Chapter 9

Antibiotic Susceptibility Testing of *Brucella* Species - Old and New Drugs

Max Maurin

Additional information is available at the end of the chapter

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Abstract

Brucella species cause brucellosis in humans and animals, a zoonosis that can manifest not only as acute or chronic diseases but also as silent infections persisting throughout life with recurrences potentially occurring after several decades. *In vitro* and *in vivo* methods have been developed to evaluate the bacteriostatic and bactericidal activity of antibiotics against *Brucella* sp. Especially eukaryotic cells and animal models have been used to evaluate the ability of antibiotics, alone or in combination, to eradicate these bacteria from their intracellular reservoir. Although treatment recommendations have been established for common clinical forms of brucellosis, optimized therapeutic alternatives are still needed for severe forms of the disease, and for infections occurring in young children and pregnant women. Moreover, acquired resistance to first-line treatments of brucellosis is a current concern. This chapter will summarize current knowledge on *in vitro* and *in vivo* interactions between *Brucella* species and antibiotics and new therapeutic strategies that have been evaluated.

Keywords: *Brucella*, brucellosis, antibiotic susceptibility testing, antibiotic resistance, treatment

1. Introduction

Most *Brucella* species are highly infectious in humans and thus are considered class 3 biological agents [1–3] and potential biological threat agents by the CDC (class B) [4,5]. Because of a high risk of human infections, especially through inhalation of infectious aerosols, the *Brucella* cultures should be handled in a biosafety level 3 laboratory. Also, in many countries, detention of these pathogens is now subject to strict regulations. The clinical symptoms of brucellosis are often unspecific. Therefore, the diagnosis may be delayed, especially in geographic areas where the disease is rare and thus often not evoked by physicians in febrile patients. A definite

diagnosis of brucellosis relies on isolation of *Brucella* sp. from infected patients, mainly from blood samples during the first few weeks following the onset of symptoms. Serological methods lack specificity, and only represent a stopgap for brucellosis diagnosis. PCR-based techniques are useful to detect *Brucella* DNA in clinical samples, especially in patients with suppurated secondary locations. A specific antibiotic therapy should be started as soon as possible to avoid severe complications (including neurological and cardiac involvement), and evolution to a chronic debilitating disease. However, current treatment alternatives are still scarce in adult patients, and even more limited in young children and pregnant women. Although rarely fatal, brucellosis remains a major public health problem worldwide, and a significant economic burden in livestock because of its abortive nature. This chapter will summarize current knowledge on antibiotic susceptibilities of *Brucella* species and treatment alternatives for human brucellosis. In the first part of this chapter, experimental models currently used for the evaluation of the activity of antibiotics against *Brucella* species will be presented, including *in vitro* models with or without eukaryotic cells, and animal models. The results obtained in these experimental models will be summarized and tentatively correlated with each other and with current knowledge on the clinical efficacy of antibiotics in brucellosis patients. The available data on antibiotic resistances in *Brucella* species will be presented, with their potential impact in clinical situations. The second part of this chapter will present current strategies for the development of new therapeutic alternatives for human brucellosis. These may include the development of new drugs inhibiting the intracellular growth of *Brucella* sp., reducing the virulence of this pathogen or enhancing the host response to *Brucella* infection.

2. Experimental models for evaluation of the activity of antibiotics against *Brucella* spp.

Routine antibiotic susceptibility testing (AST) of *Brucella* sp. is not currently advocated because of lack of acquired resistances to clinically useful antibiotics and a high risk of laboratory-acquired brucellosis [6,7]. Three types of experimental models have been used to assess the activities of antibiotics against *Brucella* sp.: AST in cell-free liquid or solid media (minimum inhibitory concentration (MICs)), AST in *Brucella*-infected eukaryotic cell models, and *Brucella*-infected animal models. We will summarize data obtained in these three models and their respective predictive value of the clinical efficacy of antibiotics in brucellosis patients.

2.1. AST in axenic media

2.1.1. Bacteriostatic activity by class of antibiotics

Current reference methods for the *in vitro* determination of the minimum inhibitory concentration (MIC) of antibiotics against bacteria in axenic media do not apply to the fastidious and slow growth of *Brucella* species. The method recommended by the Clinical and Laboratory Standards Institute (CLSI) includes the use of *Brucella* medium (pH 7.1), inoculated with a 0.5 McFarland standard inoculum, and an incubation at 35 ± 2°C in aerobic atmosphere for 48 h

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before reading MICs [8]. An incubation in 5% CO_2-enriched atmosphere may be required for some *Brucella* strains, but this usually increases MIC levels [9–11]. Using this method, susceptibility breakpoints for *Brucella* are ≤ 8 mg/L for streptomycin; ≤ 4 mg/L for gentamicin; ≤ 1 mg/L for tetracycline, doxycycline, and rifampicin; and ≤ 2/38 mg/L for the combination of trimethoprim (TMP) plus sulfamethoxazole (SMX) (i.e., cotrimoxazole).

In the literature, however, the multiplicity of methods used for MIC determination for *Brucella* strains shows a lack of standardization (Tables 1 and 2). MICs were determined using either the Kirby–Bauer agar disk diffusion method, the E-test strip method, a broth dilution or microdilution method, or the agar dilution method. Culture media have included Brucella broth, Mueller Hinton agar with or without 5% sheep blood, Mueller Hinton broth supplemented with 1% polyvitex with or without 1% hemoglobin, Trypticase soy broth, and Iso-Sensitest® Agar (Oxoid, CM47L). The tested bacterial inoculum was expressed in McFarland standard, cfu/mL, or cfu per spot, often without any correspondence between units, and it varied between studies (e.g., 10^5–10^6 cfu/mL, 0.5–1 McFarland). The incubation atmosphere varied from 35°C to 37°C, with 0%–10% CO_2. The incubation time before reading MICs varied from 24 h to 48 h. Obviously, all these parameters may change MIC levels. Several studies have shown that a high bacterial load and a low pH of the culture medium increase MICs by two to four times or even more [9,11–15].

A first interesting finding is the variability in susceptibility to beta-lactams among *Brucella* strains (Table 2). In cell-free media, MICs to ampicillin varied from 0.02 mg/L to 8 mg/L, with MIC90 of 2–4 mg/L [9,16–19]. For ceftriaxone, a third-generation cephalosporin, MICs varied from 0.064 mg/L to 4 mg/L, with MIC90 of 0.5–1 mg/L [20–23]. For thienamycin, a carbapenem compound, MICs varied from 0.1 mg/L to 2 mg/L, with an MIC90 of 2 mg/L [24]. These variations were also observed for other beta-lactams tested, although *Brucella* species were less susceptible to aztreonam [20]. Because heterogeneity in MICs was observed whatever the methodology used, they may represent true variations in genetic backgrounds among *Brucella* strains. However, beta-lactamases have never been characterized in these species, neither variations in penicillin-binding proteins (PBP).

The tetracyclines display the lowest MICs against *Brucella* spp. in cell-free media (Table 1). However, these MICs greatly vary according to the methodology used. Doxycycline, which is currently recommended as first-line treatment of brucellosis, displayed MICs ranging from 0.6 to 0.25 mg/L (MIC90 of 0.12–0.25 mg/L), when using the agar dilution method with either the CM47L medium or Mueller Hinton agar, supplemented with hemoglobin and polyvitex (1% each) [23,25]. The broth microdilution method gave MICs ranging from 0.01 to 0.5 mg/L (MIC90 of 0.06–0.3 mg/L), using various media and incubation conditions [9,10,19]. However, MICs up to 8 mg/L were reported in a Turkish study of 43 human strains of *B. melitensis* [11]. The only significant difference in the methodology might have been the use of a high bacterial inoculum (i.e., 10^5–10^6 cfu per well). The E-test method, using sheep blood-supplemented Mueller Hinton agar and a 0.5 McFarland standard inoculum, gave MICs ranging from 0.023 to 0.5 mg/L (MIC90 of 0.064–0.38 mg/L) [21,22,26–28]. Using the E-test method, much higher MICs ranging from 8 to 32 mg/L were recently reported for 19 Chinese strains of *B. melitensis*, although the incubation time before the MIC reading was only 24 h [17]. Tigecycline was no

more effective than doxycycline, with MICs ranging from 0.019 to 0.5 mg/L using the E-test method [26,27,29].

