BAOJ Microbiology

Amir Hosseinabadi, et al., BAOJ Microbio 2017 2: 3
2: 021

Research Article

Evaluation Bactericidal Effect of Horse Milk against *Brucella*

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Abstract

Brucellosis is a highly infectious disease affecting both animals and humans. The current standard tools for the diagnosis of this bacterial infection are serological and microbiological. Brucellosis is zoonosis diseases that are caused by four species of *Brucella,* have a high morbidity that can cause a highly contagious disease in sheep, goats, cattle and one-humped camels. The bacterial strain were sequenced by PCR and was *Brucella melitensis*. The aim of this study was to evaluate the role of horse milk against *Brucella melitensis*. Horse milk was prepared from Tehran Jockey Club. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Brucella* spp. were determined by broth macrodilution and agar well diffusion methods.

Introduction

Brucella is a facultative intracellular pathogen and one of the etiological agents of brucellosis which is a highly common bacterial zoonosis worldwide that can infect humans and animals and cause economic losses [1,2]. It is considered as an endemic disease in Mediterranean Middle East, Western Asia, Africa, and Latin America [3] Persian Gulf [1]. Although Iran have put into effect programs to control and eradicate brucellosis, the disease still occurs in the country [4,5]. *Brucella* species are often categorized according to the principal farm animal they infect [2. Infection with *Brucella abortus* cause several consequences in horses. The most common disease is supra spinouts bursitis (fistulous withers), which results from the apparent predilection of the organism for synovial structures [6]. This is marked by a painful swelling over the withers which may open and drain purulent material [7]. Supra atlantal bursitis (poll evil)may also be caused by *B. abortus* infection [1]. Moreover, *B. abortus* infection is an infrequent cause of abortion in mares and infertility in the stallion [8].

Great efforts were being made to eradicate *B. melitensis* all over the world [5]. After invasion of the lymphoid system, the bacteria are developed within mononuclear phagocytes, and the infected cells play a crucial role in the dissemination of the bacteria in specific locations of the body such as spleen, brain, heart, and bones. *Brucella* species virulence and chronic infections are thought to be due to their ability to escape killing mechanisms within macrophages, such as lysosomal enzymes and products of the oxidative burst. In spite of the improvements in food hygiene and food production techniques, food safety is an increasingly important public health issue. For this reason, to produce safe foods new methods are still needed, to possibly in combination with the existing methods, reduce or inhibit food borne pathogens. Because of increasing pressure from consumers and legal authorities, food industry has tended to reduce the use of chemical preservative since their products to either completely nil or to adopt more natural alternatives for the maintenance or extension of product shelf life. The aim of this study was to screen *in vitro* antibacterial activity of mare milk against *B. melitensis* isolates.

Materials and Methods

Mare Milk Collection

Horse milk was prepared from Inqilab Jockey Club of Tehran, then was transferred to the laboratory.

Bacterial Strain

The *B. melitensis* strain used in this study was isolated from aborted sheep fetus with acute brucellosis. It was identified to the species level by conventional methods (the requirement for CO for growth, production of HS, urease production, sensitivity to fuchsine and thionine, and agglutination with specific antiserum). Extraction of DNA carried out and then IS711specific gene amplified by Polymerase Chain Reaction by IS711specific gene. A class II biological safety cabinet was used. This strain was stored in skim milk at −70 °C and twice sub cultured before starting the study.

Bacterial Culture

For infection experiments, *B.melitensis* was grown for 48 h in 2YT (peptone; 16 g, sodium Chloride; 5 g, meat extract; 10 g, distilled water;1 liter, (Difco, BD, Spars, MD) with 5% sterile.

Horse serum. Bacteria were suspended in a sterile phosphate-buffered saline (PBS). Abundance of *B.melitensis* in PBS was

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Sub Date: December 30, 2016, **Acc Date:** January 6, 2017, **Pub Date:** January 6, 2017.

Citation: Amir Hosseinabadi, Asma Razzhaghi, Abbas Bahador, Davoud Esmaeili and Mohsen Korani (2017) Evaluation Bactericidal Effect of Horse Milk against Brucella. BAOJ Microbio 2: 021.

monitored by recording optical density (OD) at 590 nm.

The exact number of bacteria colony forming units (CFU)was assessed by viable count on 2YT agar (20g/L) plates. Plates were placed in an incubator for48 h at 37°C with 10% CO_2 tension adjusted automatically.

Antibacterial Susceptibility Assay

The test isolate was grown in Muller-Hinton Broth (MHB, Merck) medium at 37 °C for 22 h. Final inoculum bacterial numbers were adjusted to 10^8 CFU/ml with reference to the McFarland turbidimetry [9,10]. A total of 0.1 ml of bacterial suspension was poured on each plate containing Muller-Hinton Agar(MHA, Merck). The lawn culture was prepared by sterile cotton swab and allowed to remain in contact for 1 min. Four concentrations of mare milk (50, 100, 200, and 400 mg/ml) was prepared. The sterile filter paper discs (6-mm diameter) were saturated by 50 µl of mare milk and then were placed on lawn cultures [11]. The Petri dishes were subsequently incubated at 37 °C for 24 h and the inhibition zone around each disc was measured in mm. As positive controls, discs (Difco, USA) containing streptomycin 10 µg, tetracycline 30 µg, gentamicin10 µg, doxycycline 30 µg, oxacillin 1 µg, colistin10 µg, nafcillin 1 µg, and methicillin 5 µg were used. Different discs impregnated with 80% ethanol and 80% methanol were also included to test if they had an inhibitory effect on the test bacteria.

