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RESEARCH ARTICLE

Evaluation of Adding Erythritol to Farrell Medium for Primary Isolation of the *Brucella melitensis* Strains

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ABSTRACT

This study aims to evaluate the addition of erythritol to Farrell medium (FM) for the primary isolation of *B.melitensis* strains. Selective media, particularly FM, is commonly used for *Brucella* isolation. Isolation is the gold standard for the diagnosis of brucellosis. The isolation success of selective media depends on their ingredients. Isolation rate may decrease particularly in contaminated samples; therefore, the addition of components like erythritol that augments the growth of *Brucella* species on media can support isolation. In this study, organ samples associated with 14 small ruminant abortion cases caused by *B.melitensis* strains were utilized. Organ suspensions were inoculated to FM and Farrel medium with erythritol (F+ER) simultaneously. The media's isolation and inhibition performance was observed during incubation. The addition of erythritol was evaluated based on whether it provides earlier isolation with the help of increasing the colony growth rate and colony size. According to the findings, F+ER is more advantageous for 12 samples among 14 through isolation as it enables faster colony growth and larger colonies. The results confirm the recommendation of adding erythritol particularly in the samples with inadequate contaminant inhibition. Therefore, high isolation performance can be achieved through erythritol addition to the selective media like FM.

Keywords: *Brucella melitensis*, erythritol, Farrell, isolation, selective medium.

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Brusella melitensis Şuşlarının İlk İzolasyonu İçin Farrell Besiyerine Eritritol İlavesinin Değerlendirilmesi

ÖZ

Bu çalışmanın amacı, *B.melitensis* şuşlarının ilk izolasyonu için selektif Farrel besiyerine (FB) eritritol ilavesini değerlendirmektir. Selektif besiyerleri ve özellikle FB, *Brusella* (B.) etkenlerinin izolasyonunda yaygın olarak kullanılmaktadır. Bruselloz teşhisinde altın standart bakteri izolasyonudur. Selektif besiyerlerinin izolasyon başarısı içeriklerine bağlıdır. Özellikle kontamine örneklerde izolasyon yüzdesi düşebilmektedir. Bu bakımdan *Brusella* türlerinin besiyerlerinde üremelerini provoke edecek eritritol gibi bileşenlerin selektif besiyerlerine ilavesi izolasyona fayda sağlayabilir. Çalışmada *Brusella melitensis* şuşlarından kaynaklı 14 küçük ruminant atık vakasına ait iç organ numuneleri kullanılmıştır. Örneklerden hazırlanan suspansiyonlar eş zamanlı olarak FB ve eritritol ilaveli Farrel besiyerine (F+ER) inokule edilmiştir. İnkubasyon boyunca besiyerleri gözlemlenmiş ve besiyerlerinin gösterdiği izolasyon ve inhibisyon performansları takip edilmiştir. Özellikle eritriol ilavesi, koloni gelişim hızı ve koloni çapı büyüklüğü sağlayarak daha erken izolasyon imkanı verebilmesi açısından karşılaştırılmıştır. 14 numune arasında 12'si için F+ER besiyerinde koloni gelişim hızı veya koloni çapı büyüklüğüne göre izolasyon için avantaj elde edilmiştir. Sonuçlar selektif besiyerine eritritol ilavesinin tavsiye edilmesini özellikle kontaminant inhibisyonun yetersiz kaldığı numuneler için desteklemektedir. Bu nedenle çalışmada olduğu gibi eritritolun, FB gibi selektif besiyeri formulasyonlarına dahil edilmesi ile yüksek izolasyon başarısının elde edilmesi mümkün görünmektedir.

Anahtar Kelimeler: *Brusella melitensis*, eritritol, Farrell, izolasyon, selektif besiyeri.