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, $\%CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
Aminoglycosides						
Streptomycin	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	2.5	0.15->100	[9]
	Israel/H	Bru, 31, 1978–82	Agar dilution (CM47L, 10^4/mL, 10%, 37°C, 48 h)	2	0.125-4	[12]
		Bru, 31, 1978–82	Agar dilution (CM47L, 10^5/mL, 10%, 37°C, 48 h)	8	0.06-8	[12]
		Bru, 31, 1978–82	Agar dilution (CM47L, 10^6/mL, 10%, 37°C, 48 h)	8	0.25-8	[12]
	Spain/H	Bm, 95, 1980–84	Agar dilution (CM47L, 10^5cfu/spot, 0%, 37°C, 48 h)	0.5	0.12-1	[23]
	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	4	1-4	[18]
	Saudi Arabia	Bm, 47, (1989)	Broth dilution (BB,5 × 10^5cfu/mL, 0%, 35°C, 48 h)	2.5	0.15-5	[13]
	Israel/H	Bm, 86, (1991)	Broth microdilution (BB, 5 × 10^5cfu/mL,5%,37°C,48 h)	3.1	NA	[33]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/7,$10^{5\text{-}6}$cfu,0%,35°C,48 h)	2	0.25-8	[11]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/5,$10^{5\text{-}6}$cfu,0%,35°C,48 h)	128	8-256	[11]
	Spain/HA	Bru, 62, (1993)	Agar dilution (MH-HP,10^4cfu/spot,10%,35°C,48 h)	4	0.1-4	[30]
	Spain/H	Bm, 160, 1997	Agar dilution (MH-HP,10^4cfu/spot,10%,35°C,48 h)	8	4-16	[25]
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	2	0.5-2	[19]
	Greece/HA	Bru, 74, 1999–2005	E-test (SB-MH, 0.5 McFd, 5%, 35°C, 48 h)	2	0.125-4	[16]
	Egypt/H	Bm, 355, 1999–2007	E-test (SB-MH, 0.5 McFd, 5%, NA, 48 h)	2	0.125-3	[22]

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, $\%CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
	Syria	Bm, 100, 2004–07	Broth microdilution (BB/7, 5 × 10^6 cfu/mL,37°C, 48 h)	>128	64->128	[15]
	Syria	Bm, 100, 2004–07	Broth microdilution (BB/5, 5 × 10^6 cfu/mL,37°C, 48 h)	>128	>128	[15]
	Turkey/H	Bru, 56, 2008–09	E-test (SB-MH, 0.5 McFd, 0%, 35°C, 48 h)	1	0.064-1.5	[27]
	Turkey/H	Bm, 73, 2009–11	E-test (SB-MH, 0.5 McFd, NA, 37°C, 48 h)	1	0.5-1.5	[26]
	Turkey/H	Bm, 76, 2001–06	E-test (SB-MH, 1 McFd, 0%, 35°C,48 h)	1	0.064-1.5	[29]
	US/H	Bru, 39, (2010)	Broth microdilution (BB, NA, 0%, 35°C, 48 h)	2	1-8	[10]
		Bru, 39, (2010)	Broth microdilution (BB, NA, 5%, 35°C, 48 h)	4	2-16	[10]
Gentamicin	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	0.3	0.02-2.5	[9]
	Israel/H	Bru, 31, 1978–82	Agar dilution (CM47L, 10^4/mL, 10%, 37°C, 48 h)	0.25	0.03-0.25	[12]
		Bru, 31, 1978–82	Agar dilution (CM47L, 10^5/mL, 10%, 37°C, 48 h)	1	0.03-1	[12]
		Bru, 31, 1978–82	Agar dilution (CM47L, 10^6/mL, 10%, 37°C, 48 h)	2	0.03-2	[12]
	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	1	0.25-2	[18]
	Saudi Arabia	Bm, 116, (1995)	Broth dilution (MH,$10^{5\text{-}6}$cfu/mL, 5%, 35°C, 48 h)	0.5	<0.25-0.5	[31]
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	1	0.5-2	[19]
	Greece/HA	Bru, 74, 1999–2005	E-test (SB-MH, 0.5 McFd, 5%, 35°C, 48 h)	2	0.03-1.5	[16]
	Egypt/H	Bm, 355, 1999–2007	E-test (SB-MH, 0.5 McFd, 5%, NA, 48 h)	1	0.094-3	[22]
	Peru/H	Bm, 48, 2000–06	E-test (SB-MH, 0.5 McFd, NA, NA, 48 h)	0.25	0.032-0.25	[28]

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, %$CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
	Turkey/H	Bm, 76, 2001–06	E-test (SB-MH, 1 McFd, 0%, 35°C,48 h)	0.5	0.064-0.75	[29]
	US/H	Bru, 39, (2010)	Broth microdilution (BB, ND, 0%, 35°C, 48 h)	2	0.5-2	[10]
		Bru, 39, (2010)	Broth microdilution (BB, ND, 5%, 35°C, 48 h)	4	0.5-8	[10]
	China/H	Bm, 19, 2010–12	E-test (BA-MH, 0.5 McFd, 5%, 35°C, 24 h)	0.75	0.5-0.75	[17]
Tobramycin	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	2	0.5-4	[18]
Kanamycin	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	2.5	0.02-5	[9]
Amikacin	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	4	1-4	[18]
	China/H	Bm, 19, 2010–12	E-test (BA-MH, 0.5 McFd, 5%, 35°C, 24 h)	12	4-12	[17]
Tetracyclines						
Tetracycline	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	0.04	0.001-0.15	[9]
	Israel/H	Bru, 31, 1978–82	Agar dilution (CM47L, 10^4/mL, 10%, 37°C, 48 h)	0.25	≤0.06-0.5	[12]
		Bru, 31, 1978–82	Agar dilution (CM47L, 10^5/mL, 10%, 37°C, 48 h)	0.5	≤0.06-0.5	[12]
		Bru, 31, 1978–82	Agar dilution (CM47L, 10^6/mL, 10%, 37°C, 48 h)	1	≤0.06-2	[12]
	Spain/H	Bm, 95, 1980–84	Agar dilution (CM47L, 10^5cfu/spot, 0%, 37°C, 48 h)	0.25	0.6-0.25	[23]
	Spain/H	Bm, 98, (1982)	Agar dilution (CM47L, 10^5cfu/spot, 0%, 37°C, 48 h)	0.39	0.1-0.5	[24]
	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	0.25	≤0.13-0.25	[18]
	Spain/H	Bm, 358, 1987–89	Agar dilution (CM471, 10^5cfu/spot, 0%, 37°C, 48 h)	0.25	0.06-0.5	[32]

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, $\%CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
	Saudi Arabia	Bm, 47, (1989)	Broth dilution (BB,5 × 10^5cfu/mL, 0%, 35°C, 48 h)	0.04	0.001-0.6	[13]
	Spain/HA	Bru, 62, (1993)	Agar dilution (MH-HP, 10^4cfu/ spot, 10%, 35°C, 48 h)	0.2	0.01-0.2	[30]
	Saudi Arabia	Bm, 116, (1995)	Broth dilution (MH, 10^{5-6}cfu/mL, 5%, 35°C, 48 h)	0.5	<0.25-0.5	[31]
	Greece/HA	Bru, 74, 1999–2005	E-test (SB-MH, 0.5 McFd, 5%, 35°C, 48 h)	0.5	0.03-1.5	[16]
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	0.25	0.125-0.5	[19]
	Egypt/H	Bm, 355, 1999–2007	E-test (SB-MH, 0.5 McFd, 5%, NA, 48 h)	0.19	0.023-0.75	[22]
	Syria	Bm, 100, 2004–07	Broth microdilution (BB/7, 5 × 10^6 cfu/mL, 37°C, 48 h)	16	0.25-16	[15]
	Syria	Bm, 100, 2004–07	Broth microdilution (BB/5, 5 × 10^6 cfu/mL, 37°C, 48 h)	16	0.25-16	[15]
	US/H	Bru, 39, (2010)	Broth microdilution (BB, ND, 0%, 35°C, 48 h)	0.25	0.06-0.5	[10]
		Bru, 39, (2010)	Broth microdilution (BB, ND, 5%, 35°C, 48 h)	0.25	0.03-0.5	[10]
Doxycycline	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	0.3	0.01-0.3	[9]
	Spain/H	Bm, 95, 1980–84	Agar dilution (CM47L, 10^5cfu/ spot, 0%, 37°C, 48 h)	0.12	0.6-0.25	[23]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/ 7,10^{5-6}cfu,0%, 35°C,48 h)	<0.125	<0.125-8	[11]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/ 5,10^{5-6}cfu,0%, 35°C,48 h)	2	<0.125-8	[11]
	Spain/H	Bm, 160, 1997	Agar dilution (MH-HP, 10^4cfu/ spot, 10%, 35°C,48 h)	0.25	0.12-0.25	[25]
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	0.25	0.063-0.5	[19]
	Egypt/H	Bm, 355, 1999–2007	E-test (SB-MH, 0.5 McFd, 5%, NA, 48 h)	0.25	0.016-0.5	[22]

