

***In Vitro* Activities of Antimicrobials Against *Brucella abortus* Isolates from Cattle in Korea During 1998–2006**

Heo, Eun-Jeong, Sung-Il Kang, Jong-Wan Kim, Moon Her, Donghee Cho, Yun-Sang Cho, In-Yeong Hwang, Jin-San Moon, Sung-Hwan Wee, Suk-Chan Jung, and Hyang-Mi Nam*

Animal, Plant and Fisheries Quarantine and Inspection Agency, Anyang 430-824, Korea

Received: September 8, 2011 / Revised: November 20, 2011 / Accepted: November 27, 2011

***In vitro* activities of 13 antibiotics were assessed against 85 *Brucella abortus* isolates from naturally infected cattle in the Republic of Korea during 1998–2006, using broth microdilution test. Tetracyclines showed the most excellent activity against *B. abortus*, displaying MIC values of 0.5 µg/ml or below. In particular, minocycline showed the lowest MIC_{50/90} values (0.125/0.125 µg/ml) in this study. Among four fluoroquinolones tested, ciprofloxacin (MIC_{50/90}, 0.5/1 µg/ml) and norfloxacin (MIC_{50/90}, 8/8 µg/ml) had the most and the least activities, respectively. Gentamicin (MIC_{50/90}, 1/1 µg/ml) was more effective than streptomycin, erythromycin, rifampin, and chloramphenicol (MIC_{50/90}, 2/2 µg/ml).**

Keywords: Antimicrobial susceptibility, *Brucella abortus*, cattle

Brucellosis is a zoonotic disease in many countries around the world [7]. Bovine brucellosis had remained a sporadic problem for a long time in the Republic of Korea (referred to as ROK hereafter) until 1984, when the incidence of the disease started to increase [30]. With increasing of bovine brucellosis in the ROK, the first case of human brucellosis was also reported in 2002 [24]. Since then human brucellosis has emerged as an important public health problem in ROK. According to data obtained from the Korea Centers for Disease Control and Prevention (<http://www.cdc.go.kr/kcdchome/jsp/observation/stat/rgt/STATRGT0003List.jsp>), 47 human cases were reported in 2004, 158 cases in 2005, 215 in 2006, 101 in 2007, 58 in 2008, 24 in 2009, and 31 in 2010. Although currently *Brucella melitensis* remains the principal cause of human brucellosis worldwide [18, 19],

only *B. abortus* has been reported as the cause of human brucellosis in the ROK.

As *Brucella* species are intracellular pathogens, the treatment requires a long duration with agents that may efficiently penetrate macrophages [12]. The World Health Organization (WHO) has recommended the combination of doxycycline and rifampin for the treatment of human brucellosis [31]. Since then, no major changes in the therapeutic modalities have taken place, although there have been many studies on the treatment of brucellosis for humans [4]. *In vitro* susceptibilities of these antibiotics may change over time and from one geographical region to another [5]. Despite that human brucellosis continues to occur primarily in livestock-related workers [25], few studies have been conducted to investigate the antimicrobial susceptibility of *B. abortus* isolated from naturally infected cattle in the ROK. Although a study was reported recently [16], the method used was disc diffusion and the strains tested originated from only a limited part of the country. The purpose of this study was, therefore, to assess the antimicrobial susceptibility of *B. abortus* strains isolated from naturally infected cattle in nationwide Korea during 1998–2006.

To assess the antimicrobial susceptibility, 85 *B. abortus* isolates were selected from a total of 743 isolates recovered from naturally infected cattle of farms located nationwide in Korea during 1998–2006. The selected isolates represented isolates from different farms and animals in different regions. The number of *B. abortus* isolates selected by different geographical locations was as follows: 19 strains from Gyeonggi area, 11 from Gangwon area, 9 from Gyungbuk area, 9 from Gyungnam area, 5 from Chungbuk area, 12 from Chungnam area, 11 from Jeonbuk area, 7 from Jeonnam area, and 2 from Jeju area. These *B. abortus* isolates were recovered from milk and blood samples of infected cattle that were serologically positive and/or showed clinical signs, including abortion. Identification and confirmation

*Corresponding author

Phone: +82-31-467-1772; Fax: +82-31-467-1778;
E-mail: namhm@korea.kr

of the isolates were performed using biochemical tests [23] and PCR system targeting the *Brucella* 16S rRNA gene, as previously described [10, 27].