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INTRODUCTION

Brucellosis, caused by *Brucella* spp., is one of the most common zoonotic diseases (Songer and Post 2012). According to the World Health Organization (WHO), brucellosis is a worldwide zoonotic infection which causes important problems of health by risking public health and of economy through loss in husbandry (Godfroid et al 2005, Yumuk and O'Callaghan 2012). The roots of this disease might be traced in the 5th plague of Egypt around 1600 BC (Pappas et al. 2006, Seleem et al. 2010). In addition, in the WHO laboratory biosecurity manual, it is revealed that *Brucella* organisms belong to risk group 3 microorganisms (Perez-Sancho et al. 2015, WHO 2004). According to the WHO, all around the world, there are 500,000 brucellosis cases reported annually (Doganay and Aygen 2003, Perez-Sancho 2015). As *Brucella* species can be transmitted through aerosol way, it is also classified as a potential bioteror agent (Godfroid et al 2005*,* Songer and Post 2012). Bacteria isolation is considered to be a gold standard for the diagnosis of brucellosis (OIE 2009). However, no method other than classical bacteriology has been recommended until now, which is sufficiently sensitive for all kinds of biological samples (Garin-Bastuji 2006). For brucellosis eradication programs, test&slaughter and vaccination are important implementations (Her et al. 2010). What is more, the investigation of the epidemiological source of the disease is equally important (Krstevski 2015). The source and the spread of the infection can be determined with the help of isolation and identification of the etiological agent, which is important for this investigation. Therefore, as the diagnostic material includes plenty of fast growing organisms, it is necessary to use a selective medium for *Brucella* spp. isolation (Marin et al. 1996a, Stack et al. 2002). There are different types of selective media which include different basal media, antibiotic mixture, and concentration (Hornsby et al. 2000). According to Marin et al. (1996b) and Vicente et al. (2014), each medium has a specific impact on the species of *Brucella*, its biovars and contaminants because of the differences in media. After the first selective medium was developed, the ecological range of *Brucella* genus has expanded as a result of the identification of new species and strains in different hosts (Godfroid et al. 2005, Pappas 2010, Yumuk and O'Callaghan 2012). In order to address the problems associated with the transmission of the disease, it is important to isolate and identify the etiological agent (Her et al. *2010)*. Thus, the selective media having an important role in isolation are fundamental for bacteriological isolation as a gold standard as well. The World Organization for Animal Heath (OIE), in its Chapter of Cattle Brucellosis, also suggests using two different media at the same time in order to sensitivity of isolation (Marin et al. 1996b, OIE 2012*),* one of which is FM (Karagul and Ikiz 2017, OIE 2012). In the same way, in their study, Nardi Junior et al. (2015) compared and contrasted *Brucella* agar, Farrell, and CITA media and, despite the similarities between their isolation numbers, FM was found to be the best regarding the inhibition of the contaminants, and it is also considered to be the best selective medium for microbiological diagnosis. De Miguel et al. (2011) defines FM as the most common selective medium for the bacterial diagnosis of brucellosis as it can inhibit most of the contaminants.

In animals, abortion is an expected complication of brucellosis in that organisms tend to be settled in the animals' placenta, where erythritol, a growth stimulant of *B.abortus*, exists (Corbel 2006). Erythritol, which is a four carbon atom sugar alcohol (polyol), is found in some fruits in small amounts, and it is also found in high concentration in foetal tissues of ruminants (Garcia Lobo and Sangari Garcia 2005). As erythritol is absent in humans, it is believed to make human placenta more resistant against brucellar placentitis. (Madkour 2001). Among all the bacterial species, members of *Brucella* genus are the ones that are most closely related to erythritol. The Brucellae can not only utilize erythritol, but they prefer to use it instead of other sugars. A recent report illustrated that the erythritol concentration in foetal sheep blood was 60 times higher than the one in the maternal blood (Garcia Lobo and Sangari Garcia 2005).

This study aims to evaluate the benefits of adding erythritol to FM, which is commonly used for *Brucella* isolation. It is particularly aimed to elaborate on whether the addition of erythritol leads to an earlier isolation by the help of faster growth rate and larger colony size.

MATERIAL and METHOD

Organ samples associated with 14 small ruminant abortion cases caused by *B.melitensis* strains were utilized in this study. The number of samples belonging to sheep or goat abortions is 10 and 4, respectively. The media included in this study are FM, F+ER and Tryptic Soy Agar (TSA) as a non-selective medium. The content of FM (Farrell 1974), includes Brucella Medium Base with Calf Sera (BMB-CS), Bacitracin (25,000 IU/liter), Polymyxin (5,000 IU/liter), Nalidixic acid (5mg/liter), Natamycin (50 mg/liter, Vancomycin (20mg/liter). F+ER also contains erythritol (1gr/liter). For this study, the basal medium needed for the selective media was prepared and sterilized through autoclaving (MVR-121°C ± 3°C, 15-20 minutes). Erythritol solution composed of 10 gr of erythritol and 10 ml distilled water was sterilized by filtration. Antibiotics, sterile new born calf sera and erythritol (1ml) were added to the media at about 56 ^{o} C depending upon their contents (Alton

et al. 1988). Solidified media were incubated at 37 °C for 48 h for sterility control (ISO/TS 2009).