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, $\%CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
	Peru/H	Bm, 48, 2000–06	E-test (SB-MH, 0.5 McFd, NA, NA, 48 h)	0.38	0.032-0.5	[28]
	Turkey/H	Bm, 76, 2001–06	E-test (SB-MH, 1 McFd, 0%, 35°C,48 h)	0.125	0.016-0.19	[29]
	Syria	Bm, 100, 2004–07	Broth microdilution (BB/7, 5 × 10^6 cfu/mL, 37°C, 48 h)	16	0.5-16	[15]
	Syria	Bm, 100, 2004–07	Broth microdilution (BB/5, 5 × 10^6 cfu/mL, 37°C, 48 h)	8	0.5-8	[15]
	Italy/H	Bru, 20, 2005–06	E-test (SB-MH, 0.5 McFd, 5%, 37°C, 48 h)	ND	0.06-0.125	[21]
	Turkey/H	Bru, 56, 2008–09	E-test (SB-MH, 0.5 McFd, 0%, 35°C, 48 h)	0.064	0.023-0.125	[27]
	Turkey/H	Bm, 73, 2009–11	E-test (SB-MH, 0.5 McFd, NA, 37°C, 48 h)	0.094	0.023-0.19	[26]
	US/H	Bru, 39, (2010)	Broth microdilution (BB, ND, 0%, 35°C, 48 h)	0.25	0.06-0.5	[10]
		Bru, 39, (2010)	Broth microdilution (BB, ND, 5%, 35°C, 48 h)	0.5	0.03-1	[10]
	China/H	Bm, 19, 2010–12	E-test (BA-MH, 0.5 McFd, 5%, 35°C, 24 h)	32	8-32	[17]
Minocycline	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	0.3	0.01-1.25	[9]
	Israel/H	Bm, 86, (1991)	Broth microdilution (BB, 5 × 10^5cfu/mL, 5%, 37°C, 48 h)	0.4	NA	[33]
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	0.125	0.063-0.25	[19]
Tigecycline	Turkey/H	Bru, 56, 2008–09	E-test (SB-MH, 0.5 McFd, 0%, 35°C, 48 h)	0.094	0.019-0.25	[27]
	Turkey/H	Bm, 73, 2009–11	E-test (SB-MH, 0.5 McFd, NA, 37°C, 48 h)	0.125	0.047-0.19	[26]
	Turkey/H	Bm, 76, 2001–06	E-test (SB-MH, 1 McFd, 0%, 35°C, 48 h)	0.094	0.023-0.5	[29]
	Turkey/H	Bm, 38, (2010)	E-test (SB, NA, NA, 35°C, 48 h)	0.5	0.032-0.5	[76]

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, $\%CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
	Turkey/H	Bm, 38, (2010)	E-test (BA, NA, NA, 35°C, 48 h)	1	0.0125-1	[76]
Rifampin	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	1.25	0.02-12.5	[9]
	Spain/H	Bm, 98, (1982)	Agar dilution (CM47L, 10^5cfu/spot, 0%, 37°C, 48 h)	0.5	0.06-1	[24]
	Spain/H	Bm, 95, 1980–84	Agar dilution (CM47L, 10^5cfu/spot, 0%, 37°C, 48 h)	2	0.12-4	[23]
	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB, 5 × 10^5cfu/mL, 6%, 35°C, 48 h)	1	0.06-1	[18]
	Saudi Arabia	Bm, 47, (1989)	Broth dilution (BB, 5 × 10^5cfu/mL, 0%, 35°C, 48 h)	1.25	0.02-2.5	[13]
	Israel/H	Bm, 86, (1991)	Broth microdilution (BB, 5 × 10^5cfu/mL, 5%, 37°C, 48 h)	4	NA	[33]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/ 7,$10^{5\text{-}6}$cfu, 0%, 35°C, 48 h)	2	1-32	[11]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/ 5,$10^{5\text{-}6}$cfu, 0%, 35°C, 48 h)	1	<0.125-1	[11]
	Spain/HA	Bru, 62, (1993)	Agar dilution (MH-HP, 10^4cfu/spot, 10%, 35°C, 48 h)	1	0.1-4	[30]
	Saudi Arabia	Bm, 116, (1995)	Broth dilution (MH, $10^{5\text{-}6}$cfu/mL, 5%, 35°C, 48 h)	1	0.25-1	[31]
	Spain/H	Bm, 160, 1997	Agar dilution (MH-HP, 10^4cfu/spot, 10%, 35°C, 48 h)	1	0.5-1	[25]
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	2	0.5-4	[19]
	Greece/HA	Bru, 74, 1999–2005	E-test (SB-MH, 0.5 McFd, 5%, 35°C, 48 h)	1	0.09-1.5	[16]
	Egypt/H	Bm, 355, 1999–2007	E-test (SB-MH, 0.5 McFd, 5%, NA, 48 h)	4	0.25-6	[22]
	Peru/H	Bm, 48, 2000–06	E-test (SB-MH, 0.5 McFd, NA, NA, 48 h)	0.75	0.19-1	[28]
	Turkey/H	Bm, 76, 2001–06	E-test (SB-MH, 1 McFd, 0%, 35°C,48 h)	1.5	0.064-3	[29]

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, %$CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
	Syria	Bm, 100, 2004–07	Broth microdilution (BB/7, 5 × 10^6 cfu/mL,37°C, 48 h)	64	2-64	[15]
	Syria	Bm, 100, 2004–07	Broth microdilution (BB/5, 5 × 10^6 cfu/mL,37°C, 48 h)	64	2-64	[15]
	Italy/H	Bru, 20, 2005–06	E-test (SB-MH, 0.5 McFd, 5%, 37°C, 48 h)	ND	0.75-2	[21]
	Turkey/H	Bru, 56, 2008–09	E-test (SB-MH, 0.5 McFd, 0%, 35°C, 48 h)	2	0.5-2	[27]
	Turkey/H	Bm, 73, 2009–11	E-test (SB-MH, 0.5 McFd, NA, 37°C, 48 h)	2	0.38-3	[26]
	US/H	Bru, 39, (2010)	Broth microdilution (BB, ND, 0%, 35°C, 48 h)	2	0.25-2	[10]
		Bru, 39, (2010)	Broth microdilution (BB, ND, 5%, 35°C, 48 h)	2	0.25->8	[10]
	China/H	Bm, 19, 2010–12	E-test (BA-MH, 0.5 McFd, 5%, 35°C, 24 h)	2	0.06-2	[17]
Rifapentine	Spain/HA	Bru, 62, (1993)	Agar dilution (MH-HP, 10^4cfu/spot, 10%, 35°C, 48 h)	1	0.2-4	[30]

NA, data not available; Bm, *B. melitensis*; Bab, *B. abortus*; Bru, *Brucella* sp.

[£]Studies have been classified according to the period of isolation of the studied *Brucella* strains (e.g., 2010–12) and the date of the corresponding publication (e.g., (1993)) when the latter was unavailable.

[$]Method: Kirby–Bauer disk diffusion method (Kirby–Bauer); E-test strip method (E-test);

Medium: Mueller Hinton agar with 5% sheep blood (SB-MH) or unspecified percentage and type of blood (BA-MH); 5% sheep blood agar (SB); Brucella broth (BB); Brucella agar (BA); Trypticase soy broth (TSB); Iso-Sensitest® Agar CM47L (CM47L); Mueller Hinton broth supplemented with 1% polyvitex with (HP) or without (P) 1% hemoglobin, at pH 7 (/7) or pH 5 (/5); The bacterial inoculum used for antibiotic susceptibility testing is specified in cfu/mL or according to McFarland standards (McFd). Host: human (H), cattle (C), unspecified or various animals (A).

Table 1. Antibiotic susceptibilities of *Brucella* sp. to aminoglycosides, tetracyclines, and rifampin, as determined in cell-free media.

Rifampicin is the second most active compound against *Brucella* sp. in cell-free medium (Table 1). MICs ranged from 0.06 to 4 mg/L with the agar dilution method [23–25,30], 0.06 to 4 mg/L with the broth dilution method [10,11,13,18,19,31], and 0.06 to 6 mg/L with the E-test method [16,17,21,22,26–29]. Higher MICs (up to 12.5 mg/L) were reported in one study using *Brucella* broth and a 10% CO_2 atmosphere incubation [9]. Rifampicin MICs were lower at acidic pH [11], but higher at increasing concentrations of CO_2 [10,11].