MIC and MBC Determination

In this study we tested isolated strains with Disc Diffusion method (antibigram disc- Merck) by minocycline, doxycycline, co-amoxiclave, rifampin, ciprofloxacin, streptomycin, cotrimoxazole, ceftriaxone, tetracycline, tigecyclin, gentamicin for susceptibility test. Since, there is not control for report of antibiogram test of *Brucellaspp*.. We used the *Haemophilus* influenzae (ATCC 49247) for antibiogram control. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for horse milk were determined against *B. melitensis*. MIC was determined by the macro broth dilution assay method [12]. In the tube dilution assay, standard bacterial suspension and different concentrations mare milk (5, 10, 20, 40, 80,160, and 200 mg/ml) were added to tubes containing1 ml MHB. These tubes were incubated at 37 °C for 24 h. The first tube in the above series with no sign of visible growth was considered as the MIC. MBC was determined by culturing one standard loop of the tubes with no apparent growth on MHA and subsequent incubation at 37 °C for 24 h. The least concentration that inhibited colony formation on agar was taken as the MBC for these extracts.

Result

The result of susceptibility test for antibiotic resistant of *Brucella melitensis* showed in table 1 by measuring inhibition according to CLSIM100-S172014.The strain was resistant to rifampin. The MBC result by colony Forming Unit of bactericidal Horse milk in comparison with control are significant ($p<01$) in chart 1. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for horse milk were determined against *B. melitensis*. MIC was determined by the macro broth.

Discussion

Brucellosis remains the most common zoonotic infection worldwide [13] and although eradication programmers have managed to diminish its prevalence in most developed countries, the disease is re-emerging in new global foci, is still present in certain areas of the developed world and, moreover, is continuously imported into non-endemic areas through international travel or infected food products [14]. The importance of human disease lies in the fact that it can induce chronic morbidity [15] and it demands complex and protracted treatment schedules that still are not always effective in eradicating the infection. Since global eradication of animal brucellosis will not be feasible in the near future owing to socio-economic and political factors, and since the evolution of an adequate human vaccine currently seems a utopia [16], there exists a need for optimal antibiotic treatment schedules. These would ideally minimize the percentage of treatment failures and relapses whilst simultaneously being affordable for populations of low socio-economic status as well as being convenient in order to ensure adequate patient adherence.

Antibiotic Name	Disc	Concertation	Resistance	Intermediate	Sensitive
Tigecyclin	TIG	15	0	0	30
Co-amoxiclave	AMC	15	8	2	20
Rifampin	RA	10	10	6	14
Ciprofloxacin	CP	5	3	5	25
Streptomycin	S	15	3	3	23
Cotrimoxazole	SXT	20	2	3	22
Ceftriaxone	CRO	30	5	2	23
Gentamicin	GM	10	0	0	29
Doxycycline	D	30	0	2	30
Minocycline	MIN	30	0	0	30
Tetracycline	TE	30	0	0	30

Table1: antibiotic susceptibility test

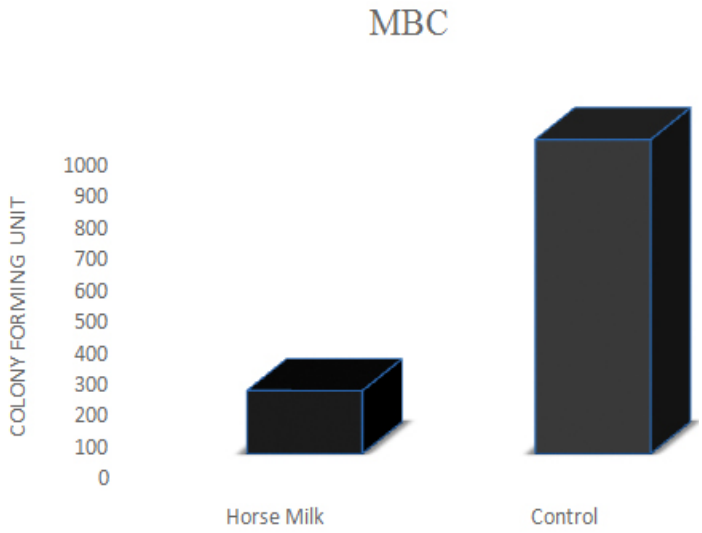

Chart 1: The MBC of Horse milk in comparison with control group.