The suspensions obtained from organ samples were prepared in a 1/10 dilution with phosphate buffered saline in a biosafety cabinet (Vicente et al. 2014). Organ suspensions were inoculated to media and incubated in 37°C, 5-10% CO_2 condition for 5-8 days. At the end of the incubation period, Brucella strains isolated were identified through a conventional biotyping method. Biotyping was carried out according to CO_2 requirement, H_2S production, growth in media which contain thionin (20μg/ml), basic fuchsin (20μg/ml), safranine (100μg/ml), penicillin (5 IU/ml), streptomycin (2,5μg/ml), and erythritol (1mg/ml) sensitivity, lysis with Tibilisi and R/C phages and agglutination with monospecific A and M antisera (Alton et al. 1988).

In addition, the growth level of contaminant microorganisms was observed. The inhibition ability of the selective media against contaminant microorganisms was categorized as total inhibition (TI) or partial inhibition (PI) with regards to contaminant growth diffuseness by counting colony-forming units (CFUs) (ISO/TS 2009). After the contaminant colonies were counted, the inhibition ability of the media was identified with the help of specifying the range of contaminant burden. The ranges were divided 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 (ISO/TS 2009, Jones et al. 1975, O'Grady et al. 2014). The growth of *Brucella* colonies was observed during incubation. The colony diameter of the observed colonies was measured. FM and F+ER were compared and contrasted based on the day of incubation on which the colony growth appeared for the first time.

RESULTS

Inoculation of 11 organ samples caused gross contamination on TSA. It was observed that contaminants were totally inhibited on FM and F+ER media for 11 samples. The inhibition abilities of FM and F+ER to suppress the contaminants and the contamination level on TSA are listed in Table 1.

As it is shown in Table 1, all of the inoculations on F+ER led to isolation. The isolates were then identified through biotyping procedure. The inoculation of sample 5 did not end up with a pure Brucella growth; therefore, the passage of this sample from FM was contaminated. The identification of sample 5 taken from FM as could not be carried out via biotyping because of this contamination.

The growth rate of Brucella colonies varied in FM and in F+ER. In Table 2, the data about the day of the incubation on which colonies were observed for the first time and colony growth and size were listed. , it was possible to recognize and measure the colonies on F+ER for 8 samples but not on FM, on the same day of incubation. 5 samples were observed both on FM and F+ER on the same day of incubation. However, for 4 samples out of 5 the colonies were larger on F+ER than the ones on FM. There is only l sample (no: 8) on which larger Brucella colonies were observed on FM. Sample 9 was the only one which showed equal colony growth rate on both FM and F+ER.

The colony growth on the days following incubation was checked. It was apparent that colonies on F+ER became larger than the colonies on FM. In some samples (sample 4, 6, 8), the differences of colony size between FM and F+ER was observable only on the first days of incubation. In these samples, profusion of Brucella growth spread on the whole surface of media on the following days. It was the reason for not being able to measure a single colony and compare the sizes.

DISCUSSION

For 8 samples out of 14, F+ER enables us to passage the *Brucella* colonies one day earlier. It also provides an easy passaging for 4 samples as it gives the chance of gathering larger colonies with longer colony diameter. *Brucella* bacteria are fastidious microorganisms and require a longer incubation period than the contaminants growing fast in the samples (Alton et al. 1988, Marin et al. 1996, Stack et al. 2002). For growth on primary isolation, most strains need complex media which contain multiple amino acids, thiamin, biotin, nicotinamide and pantothenic acid (Corbel 2001). In addition, it was also stated that seeing colonies on selective media might take a few more days than the usual incubation period on the nonselective media (Alton et al. 1988). In a study by Hunter and Kearns (1977), in the medium they used, which included erythritol, the colonies were larger and noticeable after only 3-day incubation period. It can be stated that addition of erythritol to FM for isolation of *B.melitensis* strains contributes positively to the isolation success. Contrary to other studies (Her et al. 2010, Hunter and Kears 1977), the effect of erythritol addition on isolation and colony growth was elaborated by using *B.melitensis* strains in this study.