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The aminoglycosides are also highly active *in vitro* against *Brucella* sp. (Table 1). Streptomycin has long been used as first-line treatment of brucellosis, whereas gentamicin is now used in most countries because the former antibiotic is no longer available. MICs to streptomycin varied from 0.12 to 4 mg/L with the agar dilution method [23,30], although a more recent study from Spain reported higher MIC levels (4–16 mg/L) for 160 human strains of *B. melitensis* [25]. MICs varied from 0.1 to 16 mg/L with the broth dilution method [10,11,13,18,19,25]. An acidic pH of the broth medium and/or an incubation in 5%–10% CO_2-enriched atmosphere were associated with higher MICs [9–11]. Higher MIC levels (≥256 mg/L) were, however, occasionally reported with this technique [9,11]. MICs ranged from 0.06 to 4 mg/L with the E-test method [16,22,27,29]. Gentamicin displayed lower MICs, ranging from 0.02 to 2.5 mg/L [9,10,18,31] with the broth dilution method, and 0.03 to 3 mg/L with the E-test method [16,17,22,28,29].

The combination of trimethoprim (TMP) and sulfamethoxazole (SMX) was usually tested at a ratio of 1:19, and only TMP MICs were reported (Table 2). These varied from 0.06 to 4 mg/L with the agar dilution method [23,30], 0.006 to 4 mg/L with the broth microdilution method [10,18,31], and 0.06 to 1.5 mg/L with the E-test method [16,21,22,26–29]. Similar MIC ranges (0.8–3.2 mg/L) were obtained when using TMP/SMX at a ratio of 1:5 [17]. In contrast, higher MICs (5–25 mg/L of TMP) were reported in a study from Saudi Arabia [13], using a broth dilution method with high-volume (5 mL) medium culture and a high bacterial inoculum (2.5×10^6 cfu per test).

Antibiotics	Country /host	Collected isolates: species, n, period[ε]	Method[$] (medium, inoculum, $\%CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
β-lactams						
Penicillin G	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	25	0.3->100	[9]
	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	4	0.25-8	[18]
Ampicillin	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	2.5	0.02-5	[9]
	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	4	0.25-8	[18]
	Greece/HA	Bru, 74, 1999–2005	E-test (SB-MH, 0.5 McFd, 5%, 35°C, 48 h)	2	0.09-3	[16]
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	4	0.125-4	[19]
	China/H	Bm, 19, 2010–12	E-test (BA-MH, 0.5 McFd, 5%, 35°C, 24 h)	2	1.5-2	[17]

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, %$CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
Carbenicillin	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	50	0.6->100	[9]
Cephalothin	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	100	0.3->100	[9]
	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	32	1-64	[18]
Cefoxitine	Spain/H	Bm, 98, (1982)	Agar dilution (CM47L, 10^5 cfu/spot, 0%, 37°C, 48 h)	64	8-128	[24]
	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	16	2-16	[18]
Cefuroxime	Spain/H	Bm, 83, (1986)	Agar dilution (CM47L, 10^5 cfu/spot, 0%, 37°C, 48 h)	32	8-64	[20]
Ceftizoxime	Spain/H	Bm, 83, (1986)	Agar dilution (CM47L, 10^5 cfu/spot, 0%, 37°C, 48 h)	1	0.5-1	[20]
Cefoperazone	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	16	≤1-16	[18]
	Spain/H	Bm, 83, (1986)	Agar dilution (CM47L, 10^5 cfu/spot, 0%, 37°C, 48 h)	32	4-64	[20]
Cefotaxime	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL, 6%,35°C,48 h)	2	≤0.5-4	[18]
	Spain/H	Bm, 83, (1986)	Agar dilution (CM47L, 10^5 cfu/spot, 0%, 37°C, 48 h)	2	≤0.5-2	[20]
Ceftriaxone	Spain/H	Bm, 95, 1980–84	Agar dilution (CM47L, 10^5cfu/spot, 0%, 37°C, 48 h)	0.5	0.12-1	[23]
	Spain/H	Bm, 83, (1986)	Agar dilution (CM47L, 10^5 cfu/spot, 0%, 37°C, 48 h)	1	≤0.25-1	[20]
	Egypt/H	Bm, 355, 1999–2007	E-test (SB-MH, 0.5 McFd, 5%, NA, 48 h)	1	0.064-4	[22]
	Italy/H	Bru, 20, 2005–06	E-test (SB-MH, 0.5 McFd, 5%, 37°C, 48 h)	NA	0.064-0.38	[21]
Ceftazidime	China/H	Bm, 19, 2010–12	E-test (BA-MH, 0.5 McFd, 5%, 35°C, 24 h)	8	2-8	[17]
Moxalactam	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL, 6%,35°C,48 h)	16	1-16	[18]

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, $\%CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
	Spain/H	Bm, 83, (1986)	Agar dilution (CM47L, 10^5cfu/ spot, 0%, 37°C, 48 h)	16	4-16	[20]
Aztreonam	Spain/H	Bm, 83, (1986)	Agar dilution (CM47L, 10^5cfu/ spot, 0%, 37°C, 48 h)	>256	64->256	[20]
Thienamycin	Spain/H	Bm, 98, (1982)	Agar dilution (CM47L, 10^5cfu/ spot, 0%, 37°C, 48 h)	2	0.1-2	[24]
TMP/SMX*	Spain/H	Bm, 98, (1982)	Agar dilution (CM47L, 10^5cfu/ spot, 0%, 37°C, 48 h)	6.25	0.39-6.25	[24]*
	Spain/H	Bm, 95, 1980–84	Agar dilution (CM47L, 10^5cfu/ spot, 0%, 37°C, 48 h)	0.25	0.06-0.5	[23]
	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	1	≤0.25-1	[18]
	Saudi Arabia	Bm, 47, (1989)	Broth dilution (BB,5 × 10^5cfu/mL, 0%, 35°C, 48 h)	5	5-25	[13]
	Spain/HA	Bru, 62, (1993)	Agar dilution (MH-HP,10^4cfu/ spot,10%,35°C,48 h)	4	0.1-4	[30]
	Saudi Arabia	Bm, 116, (1995)	Broth dilution (MH, $10^{5\text{-}6}$cfu/mL, 5%, 35°C, 48 h)	1	<0.25-1	[31]
	Greece/HA	Bru, 74, 1999–2005	E-test (SB-MH, 0.5 McFd, 5%, 35°C, 48 h)	0.75	0.032-1.5	[16]
	Egypt/H	Bm, 355, 1999–2007	E-test (SB-MH, 0.5 McFd, 5%, NA, 48 h)	0.19	0.006-0.75	[22]
	Peru/H	Bm, 48, 2000–06	E-test (SB-MH, 0.5 McFd, NA, NA, 48 h)	0.15	0.012-0.64	[28]
	Turkey/H	Bm, 76, 2001–06	E-test (SB-MH, 1 McFd, 0%, 35°C,48 h)	0.094	0.016-0.125	[29]
	Italy/H	Bru, 20, 2005–06	E-test (SB-MH, 0.5 McFd, 5%, 37°C, 48 h)	ND	0.012/0.064	[21]
	Turkey/H	Bru, 56, 2008–09	E-test (SB-MH, 0.5 McFd, 0%, 35°C, 48 h)	0.125	0.064-0.25	[27]
	Turkey/H	Bm, 73, 2009–11	E-test (SB-MH, 0.5 McFd, NA, 37°C, 48 h)	0.19	0.016-0.5	[26]
	China/H	Bm, 19, 2010–12	E-test (BA-MH, 0.5 McFd, 5%, 35°C, 24 h)	3.2	0.8-3.2	[17]*

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, %$CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
	US/H	Bru, 39, (2010)	Broth microdilution (BB, ND, 0%, 35°C, 48 h)	2	0.25-2	[10]
		Bru, 39, (2010)	Broth microdilution (BB, ND, 5%, 35°C, 48 h)	2	0.25-4	[10]
Chloramphenicol	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)		0.3->100	[9]
	Israel/H	Bru, 31, 1978–82	Agar dilution (CM47L, 10^4/mL, 10%, 37°C, 48 h)	2	0.125-4	[12]
		Bru, 31, 1978–82	Agar dilution (CM47L, 10^5/mL, 10%, 37°C, 48 h)	4	0.06-4	[12]
		Bru, 31, 1978–82	Agar dilution (CM47L, 10^6/mL, 10%, 37°C, 48 h)	8	0.06-8	[12]
	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	2	0.25-4	[18]
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	5	0.15-12.5	[19]
Macrolides and azalides						
Erythromycin	US/HA	Bru, 27, (1970)	Broth microdilution (BB, NA, 10%, 37°C, 48 h)	0.6	0.02-2.5	[9]
	US-Mexico/HA	Bru, 15, (1986)	Broth microdilution (TSB,5 × 10^5cfu/mL,6%,35°C,48 h)	8	0.5-8	[18]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/ 7,$10^{5\text{-}6}$cfu,0%,35°C,48 h)	128	0.5-256	[11]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/ 5,$10^{5\text{-}6}$cfu,0%,35°C,48 h)	>256	32->256	[11]
	Spain/HA	Bru, 62, (1993)	Agar dilution (MH-HP,10^4cfu/ spot,10%,35°C,48 h)	16	0.2-16	[30]
	Greece/HA	Bru, 74, 1999–2005	E-test (SB-MH, 0.5 McFd, 5%, 35°C, 48 h)	4	0.5-8	[16]
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	2	1-4	[19]
Roxithromycin	Spain/HA	Bru, 62, (1993)	Agar dilution (MH-HP,10^4cfu/ spot,10%,35°C,48 h)	16	0.1-32	[30]