The World Health Organization [17] guidelines from 1986 [18] are still considered the gold standard for the treatment of brucellosis, suggesting either the combination of doxycycline and rifampicin for 6 weeks, or the combination of doxycycline for 6 weeks with streptomycin for 2 or 3 weeks. These guidelines acknowledged the need for protracted treatment to minimize the percentage of relapses, which for previous 3 week treatment schedules reached almost 20%. The effectiveness of the combination of streptomycin with a tetracycline has been acknowledged since the early days of antibiotic use [19], and the addition of rifampicin in treatment regimens for brucellosis also has a history of more than 30 years [20]. Both regimens are cheap, with few complications and convenient dosing schedules. Yet drawbacks exist for both regimens, the most obvious being that they have not been universally accepted; in various endemic regions, optimal regimens are modified according to the experience of local specialists, both regarding the duration of treatment and the nature of the regimen, as will be discussed later. Other drawbacks include: the need for parenteral administration of streptomycin, which requires either an adequate healthcare network for outpatient treatment (which is not common in many endemic areas) or prolonged hospitalization; inadequate study of the side-effect profiles of these regimens (for example, ototoxicity and renal toxicity of streptomycin have not been consistently followed-up); the danger of inducing rifampicin resistance through its use in brucellosis, which would cause significant problems in the treatment of an infection with similar endemicity but far more severe morbidity and mortality, namely brucellosis [21] the effect the extended treatment schedule has on patient adherence to treatment (especially since symptom resolution is usually brisk); and most importantly the fact that relapses also occur with these regimens, with the average percentage in most studies nearing 10%. This relapse percentage has often been considered acceptable compared with relapse percentages observed with other treatment regimens or shorter duration of treatment, yet it still refers to a considerable number of patients and should be targeted for further decrease. The advantages and disadvantages of the suggested WHO regimens are summarized in Table 2. Using these regimens as the gold standard, efforts to implement a new approach to the antibiotic treatment of brucellosis should target minimizing relapses, treatment failures and duration of treatment without a reciprocal increase in relapses or antibiotic dosage, preferably with the use of a single antibiotic. Usually horses resistant to brucellosis. We decide to study milk products against *brucella*. *Brucella* was excreted by milk but horses milk have probably compounds that enable inhibit *brucella***.**

Alternative Approaches

Instead of hoping for a new, more efficient regimen, viewing the whole pathogenesis picture might allow for a different approach to the treatment of brucellosis. Since the acidic intracellular environment where *Brucella* reside is the main determinant of the impaired ability of antibiotics to eradicate human disease, one could attempt to modify this environment, thus allowing for enhanced effectiveness of the commonly used antibiotic classes and, furthermore of macrolides. How can this environment be altered? The experience from other intracellular bacteria with a propensity for chronic invasions and infection, such as *Coxiella burnetii*, might be useful; it has been proven that a similar acidic environment exists for *C. burnetii* and that this environment decreases microbial antibiotic susceptibility. The addition of hydroxychloroquine in the classical treatment of chronic of ever improved the outcome [22] by neutralizing this environment and therefore enhancing antibiotic action. A similar approach in brucellosis might prove important, by possibly allowing the use of a single antibiotic agent, decreasing the overall duration of therapy, or allowing for the implementation of other antibiotic classes currently considered inactive. However, such an approach first needs to be experimentally outlined and subsequently extensively studied clinically. Immunomodulation techniques have been studied in models and in human cases of chronic brucellosis, in the context of viewing chronic disease as a result of inadequate immune response. Studies with Levamisole [23], interferon [24] and bacterial extracts [25] exist, and Spink and Hall [26] studied corticosteroid administration as early as 50 years ago, yet clarification of the true nature of chronic brucellosis should precede such experimental processes. In a similar manner, one can expect that evolution of the understanding of the molecular pathogenesis of the disease may allow for identification of possible vaccine targets in the future, although the development of such a vaccine is hampered by the enormous cost and the minimal morbidity, as well as the developing world-endemicity of the disease that has attenuated international scientific interest on brucellosis [27].

At the Present Time Six Species of Brucella are Recognized

B. abortus, *B. melitensis*, *B. suis*, *B. ovis*,*B.canis* and *B. neotoma* [28]. Horses appear to be more resistant to *Brucella* infection than cattle, swine, and goats [29]. Two *Brucella* species have been isolated in horses, namely *B.abortus* and *B. suis* biovars 1 [30] and 3 [31]. In Iran, *B.abortus* and *B. melitensis* are more prevalent [32] *B. suis*, *B.*

neotoma, *B. ovis* were not isolated in Iran [33,34].

The principal serological test used for brucellosis diagnosis is the RBPT(Rose Bengal Plate Test), which is a screening test with high (>99%) sensitivity and low specificity in humans [3] as well as in bovines [35]. Although no single test provides 100% sensitivity and specificity, SAT(Serum Agglutination Test) still remains the alternative test and is the test used for verification as it is a standard method for the diagnosis of brucellosis [36]. The sensitivity and specificity of the SAT are 95.6 and 100%, respectively [37,38]. 2-mercaptoethanol test has been used in cattle for the serological diagnosis of brucellosis.

Antibiotic first Line	Advantage	Disadvantage
Tetracycline	Low cost Few adverse effects High efficacy	Disease relapse
Antibiotic second Line	Advantage	Disadvantage
Rifampin SXT	Convenient	Antimicrobial resistance

Table 2 : Advantage and disadvantages of treatment brucellosis in human

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