A possible energy source for many strains is erythritol. *B.melitensis* can oxidize amino acids but few carbohydrates other than D-glucose and erythritol (Corbel 2001). Growth analysis of *Brucella* spp. in media which contain erythritol revealed that Brucella spp. will use erythritol as a source of carbon

preferentially over the presence of glucose in the media (Anderson and Smith 1965, Petersen et al. 2013). Stack et al. (2002) pointed out that they could not identify the *Brucella* bacteria colonies in some of the milk samples which are artificially infected. They suggested that the reason was that contaminants in the milk samples hid the *Brucella* colonies. In this sense, the high burden of contaminants in the samples might lead to a decrease in the isolation performance. Therefore, enhancing the growth of *Brucella* colonies as, it is in this study, might overcome this difficulty. Karagul and Ikiz (2017) stated that although a medium in their study had the lowest inhibition ability; it did not have the lowest isolation rate. This fact might be interpreted as the positive effect of the erythritol component it included.

Erythritol is also mentioned as a sugar alcohol which has an impact on the tissue tropism of *Brucella* bacteria in ruminants (Garcia Lobo and Sangari Garcia 2005, Her et al. 2010, Keppie et al. 1965). It was explained that the presence of erythritol in the placentas of goats, cows, and pigs was the reason for the existence of Brucella in these sites and the following accumulation of big amounts of bacteria, which eventually leads to abortion (Keppie et al. 1965, Petersen et al. 2013). However, it is stated that erythritol does not stimulate (Poester et al. 2013, Seleem et al. 2008,) but hinders the growth of S19 strain (Garcia Lobo and Sangari Garcia 2005, Sperry and Robertson 1975). It was also shown that all of the erythritol degradation enzymes in S19 were identified, except for the D-erythrulose 1-phosphate dehydrogenase (Sperry and Robertson 1975).

At first, the ability of *B.abortus* to use erythritol was believed to be directly related to virulence, as shown by the fact that the *B.abortus* vaccine strain S19 was recognized as deficient in the use of erythritol (Petersen et al. 2013). The findings of the study by Karagul and Ikiz (2018) revealed that adding erythritol component to the selective media will contribute to the growth of *Brucell*a strains except S19 and it might be suggested as a way of augmenting isolation sensitivity.

Alton et al. (1988) suggested passaging *Brucella* suspect colonies before the contaminants spread on the media's surface and they also recommended checking them three days after the incubation. However, contaminants might cover the surface of the medium completely earlier than the end of 3 days (Karagul and Ikiz 2017). In this study, it was possible to recognize the *Brucella* colonies on F+ER for some samples at the end of the second day of incubation. It is very helpful that may be used to support the Brucella isolation in the samples with high contaminant burden. In another study, the colony growth rate of 14 reference strains out of 20 was observed to be fastest in Brucella medium including erythritol (Karagul 2016).

Contaminants were totally inhibited on both of the FM and F+ER for most of the samples. Therefore, the effect of adding erythritol has just offered a chance for earlier isolation. Total inhibition of contaminants on F+ER also confirms that erythritol addition does not have a negative influence on the inhibition ability of the medium. The most effective medium was FM while controlling unwanted organisms, especially when there is a high level of contamination (Hunter and Kearns 1977). Considering this, Karagul and Ikiz (2017, 2018) stated that the isolation rate can diminish in the samples in which contaminant organism burden rises up qualitatively and quantitatively while the number of the target bacteria lowers. While developing media, components such as erythritol which provokes growth and antimicrobial agents which provide inhibition can be added to the media (Karagul and Ikiz 2018). In this study, isolation could not be achieved by FM in the sample (no: 5), in which the contaminants were partially inhibited. Even if the F+ER had inhibited the contaminants partially too, the isolation was carried out. In this respect the isolation from this sample on F+ER might show the influence of erythritol on the isolation success. A study supporting this influence recommended using a combination of the contaminant inhibitors of FM in addition to the more enriched base of Barrow & Peel's Medium which includes erythritol for a better medium (Hunter and Kearns 1977).

The link between *Brucella* spp. and erythritol has been apparent recently. It was shown by some studies that there was a correlation between *B.abortus* strains's ability to utilize erythritol and the virulence of the strain with smaller utilization rates which lead to lower virulence growth (Petersen et al. 2013). Therefore, using a selective medium containing erythritol might provide some preliminary data in terms of the relationship between virulence and eryhtritol.