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, $\%CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
Dirithromycin	Spain/HA	Bru, 62, (1993)	Agar dilution (MH-HP,10^4cfu/spot,10%,35°C,48 h)	16	0.5-16	[30]
Clarithromycin	Spain/HA	Bru, 62, (1993)	Agar dilution (MH-HP,10^4cfu/spot,10%,35°C,48 h)	8	0.06-8	[30]
Azithromycin	Spain/H	Bm, 358, 1987–89	Agar dilution (CM471, 10^5cfu/spot, 0%, 37°C, 48 h)	1	0.03-2	[32]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/7,$10^{5\text{-}6}$cfu,0%,35°C,48 h)	1	<0.125-4	[11]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/5,$10^{5\text{-}6}$cfu,0%,35°C,48 h)	>256	16->256	[11]
	Spain/HA	Bru, 62, (1993)	Agar dilution (MH-HP, 10^4cfu/spot, 10%,35°C, 48 h)	2	0.1-4	[30]
	Saudi Arabia	Bm, 116, (1995)	Broth dilution (MH,$10^{5\text{-}6}$cfu/mL, 5%, 35°C, 48 h)	0.5	<0.25-2	[31]
	Peru/H	Bm, 48, 2000–06	E-test (SB-MH, 0.5 McFd, NA, NA, 48 h)	0.5	0.064-0.5	[28]
	Turkey/H	Bm, 73, 2009–11	E-test (SB-MH, 0.5 McFd, NA, 37°C, 48 h)	8	0.75-16	[26]
Fluoroquinolones						
Norfloxacin	Greece/HA	Bru, 74, 1999–2005	E-test (SB-MH, 0.5 McFd, 5%, 35°C, 48 h)	3	0.125-4	[16]
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	8	4-16	[19]
Ofloxacin	Saudi Arabia	Bm, 47, (1989)	Broth dilution (BB,5 × 10^5cfu/mL, 0%, 35°C, 48 h)	0.02	0.02-0.3	[13]
	Israel/H	Bm, 86, (1991)	Broth microdilution (BB, 5 × 10^5cfu/mL,5%,37°C,48 h)	2.5	ND	[33]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/7,$10^{5\text{-}6}$cfu,0%,35°C,48 h)	1	<0.125-4	[11]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/5,$10^{5\text{-}6}$cfu,0%,35°C,48 h)	>16	4->16	[11]
	Spain/H	Bm, 160, 1997	Agar dilution (MH-HP,10^4cfu/spot,10%,35°C,48 h)	2	1-2	[25]

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, %$CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	2	0.5-2	[19]
Levofloxacin	Spain/H	Bm, 160, 1997	Agar dilution (MH-HP,10^4cfu/spot,10%,35°C,48 h)	0.5	0.5	[25]
	Greece/HA	Bru, 74, 1999–2005	E-test (SB-MH, 0.5 McFd, 5%, 35°C, 48 h)	0.5	0.06-0.75	[16]
	China/H	Bm, 19, 2010–12	E-test (BA-MH, 0.5 McFd, 5%, 35°C, 24 h)	8	2-8	[17]
Ciprofloxacin	Spain/H	Bm, 95, 1980–84	Agar dilution (CM47L, 10^5cfu/spot, 0%, 37°C, 48 h)	0.5	0.12-0.5	[23]
	Saudi Arabia	Bm, 47, (1989)	Broth dilution (BB,5 × 10^5cfu/mL, 0%, 35°C, 48 h)	1.25	1.25-2.5	[13]
	Israel/H	Bm, 86, (1991)	Broth microdilution (BB,5 × 10^5cfu/mL,5%,37°C,48 h)	0.8	NA	[33]
	Spain/H	Bm, 34, (1991)	Agar dilution (MH-HP/7,10^3cfu/spot,10%,35°C,48 h)	0.5	0.25-0.5	[14]
	Spain/H	Bm, 34, (1991)	Agar dilution (MH-HP/5,10^3cfu/spot,10%,35°C,48 h)	1	0.5-1	[14]
	Spain/H	Bm, 34, (1991)	Agar dilution (MH-HP/7,10^4cfu/spot,10%,35°C,48 h)	0.5	0.25-0.5	[14]
	Spain/H	Bm, 34, (1991)	Agar dilution (MH-HP/5,10^4cfu/spot,10%,35°C,48 h)	1	0.5-1	[14]
	Spain/H	Bm, 34, (1991)	Agar dilution (MH-HP/7,10^6cfu/spot,10%,35°C,48 h)	1	0.5-1	[14]
	Spain/H	Bm, 34, (1991)	Agar dilution (MH-HP/5,10^6cfu/spot,10%,35°C,48 h)	2	1-2	[14]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/7,$10^{5\text{-}6}$cfu,0%,35°C,48 h)	2	<0.125-8	[11]
	Turkey/H	Bm, 43, 1991–94	Broth microdilution (MH-P/5,$10^{5\text{-}6}$cfu,0%,35°C,48 h)	>16	2->16	[11]
	Spain/H	Bm, 160, 1997	Agar dilution (MH-HP,10^4cfu/spot,10%,35°C,48 h)	1	0.25-1	[25]
	Korea/C	Bab, 85, 1998–2006	Broth microdilution (TSB, 0.5 McFd, 5%, 37°C, 48 h)	1	0.25-4	[19]

Antibiotics	Country /host	Collected isolates: species, n, period[£]	Method[$] (medium, inoculum, $\%CO_2$ temperature and hours of incubation)	MIC_{90} (mg/L)	MIC ranges (mg/L)	Reference
	Greece/HA	Bru, 74, 1999–2005	E-test (SB-MH, 0.5 McFd, 5%, 35°C, 48 h)	0.5	0.016-0.75	[16]
	Egypt/H	Bm, 355, 1999–2007	E-test (SB-MH, 0.5 McFd, 5%, NA, 48 h)	0.38	0.125-0.75	[22]
	Peru/H	Bm, 48, 2000–06	E-test (SB-MH, 0.5 McFd, NA, NA, 48 h)	0.25	0.064-0.25	[28]
	Turkey/H	Bm, 76, 2001–06	E-test (SB-MH, 1 McFd, 0%, 35°C,48 h)	0.38	0.064-0.5	[29]
	Syria	Bm, 100, 2004–07	Broth microdilution (BB/7, 5 × 10^6 cfu/mL,37°C, 48 h)	4	0.125-8	[15]
	Syria	Bm, 100, 2004–07	Broth microdilution (BB/5, 5 × 10^6 cfu/mL,37°C, 48 h)	8	0.125-8	[15]
	Italy/H	Bru, 20, 2005–06	E-test (SB-MH, 0.5 McFd, 5%, 37°C, 48 h)	ND	0.094-0.5	[21]
	Turkey/H	Bm, 73, 2009–11	E-test (SB-MH, 0.5 McFd, NA, 37°C, 48 h)	0.19	0.125-1	[26]
Sparfloxacin	Israel/H	Bm, 86, (1991)	Broth microdilution (BB, 5 × 10^5cfu/mL,5%,37°C,48 h)	1.5	NA	[33]
Moxifloxacin	Spain/H	Bm, 160, 1997	Agar dilution (MH-HP,10^4cfu/spot,10%,35°C,48 h)	1	1	[25]
	Turkey/H	Bm, 76, 2001–06	E-test (SB-MH, 1 McFd, 0%, 35°C,48 h)	0.25	0.032-0.25	[29]

NA, data not available; Bm, *B. melitensis*; Bab, *B. abortus*; Bru, *Brucella* sp.

[£]Studies have been classified according to the period of isolation of the studied *Brucella* strains (e.g., 2010–12) and the date of the corresponding publication (e.g., 1993) when the latter was unavailable.

