagnosis of Brucellosis (21). In the study by Vicente et al. (18) where CITA and Farrell were compared and contrasted, both media were found to be similar in their inhibition abilities against the contaminants and they showed good results when they were used together. De Miguel et al. (21) stated in their study, in which they developed the CITA medium, that CITA could inhibit most of the contaminants and it had better isolation sensitivity than those of MTM and Farrell. In a similar study, *Brucella* agar, the Farrell and CITA media were compared and contrasted and despite the same number of isolations in each medium, Farrell had the highest inhibition ability against contaminants and it is regarded as the best selective medium for microbiological diagnosis (31).

According to the results, it can be stated that the highest *Brucella* spp. isolation for each medium was obtained in the samples in which all the contaminants were totally inhibited (TI). When all the 58 samples were evaluated, the number of the samples where all the contaminants were totally inhibited is bigger in Farrell and CITA than in the other media. In his study, Farrell classified the growth levels of contaminants as 1+, 2+ and 3+ (20). He stated that most of the *Brucella* spp. isolations were obtained in the samples with contaminant growth at 1+ level. The findings of this study, too, indicate that the increase in the inhibition ability of the selective media plays an important role in

enhancing isolation sensitivity. It was also pointed out that although the antibiotics added to the media could decrease contamination, *B. abortus* colonies might be masked by the excessive amount of contaminant growth due to the length of incubation period (29). For this reason, in such samples, the isolation rate can decline in the selective media which are not as effective as Farrell and CITA in terms of contaminant inhibition.

Brucella bacteria are fastidious microorganisms and require a longer period of incubation when we compare them with contaminants growing fast in the samples (14, 15, 25). The generation period of *Brucella* organisms, which is 2.5-3.5 hours, is considered as a long duration, too (32). It was also pointed out that it may take some more days to observe colonies on the selective media as compared to the usual incubation period on non-selective media (25). In our study, as well, we found out that when we passage the *Brucella* suspect colonies one day later, we could not identify *Brucella* colonies due to the contaminants which hid the *Brucella* colonies. For this problem, Alton et al. (25) recommended that *Brucella* suspect colonies should be passaged before the contaminants spread on the media's surface and they should be checked three days after the incubation. Using solid medium is considered to be the most satisfactory method for isolation as it can facilitate the isolation of *Brucella* colonies and it can also minimize the risk of mixing *Brucella* colonies with the other fast growing microorganisms (11, 25).

In the samples in which the contaminants were partially inhibited at the level of PI >100, the isolation percentage was the lowest or close to the lowest. The reason behind the low isolation percentage might be predicted as the contaminants covering the medium's surface. In a study by Stack et al. (15), it was stated that they could not spot the Brucella bacteria colonies in some of the artificially infected milk samples. They contend that the reason behind it was that contaminants in the milk samples disguised the *Brucella* colonies. Based on the findings of this study, it is possible to say that an increase in the inhibition ability of the media may facilitate *Brucella* spp. isolation and it may lead to an increase in the isolation sensitivity. In this sense, following and improving the inhibition abilities of the media according to the samples with different contaminant microflora might increase the isolation rate.

Her et al. (27) developed a selective medium which includes indicator neutral red for the isolation of *Brucella abortus* strains. This medium can facilitate the observation of the *Brucella* spp. colonies more easily by differentiating them from contaminants. A novel approach might be passaging the slow-growing *Brucella* bacteria with the help of the indicators before they are masked by contaminants. This approach may be a useful tool to increase the low isolation rate in samples where contaminants are inhibited inadequately. In our study, as well, lower inhibition ability level led to a lower isolation percentage; therefore, it might be a good idea to focus on the development of such kind of media. That kind of development and modification in the *Brucella* selective media as well as the findings of this study indicate the significance of contaminant inhibition for a better isolation percentage.

The medium with antibiotics, which was labeled as BM in this study, includes erythritol which stimulates the growth of *Brucella* strains. BM is composed of fewer antimicrobial agents compared to the media with high inhibition ability. Although BM has the lowest inhibition ability, it does not have the lowest isolation rate. This fact might be interpreted as the positive effect of the erythritol component it includes. The isolation rate can decrease in the samples in which contaminant organism burden increases qualitatively and quantitatively, while the number of the target bacteria decreases. In the development of media, components such as erythritol provoking growth and antimicrobial agents providing inhibition can be added to the media.

In the Modified *Brucella* selective (MBS) medium developed by Her et al. (27) for *B. abortus* strains, too, erythritol was used to provoke and improve the delayed growth of strains among antibiotic mixtures. Erythritol is also mentioned as a sugar alcohol which is effective in the tissue tropism of *Brucella* bacteria in ruminants (27, 33, 34). However, it is believed that erythritol does not stimulate (35, 36) but inhibits the growth of the S19 strain (34, 37). Conversely, S19 isolation took place in BM as weak growth in one of the samples (Table 2, no:22). Alton et al. (25) suggested that the mutation level of S19 strains against erythritol tolerance was high. They also stated that even though some suspected S19 isolates resembled S19 in other tests, they could grow in erythritol. It is pointed that the reason behind this weak growth in this sample might be what Alton et al. (25) suggested above. In light of the findings of this study, it might be stated that adding erythritol component to the selective media will provoke growth of *Brucella* strains except S19 and it might be recommended as a way of increasing isolation sensitivity.

CONCLUSION

To sum up, using two different media with high inhibition ability like Farrell simultaneously might be helpful while choosing the appropriate selective media. In the process of developing media, on the other hand, adding components that will provoke *Brucella* spp. growth should be considered. Moreover, checking the performance of media repeatedly will be beneficial for obtaining better isolation rates. In these repeated controls, qualitatively and quantitatively different microbiol burden of the field samples should also be taken into consideration.

CONFLICT OF INTEREST STATEMENT

The authors declared that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

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