Consequently, the addition of erythritol to FM, a common selective medium for *Brucella* spp isolation, provides advantage for the isolation of *B.melitensis* strains. Conducting some follow-up studies consisting of *other Brucella* species-biovar and samples with gross contaminants burden is recommended. With the help of these studies, the advantages of erythritol addition can be displayed in comparison with other media.

Table 1: Identification and inhibition results of the selective media

Sample	Animal	Count of Contaminants on TSA	Identification result for FM	Inhibition ability of FM	Identification result for F+ER	Inhibition ability of F+ER
1	Sheep	<10cfu	*B.melitensis* bv3	TI	*B.melitensis* bv3	TI
2	Sheep	>100cfu	*B.melitensis* bv3	TI	*B.melitensis* bv3	TI
3	Sheep	>100cfu[a]	Rev1	TI	Rev1	TI
4	Goat	>100cfu [a]	*B.melitensis* bv1	PI>100CFU[b]	*B.melitensis* bv1	PI(10-100)[c]CFU
5	Goat	>100cfu [a]	None[b]	PI>100CFU[b]	*B.melitensis* bv1	PI(10-100)[c]CFU
6	Sheep	10-100cfu	*B.melitensis* bv1	TI	*B.melitensis* bv1	TI
7	Sheep	>100cfu [a]	*B.melitensis* bv3	TI	*B.melitensis* bv3	TI
8	Sheep	>100cfu [a]	*B.melitensis* bv3	TI	*B.melitensis* bv3	TI
9	Sheep	>100cfu [a]	*B.melitensis* bv3	PI(10-100)[c]CFU	B.melitensis bv3	PI(10-100)[c]CFU
10	Goat	<10cfu	*B.melitensis* bv1	TI	*B.melitensis* bv1	TI
11	Sheep	>100cfu [a]	*B.melitensis* bv3	TI	*B.melitensis* bv3	TI
12	Sheep	>100cfu [a]	*B.melitensis* bv1	TI	*B.melitensis* bv1	TI
13	Sheep	>100cfu [a]	*B.melitensis* bv3	TI	*B.melitensis* bv3	TI
14	Goat	>100cfu [a]	*B.melitensis* bv1	TI	*B.melitensis* bv1	TI

a. Diffuse growth of contaminants
b. More than 100 CFU contaminants were observed
c. Between 10 and 100 CFU contaminants
TI: Contaminant were totaly inhibited.

Table 2: The growth of Brucella colonies on selective media regarding the day of incubation

Sample	FARRREL MEDIUM		FARRELL MEDIUM WITH ERYTHRITOL	
	Incubation Day: Colony size	Incubation Day: Colony size	Incubation Day: Colony size	Incubation Day: Colony size
1	3 days: None	7 days: >1mm	3 days:≥1mm	7 days: >2mm
2	5 days: ≤1mm	-	5 days: ≥1mm	-
3	5 days: ≤1mm	-	5 days: ≥1mm	-
4	2 days: None	3 days: Diffuse growth[a]	2 days: ≤0.5mm	3 days: Diffuse growth[a]
5	2 days: None	3 days: Contamination	2 days: ≤0.5mm	3 days: Diffuse growth[a]
6	2 days: None	3 days: Diffuse growth[a]	2 days: Diffuse growth[a]	3 days: Diffuse growth[a]
7	2 days: None	3 days: ≤1mm	2 days:≤1mm	3 days: ≥1mm
8	3 days: ≥1mm	6 days: Diffuse growth[a]	3 days: ≤1mm	6 days: Diffuse growth[a]
9	3 days: ≥1mm	-	3 days:≥1mm	-
10	3 days: ≥0,5mm	4 days: ≥1mm	3 days: ≥1mm	4 days: ≥2mm
11	2 days: None	3 days: ≥0,5mm	2 days: ≥0,5	3 days: ≥2mm
12	2 days: ≥0,5mm	5 days: ≤2mm	2 days: ≥1mm	5 days: ≥2mm
13	2 days: None	3 days:≤1mm	2 days: ≤0,5mm	3 days: ≥1mm
14	2 days: None	3 days:≥0,5mm	2 days: ≥0,5mm	3 days: ≥1mm

a: It was not possible to measure the colony size because of the diffuse growth on whole medium surface

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