*TMP/SMX: cotrimoxazole, trimethoprim plus sulfamethoxazole combination at 1/19 ratio, or 1/20 for reference [19] and 1/5 for reference [17].

[$]Method: Kirby–Bauer disk diffusion method (Kirby-Bauer); E-test strip method (E-test);

Medium: Mueller Hinton agar with 5% sheep blood (SB-MH) or unspecified percentage and type of blood (BA-MH); 5% sheep blood agar (SB); Brucella broth (BB); Brucella agar (BA); Trypticase soy broth (TSB); Iso-Sensitest® Agar CM47L (CM47L); Mueller Hinton broth supplemented with 1% polyvitex with (HP) or without (P) 1% hemoglobin, at pH 7 (/7) or pH 5 (/5); The bacterial inoculum used for antibiotic susceptibility testing is specified in cfu/mL or according to McFarland standards (McFd). Host: human (H), cattle (C), unspecified or various animals (A).

Table 2. Antibiotic susceptibilities of *Brucella* sp. to β-lactams, cotrimoxazole, chloramphenicol, macrolides, and fluoroquinolones, as determined in cell-free media.

MICs determined for chloramphenicol were not consistent from one study to the other (Table 2): MICs ranged from 0.3 to higher than 100 [9], 0.25 to 4 mg/L [18], and 0.15 to 12.5 mg/L [19]. However, this antibiotic was globally considered poorly effective *in vitro* against *Brucella* spp.

The macrolides also display poor *in vitro* activity against these bacteria (Table 2). MICs to erythromycin ranged from 0.2 to 16 mg/L using the agar dilution method [30], 0.5 to 8 mg/L using the E-test method [16], and 0.02 to 256 mg/L using various broth microdilution methods [9,11,18,19]. However, the very high MICs found in some studies could be related to the use of a high bacterial inoculum, especially at acidic pH [11]. Azithromycin displayed similar MIC ranges: 0.03–4 mg/L with the agar dilution method [30,32], 0.06–16 mg/L with the E-test method [26,28], and <0.12–4 mg/L with the broth microdilution method [11,31], with a deleterious effect of acidic pH [11].

In recent years, the fluoroquinolones proved to be very active against *Brucella* spp. *in vitro* (Table 2). Ciprofloxacin remains the most effective compound. MICs ranged from 0.12 to 1 mg/L using the agar dilution method [23,25], 0.016 to 1 mg/L with the E-test method [16,21,22,26,28,29], and <0.12 to 8 mg/L with the broth dilution method [11,13,19,33]. However, Garcia-Rodriguez et al. [14] demonstrated that MICs of several fluoroquinolone compounds (including ciprofloxacin), against *B. melitensis* and *B. abortus* strains, could be increased up to fourfold at acidic pH and/or in the presence of high bacterial loads. These authors also showed higher susceptibility to fluoroquinolones of *B. melitensis* compared to *B. abortus* [14].

2.1.2. Bactericidal activity by class of antibiotics

The bactericidal activity of antibiotics against *Brucella* species has been evaluated by determination of the minimal bactericidal concentrations (MBCs) and by kill-time experiments (Table 3). However, various methodologies and definitions for bactericidal activity were used in different studies. Results greatly varied according to experimental conditions, including the tested bacterial inoculum and pH of the culture medium [14]. De Rycke et al. [34] reported higher bactericidal activity of rifampicin compared to tetracycline against *B. suis*. Assuming that a bactericidal effect is at least 3-log reduction of the initial bacterial inoculum within 24–48 h incubation for *Brucella* sp., a bactericidal activity was reported by Mateu-de-Antonio et al. [35] at concentrations ranging from two to four times the MIC for the aminoglycosides (streptomycin and gentamicin), rifampicin, and the fluoroquinolone (enrofloxacin), but not for the tetracyclines (doxycycline) and the macrolides (erythromycin, clarithromycin, and roxithromycin). Only the aminoglycosides displayed early bactericidal activity (i.e., within 24 h of incubation) against *Brucella* sp. [33]. Garcia-Rodriguez et al. [14] reported lack of bactericidal activity of fluoroquinolones against 21 strains of *B. melitensis*, with ciprofloxacin MBCs ranging from 2 to ≥ 8 mg/L at neutral pH, but higher than 8 mg/L at pH 5.

2.1.3. Antibiotic combinations

The checkerboard method is considered the most accurate technique for *in vitro* evaluation of the activity of antibiotic combinations against bacteria. Results are usually expressed as the sum of fractional inhibitory concentrations (ΣFIC), which is the sum of the ratio of MIC of each

Antibiotic	Species	Number of strains	MBC ranges (mg/L)	Reference
Doxycycline	*B. melitensis, B. canis*	6	4–>16	[35]
Minocycline	*B. melitensis, B. canis*	6	1–>16	[35]
Gentamicin	*B. melitensis, B. canis*	6	0.25–1	[35]
Streptomycin	*B. melitensis, B. canis*	6	0.25–8	[35]
Ciprofloxacin	*B. melitensis, B. canis*	6	0.5–2	[35]
	B. melitensis	21	2–≥8	[14]
Ofloxacin	*B. melitensis*	21	≥8	[14]
Sparfloxacin	*B. melitensis*	21	2–≥8	[14]
Temafloxacin	*B. melitensis*	21	2–≥8	[14]
Lomefloxacin	*B. melitensis*	21	≥8	[14]
Fleroxacin	*B. melitensis*	21	≥8	[14]
Enrofloxacin	*B. melitensis, B. canis*	6	0.25–2	[35]
Rifampin	*B. melitensis, B. canis*	6	0.25–16	[35]
Erythromycin	*B. melitensis, B. canis*	6	2–>16	[35]
Spiramycin	*B. melitensis, B. canis*	6	4–>16	[35]
Clarithromycin	*B. melitensis, B. canis*	6	8–>16	[35]
Roxithromycin	*B. melitensis, B. canis*	6	16–>16	[35]

Table 3. Minimal bactericidal concentrations (MBCs) of several antibiotics against *Brucella* strains, as determined in broth culture, at pH 7.

antibiotic used in combination (MIC^{Acomb} or MIC^{Bcomb}) divided by MIC of each antibiotic (MIC^{A} or MIC^{B}, for antibiotics A and B, respectively): $\Sigma FIC = MIC^{Acomb}/MIC^{A} + MIC^{Bcomb}/MIC^{B}$. The antibiotic interactions are considered either synergistic ($\Sigma FIC \leq 0.5$, or $\Sigma FIC \leq 0.75$), additive ($\Sigma FIC > 0.5$ but ≤ 1, or $\Sigma FIC > 0.75$ but ≤ 1), indifferent ($\Sigma FIC > 1$ but ≤ 2), or antagonistic ($\Sigma FIC > 2$).

Using this method, Mortensen et al. [18] reported a synergistic effect of the combination of tetracycline with rifampicin, but indifference or antagonism with the combinations of tetracycline plus either streptomycin or gentamicin. Doxycycline plus rifampicin was reported to be synergistic in several studies [11,15,35,36]. A synergistic effect was also found for most *Brucella* strains tested for the combinations of doxycycline with either streptomycin or gentamicin [11,35,36]. The rifampicin and streptomycin combination was mainly indifferent [15]. The combinations of a fluoroquinolone (ofloxacin, ciprofloxacin, or sparfloxacin) with tetracycline, doxycycline, rifampicin, or streptomycin were mainly indifferent [11,15]. The rifampicin–azithromycin combination was also mainly indifferent [11], while ciprofloxacin plus azithromycin combination displayed variable activity (synergistic to antagonistic)

according to the strains tested [36]. Interestingly, only the combination of doxycycline with rifampicin retained its synergistic activity at pH 5 [11,15]. It is to be noted also that Rubinstein et al. [33] did not find any synergistic effect of either of the previously mentioned antibiotic combinations.