Minimum inhibitory concentrations (MICs) were determined for 85 isolates and one standard strain (*B. abortus* biovar 1 type strain, 544) using broth microdilution method described previously [22]. Briefly, each colony picked up from *Brucella* agar was cultured in Trypticase soy broth (TSB; BBL, Microbiology Systems, Cockeysville, MD, USA) at 37°C for 48 h under the condition of 0.5% CO₂. Conventional broth dilutions were prepared using serial 2-fold dilutions of the antimicrobials in TSB. The ranges of final concentrations of antimicrobials were 64 (>32) ~ 0.063 µg/ml. These dilutions were inoculated with organisms grown in TSB, and adjusted to 0.5 Macfarland turbidity using a Vitek Colorimeter (bioMérieux, Durham, NC, USA). The endpoint was considered to be the lowest concentration of antimicrobial agent that inhibited growth. Antimicrobials tested in this study included tetracyclines (tetracycline, doxycycline, minocycline), aminoglycosides (streptomycin and gentamicin), quinolones (enrofloxacin, ofloxacin, norfloxacin, and ciprofloxacin), rifampin, erythromycin, ampicillin, and chloramphenicol. Those antimicrobials were purchased from Sigma (St. Louis, MO, USA).

The MICs, MIC₅₀, and MIC₉₀ values of 13 single antibiotics against 85 *B. abortus* isolates and one standard strain are shown in Table 1. All antibiotics tested had MIC_{50/90} values of 2 µg/ml or below, except for ampicillin and norfloxacin. Norfloxacin showed the highest MIC_{50/90} values (8 µg/ml) and ampicillin the second highest MIC₉₀

(4 µg/ml), respectively. For most antibiotics, the values of MIC₉₀ were the same as the values of MIC₅₀. For ampicillin, enrofloxacin, ofloxacin, and ciprofloxacin, however, the values of MIC₉₀ were 2-fold higher than those of MIC₅₀. The three tetracyclines showed the most excellent activity against *B. abortus*, displaying MIC values of 0.063 µg/ml or below. In particular, minocycline showed the lowest MIC_{50/90} values (0.125/0.125 µg/ml) of the antibiotics tested in this study. Four kinds of fluoroquinolones showed various activities against *B. abortus*: Ciprofloxacin was the most active with MIC_{50/90} values of 0.5/1 µg/ml, whereas norfloxacin showed the least activity with MIC_{50/90} values of 8/8 µg/ml. Ofloxacin and enrofloxacin had almost similar activities with MIC_{50/90} values of 1/2 µg/ml. Two aminoglycosides tested in this study were also effective against *B. abortus*, although 3 of 85 isolates showed exceptional resistance to streptomycin. Gentamicin (MIC_{50/90} values of 1/1 µg/ml) was more effective than streptomycin (MIC_{50/90} values of 2/2 µg/ml). The rest of the antibiotics such as erythromycin, rifampin, and chloramphenicol showed almost similar activities with the same MIC_{50/90} values of 2/2 µg/ml, and ampicillin was less effective than these antibiotics (MIC_{50/90} of 2/4 µg/ml).

B. abortus is the most common brucellosis agent in both humans and animals in the ROK. Treatment for brucellosis commonly requires three principle components: adequate intracellular concentration of antibiotics, antibiotic combination, and evaluation of *in vitro* susceptibility of the antibiotics [5]. In this study, we assessed MICs of 13 different antimicrobials against *B. abortus* isolated from naturally

Table 1. Minimal inhibitory concentrations (MICs), MIC₅₀, and MIC₉₀ values of thirteen single antibiotics against *B. abortus* isolates from naturally infected cattle in Korea (N = 86)*.

Antimicrobial agent	No. of <i>B. abortus</i> at indicated MIC (µg/ml)											MIC of <i>B. abortus</i> type 1 544	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	Break-points Sensitive
	>32	32	16	8	4	2	1	0.5	0.25	0.125	0.063				
Ampicillin					11	42	22*	7	3	1		1	2	4	1 ^b
Streptomycin	3					43	35	5*				0.5	2	2	8 ^a
Gentamicin						5	78	3*				0.5	1	1	4 ^a
Doxycycline								3	45	37	1*	0.063	0.25	0.25	1 ^a
Tetracycline								4	50	32*		0.125	0.25	0.25	1 ^a
Minocycline									7	66	13*	0.063	0.125	0.125	- ^c
Erythromycin					7*	51	28					4	2	2	- ^c
Rifampin	1				4	59	19	3*				0.5	2	2	1 ^b
Chloramphenicol					1	64	21*					1	2	2	2 ^b
Enrofloxacin					1	13	69	3*				0.5	1	2	- ^c
Ofloxacin						18	64	4*				0.5	1	2	2 ^b
Norfloxacin			2	74	10*							4	8	8	- ^c
Ciprofloxacin					2	2	35	46*	1			0.5	0.5	1	1 ^b

*The number includes the MIC result of *B. abortus* type 1 544 standard strain.