The E-test method has also been used for the evaluation of the activity of antibiotic combinations [29,37]. Mueller Hinton agar plates supplemented with 5% sheep blood were inoculated with a 0.5–1 McFarland turbidity standard suspension of *Brucella* sp. The E-test strips were then successively applied to the inoculated surface so as MICs overlap at the same position. The first strip was removed after 1-h incubation, while the second was left on the agar for the 48-h incubation at 35°C. The ΣFIC index was used to interpret results: synergism (ΣFIC ≤ 0.5), additive (ΣFIC > 0.5 but ≤1), indifference (ΣFIC > 1 but ≤4), and antagonism (ΣFIC > 4). Sometimes, the additive and indifference categories were combined as indifference. However, results were not consistent between studies. Ozhak-Baysan et al. [29] reported that, among the 28 *Brucella* sp. strains tested, the combination of doxycycline with rifampicin was antagonistic for 25 (89.3%) and indifferent for the remaining three strains. The combination of streptomycin with rifampicin was synergistic, but only one *Brucella* strain was tested. Orhan et al. [36] and Kilic et al. [37] reported two different studies from Turkey, each evaluating antibiotic combinations against 16 human strains of *B. melitensis*. A synergistic effect was reported for the combination of doxycycline with rifampicin for 15/16 (93.7%) strains [36], and for tetracycline with rifampicin for 16/16 (100%) strains [37]. The combination of doxycycline plus streptomycin was synergistic for 11/16 (68.7%) strains [36], while doxycycline plus cotrimoxazole was synergistic for 6/16 (37.5%) strains but antagonistic for the same number of strains [36]. A synergistic effect was found for the combination of cotrimoxazole with rifampicin for 6/16 (37.5%) strains [36] to 14/16 (87.5%) strains [37]. Ciprofloxacin displayed a synergistic effect when combined with cotrimoxazole for 7/16 (43.7%) strains [37], or with azithromycin for 12/16 strains (75%) [36]. Tetracycline plus moxifloxacin combination gave a synergistic effect for only 4/16 (25%) strains [37]. The combination of ciprofloxacin with streptomycin was mainly indifferent [37]. It is to be stressed that Orhan et al. [36] found different results with the same *B. melitensis* strains when using the checkerboard technique. The E-test overevaluated the synergistic effect of most antibiotic combinations compared to the checkerboard technique.

Using kill-time experiments, earlier bactericidal activity was demonstrated with the combination of streptomycin with either a tetracycline (tetracycline or doxycycline), rifampicin, or a fluoroquinolone (including ciprofloxacin) [33,35]. The same was true for the combination of rifampicin with either a fluoroquinolone (especially ciprofloxacin) or a tetracycline [33]. In some studies, the combination of rifampicin with a tetracycline was no more effective than the former antibiotic alone [34]. In contrast, the combination of ciprofloxacin and minocycline was antagonistic [33].

2.2. AST in eukaryotic cell models

Brucella spp. are facultative intracellular bacteria that infect a number of eukaryotic cells, including macrophages, dendritic cells, and trophoblasts of the placenta [38]. These bacteria replicate in acidic endoplasmic reticulum-derived vacuoles. Therefore, *in vivo* efficacy of

antibiotics in *Brucella*-infected hosts is likely dependent on their activity against the intracellular and extracellular forms of this pathogen. Whereas MICs determined in cell-free media would allow detection of acquired resistances in specific *Brucella* strains, they cannot predict *in vivo* efficacy of antibiotics on their own. Eukaryotic cell models have been developed to test the activity of antibiotics against intracellular bacteria, including *Brucella* species. Mouse peritoneal macrophages and macrophage cell lines, and human monocyte-derived macrophages and macrophage cell lines (Mono Mac 6), have been most often used. These experimental models are based on *in vitro* infection of eukaryotic cells with a specific strain of *Brucella* sp., then exposure of infected cell monolayers to an antibiotic, and evaluation of residual intracellular viable bacteria to assess the antibiotic activity. The latter is usually determined by colony-forming unit (CFU) counting methodology, but quantitative real-time PCR has been used as a less fastidious alternative, although it does not differentiate viable from nonviable bacteria. Major limitations of these models include difficulties in eliminating nonphagocytized bacteria to ensure proper evaluation of intracellular antibiotic activity, lysis of eukaryotic cells before CFU determination without altering bacterial viability, and defining the most appropriate experimental conditions (especially the time of antibiotic exposure). Also, these models do not evaluate the influence of host–pathogen interactions (especially the host immune response) on intracellular activity of antibiotics.

Richardson et al. [39] first reported that streptomycin (at concentrations up to 50 mg/L) was not bacteriostatic against *B. abortus* grown in bovine cell cultures, while this antibiotic was strongly bactericidal in cell-free media. In contrast, tetracycline displayed the same activity against intracellular and extracellular bacteria. Streptomycin was no more effective when using guinea pig monocytes [40]. Filice et al. [41] demonstrated that rifampicin could induce ultrastructural damages to *B. melitensis* within mouse peritoneal macrophages. In a more recent study, using *B. abortus* strain 2308 and two cell lines (human Mono Mac 6 and J774 murine macrophages), Valderas et al. [42] demonstrated an intracellular bacteriostatic activity for tetracycline and doxycycline (at 1×MIC and 4×MIC in Mono Mac 6 and J774 cells, respectively), for rifampicin (at 0.25×MIC and 1×MIC, respectively), and for ciprofloxacin (at 1×MIC and 4×MIC, respectively). Streptomycin and gentamicin displayed no bacteriostatic activity after 24 h in these cell systems. However, these antibiotics slowly penetrate within eukaryotic cells and reach significant intracellular concentrations only after 3 days of antibiotic–cell contact [43]. A weak intracellular bactericidal activity (≤ 1-log reduction of bacterial titers) was found for rifampicin and ciprofloxacin at 4× and 8× MICs, but not for tetracycline and doxycycline [42]. Akova et al. [11] previously demonstrated the deleterious effect of acidic pH on activity of antibiotics against *Brucella* sp. It may be speculated that most antibiotics lose their bacteriostatic and/or bactericidal activity against intracellular *Brucella* sp. because these bacteria multiply in acidic cell compartments.

2.3. Animal models

Several animal models have been developed to study *in vivo* replication of *Brucella* sp., including mice, rats, guinea pigs, rabbits, and nonhuman primates [44]. The *in vivo* activity of antibiotics against this pathogen has been mainly evaluated in mice, rats, and guinea pigs.

These animals develop persistent bacteremia and a disseminated infection (especially in liver, spleen, and lungs). However, guinea pigs are the most susceptible animals, while rats usually do not develop clinical symptoms. The evaluation of *in vivo* efficacy of antibiotics was primarily based on their ability to eradicate *Brucella* from the spleen of treated animals compared to untreated controls, as determined by numeration of viable bacteria (CFU counts) in spleen tissue collected at the time of sacrifice of infected animals.

Early studies in guinea pigs demonstrated the *in vivo* activity of sulfanilamide in pigs infected with *B. melitensis* [45]. In OF1 mice and Hartley guinea pigs infected with *B. melitensis* or *B. suis*, rifampicin was able to eradicate bacteria from the spleen of most animals, while only partial decrease in spleen bacterial loads were observed with tetracycline [34,46]. In a more recent study, ICR mice were infected with *B. melitensis* 16M and received various antibiotic treatments (21 days orally or 14 days intraperitoneally) 7–14 days postinfection [47]. Rifampin (25 mg/kg/d, ip) and doxycycline (40 mg/kg/d ip) were highly effective to eradicate bacteria from the spleen, while streptomycin (75 mg/kg/d, ip), cotrimoxazole (15 mg/kg/d of TMP, ip), and ciprofloxacin (20 mg/kg/d ip) were not. Doxycycline was less effective when administrated orally at 6–80 mg/kg/d. The same authors later reported a much lower activity of rifampicin at a lower dosage (3 mg/kg/d), but a synergistic effect of the combination of streptomycin with either doxycycline or rifampicin [48]. Spiramycin, a macrolide compound, was tested in Sprague Dawley rats infected with *B. melitensis* [49]. Spiramycin (50 mg/kg/d, 21 days) alone or combined with rifampicin (50 mg/kg/d, 21 days) was found as effective as the combination of doxycycline (40 mg/kg/d, 21 days) with rifampicin. Dirithromycin, another macrolide compound, was less effective (27.3% cure rate) in mice infected with *B. abortus* S544 strain, but highly effective (81.8% cure rate) when combined with rifampicin [50]. In the same model, the fluoroquinolone levofloxacin was poorly effective when used alone (36.4% cure rate), and no more effective than rifampicin alone when combined with this antibiotic (72.7% cure rates in both cases) [50]. In Wistar albino rats infected with *B. abortus* [51], moxifloxacin (21 days) was less effective than rifampicin (cure rates in spleen of 50% and 80%, respectively). In mice infected with *B. melitensis* [52–54], doxycycline was much more effective to prevent bacterial multiplication than the fluoroquinolone compounds ciprofloxacin, moxifloxacin, gatifloxacin, trovafloxacin, and grepafloxacin, when administrated before or within hours after the bacterial challenge. Doxycycline was also effective to control *B. melitensis* infection when administrated 7–14 days following infection, whereas all the tested fluoroquinolones were ineffective. In the same animal model [54], the azalide compound azithromycin was able to control *B. melitensis* infection when administrated 2 h following the bacterial challenge. Altogether, the *Brucella*-infected animal models confirmed *in vivo* activity of rifampicin (the most effective antibiotic in all studies) and doxycycline, administrated alone or in combination. The combination of one of these two antibiotics with streptomycin was also effective. In contrast, the fluoroquinolones, cotrimoxazole, and the macrolides were unable to eradicate *Brucella* in most infected animals. A synergistic effect was found for the combination of a macrolide with rifampicin, but not for that of a fluoroquinolone with rifampicin. It is to be stressed, however, that these animal models greatly varied according to the animal species used, the *Brucella* species tested, the route and inoculum of the bacterial challenge, the dosage and duration of the tested antibiotic treatments, the time of administration of antibiotics compared to the

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bacterial challenge, and the time of antibiotic treatment evaluation. More standardization is needed to allow comparison of results obtained in different research laboratories.