^aCLSI breakpoints for *Brucella* spp.

^bCLSI breakpoints for *Haemophilus* spp. (slowly growing organism).

^cNo breakpoints in CLSI table for both *Brucella* spp. and *Haemophilus* spp.

infected cattle in the ROK. The breakpoints used for interpretation as susceptible were referenced according to the CLSI interpretive criteria for *Brucella* spp. and slow-growing bacteria (*Haemophilus* spp.) as described previously [9, 21]. As a result, all antibiotics tested proved to be effective against *B. abortus* isolates *in vitro*, although norfloxacin and ampicillin showed relatively inactivity compared with the other antibiotics. This is similar to the results obtained from a previous study, which was conducted on the antimicrobial susceptibility of *B. abortus* isolated from naturally infected cattle in the southeastern part of the ROK using the disc diffusion method [16].

In this study, tetracyclines proved to be the most effective antimicrobials against *B. abortus* *in vitro*. No *B. abortus* isolates required 0.5 µg/ml or greater amounts of tetracyclines for inhibition. This is in agreement with results from previous studies [5, 22], showing that tetracycline is the most sensitive agent to *B. abortus*. Although minocycline showed the best activity against *B. abortus* isolates in this study, doxycycline has become the most popular choice because of its longer half-life and fewer side effects [29]. Monotherapy for brucellosis is associated with a high relapse rate and thus dual therapy in different combinations is recommended [1], such as doxycycline–rifampicin [31] and doxycycline–streptomycin as first-line regimens for treatment of brucellosis [4].

Our results showed that gentamicin was more sensitive than streptomycin, which is similar to the results from several studies [13, 15]. In this study, three isolates of *B. abortus* showed exceptional resistance to streptomycin (MIC: >32). Those were isolated from cattle in the same area of the ROK, and two of them were originated from the same herd. Although no information was available on disease and treatment history of the herd, it is assumed that streptomycin may have been used for a therapeutic agent in the herd and resistance to this antimicrobial agent may have developed.

Rifampin was known to penetrate cell membranes and has been used to treat human brucellosis [3, 28]. However, this drug is not used in monotherapy because of emergence of rifampin-resistant variants [6, 20]. MIC values of rifampin have been reported ranging from 1 to 4 µg/ml [5, 17]. In this study, rifampin showed a MIC range of 0.5–4 µg/ml, except for one strain isolated in 1999. Tracing the source of the isolate, this strain was found to be originated from dairy cattle vaccinated with RB51 in 1998. No RB51 vaccine has been used since 1998 in the ROK. Thus, it was assumed that vaccine strains may have remained in some cattle even after one year or so. It was previously proved that *Brucella* AMOS PCR assay, developed to identify and differentiate specific *Brucella* species, could correctly identify and differentiate *B. abortus* isolates as field strains, vaccine strain 19 (S19), vaccine strain RB51, or the RB51 parental strain S2308 [11]. The abbreviated AMOS

assay is based on the insertion of the genetic element IS711 at a unique chromosomal locus in *B. abortus* biovars 1, 2, and 4 and the double insertion of IS711 at a specific locus in *B. abortus* RB51 [8]. Thus, we performed AMOS PCR for all the *B. abortus* isolates tested in this study to detect the RB51-specific DNA band by a previously described method [8, 11]. Of 85 *B. abortus* isolates, only the strain resistant to rifampin showed RB51-specific bands of 364 bp and 498 bp (Fig. 2), indicating that it was a RB51 vaccine strain.

Quinolones have been reported as being particularly effective agents against brucellosis *in vitro* [2]. Similar results were obtained from our study, although norfloxacin showed the least activity among the antibiotics tested in this study. We also found that erythromycin, ampicillin, and chloramphenicol had *in vitro* activity against *B. abortus*. Similar results were reported by several authors for erythromycin [26], ampicillin [15], and chloramphenicol [15, 22], although there were also some studies that described results different from ours [2, 22].

Based on the results obtained in this study, it could be concluded that *B. abortus* is most susceptible to tetracyclines, among the antibiotics tested in this study. In human brucellosis, however, single drug therapy was not recommended, and combination therapy of tetracyclines plus rifampin, especially doxycycline and rifampin, was recommended by the WHO [31]. Therefore, further studies are needed to test the combination antimicrobial susceptibility of *B. abortus* isolates for the antimicrobials selected in this study to be used in the treatment of human brucellosis in Korea.

Acknowledgment

This work was supported by the National Veterinary Research and Quarantine Service, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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