The *in vivo* efficacy of antibiotics to eradicate *Brucella* has also been evaluated in naturally infected animals. Radwan et al. [55] reported the eradication of *B. melitensis* from 480 naturally infected sheep and goats with the combination of oxytetracycline with streptomycin, as evidenced by cessation of shedding *B. melitensis* from udder secretions and absence of this bacterium in tissues at autopsy after antibiotic treatment.

3. Acquired resistances to antibiotics in *Brucella* species

In vitro selection of rifampicin-resistant mutants has been reported for *B. melitensis, B. abortus,* and *B. suis* [12,34,56]. In *B. suis*, the spontaneous rate of mutations leading to rifampicin resistance was evaluated at 2.5×10^{-9} (for a concentration of 25 mg/L) [34]. Marianelli et al. [56] characterized the genetic mechanisms involved in resistance to rifampicin in the vaccine strain *B. abortus* RB51, and in laboratory mutants derived from two *B. melitensis* isolates. They found missense mutations in two regions of the *rpoB* gene encoding subunit B of RNA polymerase, the bacterial target of rifampicin. These mutations led to a number of amino acid changes: Val154Phe, Asp526Tyr, Asp526Gly, Asp526Asn, His536Leu, His536Tyr, Arg539Ser, Ser541Leu, and Pro574Leu. A number of studies have reported wide ranges of rifampicin MICs (up to 64 mg/L) in human and animal strains of *Brucella* sp., with MIC variations between geographic regions and time periods considered [9,11,15,19,22,23,26,30,33,57,58]. *In vivo* selection of rifampicin-resistant mutants was also reported in a patient who relapsed after treatment with doxycycline and rifampicin [59]. These results have suggested the possibility of acquired resistance to rifampicin in *Brucella* species. However, there is currently no clear characterization of *rpoB* mutations leading to rifampicin resistance in *Brucella* strains isolated from humans or animals. Direct amplification and sequencing of the *rpoB* gene did not reveal any rifampicin resistance mutation in two recent studies from Turkey [60] and Spain [61], in 21 and 62 human strains of *B. melitensis*, respectively.

Very few studies have reported high doxycycline MICs (up to 32 mg/L) in animal and human strains of *Brucella* sp. [11,15,17,57]. In some studies, however, high-level MICs may have been related to the use of a high bacterial inoculum rather than true acquired resistance to tetracyclines [15,17]. Acquired resistance mechanisms to tetracyclines in *Brucella* strains have never been characterized in clinical situation, although the gene encoding the tetracycline resistance protein TetB was found in the genome of *B. abortus* [62]. The same holds true for the aminoglycoside streptomycin, with MICs > 64 mg/L in only two studies [9,15], while high MICs to gentamicin have not been reported so far. High MICs to trimethoprim–sulfamethoxazole combination have also been reported [10,13,17,57,58,63,64], but without characterization of the involved mechanisms. The MICs of macrolides, especially erythromycin [11,16,18,30], are highly variable among *Brucella* strains. For either of these antibiotics, no resistance mechanism has been characterized.

Fluoroquinolone resistance mechanisms have been characterized in *in vitro* selected resistant mutants of *B. melitensis* and *B. abortus* [65–67]. The amino acid substitutions Ala87Val and Asp91Tyr (corresponding to codon positions gyrA83 and gyrA87 in *E. coli* numbering system) were reported. However, efflux pump overexpression was also probably involved in fluoroquinolone resistance in *Brucella* sp. [65,68]. High ciprofloxacin MICs (up to 8 mg/L) have been reported for human and animal strains of *Brucella* sp. [11,15,19,30,57]. No *gyrA* mutation has been reported so far in these strains [61]. However, overexpression of efflux pumps in *Brucella* strains remains difficult to demonstrate, and could partly explain treatment failures observed with fluoroquinolones in brucellosis patients [61]. RND-type efflux pumps have been characterized in *Brucella* sp [69].

4. Correlation between laboratory data and clinical efficacy of antibiotics

In untreated patients, brucellosis may be controlled by the immune system, but relapses and chronic evolution of the disease are frequently observed [1]. The combination of immune defenses and an appropriate antibiotic therapy allows earlier amendment of clinical symptoms and more effective control of infection. The clinical experience regarding the treatment of brucellosis has established some basic principles that remain true today [70]. To reduce the risk of recurrence, at least two antibiotics should be administrated for a minimum of 4–6 weeks. The combination of doxycycline with either rifampicin or an aminoglycoside (streptomycin or gentamicin) is the most effective *in vitro* and is considered the first alternative for the treatment of brucellosis [71]. It is likely that the effectiveness of these antibiotic combinations depends on their activity on both extracellular and intracellular *Brucella*, and their ability to reach the infectious sites and remain active at local conditions (including an acidic pH in eukaryotic cell compartments, but also in organ abscesses). AST in cell-free media is poorly predictive of the *in vivo* activity of antibiotics against *Brucella* sp. However, these models have shown that the aminoglycosides and rifampin display significant bactericidal activity against extracellular *Brucella* [33,35]. Cell models have shown that rifampicin is the only antibiotic with a bactericidal activity against the intracellular form of *Brucella*, whereas the tetracyclines and the fluoroquinolones are mainly bacteriostatic [39,42]. The aminoglycosides had no activity against intracellular *Brucella* [39,42]. However, their activity was evaluated in *Brucella*-infected cell models after 24 h of antibiotic exposure, while these antibiotics significantly penetrate and concentrate in eukaryotic cells only after 3 days [43]. Thus, in patients treated with an aminoglycoside, potential intracellular activity of these antibiotics cannot be ruled out. The animal models confirmed a clear superiority of rifampicin and doxycycline for eradication of *Brucella* sp. from the spleen [34,46,48], while the fluoroquinolones were much less active [50–54]. Altogether, the superiority of the combinations of doxycycline with either an aminoglycoside or rifampicin in brucellosis patients could be related to the synergistic effect of an extracellular bactericidal activity (especially using an aminoglycoside or rifampicin) with an intracellular bactericidal activity (doxycycline or rifampicin, plus an appropriate cell immune response). A prolonged antibiotic therapy is likely needed because of poor bactericidal activity of antibiotics against intracellular *Brucella* and the need for progressive development of an efficient immune

response. Hence, brucellosis is usually a more severe disease in immunocompromised patients, often with a chronic evolution [72–74].

Nevertheless, relapse rates of 5%–15% are still reported in immunocompetent patients after administration of appropriate antibiotic therapy, sometimes several decades following the primary infection [73,75]. A first explanation could be the lack of eradication of *Brucella* by antibiotics in these relapsing patients because of interindividual variability in the inflammatory and immune responses to *Brucella* infection. It should be stressed that in animal models, the eradication rates obtained after antibiotic treatment varied from one animal species to the other, and between individuals of a same species [47,48,50–54]. Second, the infectious dose and delay in antibiotic treatment after infection may also greatly influence antibiotic activity. A typical example concerns the fluoroquinolones which were effective to control *Brucella* infection in animal models when administrated before or immediately after the infectious challenge, but not when administrated 1 or 2 weeks later [47,48,50–54]. Third, *in vivo* selection of *Brucella* mutants, resistant to antibiotics in brucellosis patients under antibiotic therapy, could also explain the therapeutic failures and relapses, especially for rifampicin and the fluoroquinolones [59,65–67]. Although *in vitro* studies have suggested that *Brucella* sp. could become resistant to first-line antibiotics used for brucellosis treatment, definite proofs of selection of acquired resistances in the clinical situation are still lacking. Interestingly, similar antibiotic susceptibilities were reported in *B. melitensis* strains isolated before and after antibiotic therapy in brucellosis patients suffering from relapses [28,76], which indicated that treatment failure was not related to development of acquired resistances to antibiotics in this pathogen.

Improving our understanding of treatment failures and relapses in brucellosis patients will necessitate not only a better standardization of assessment of the antibiotic activity using both *in vitro* and *in vivo* approaches, but also the development of new diagnostic tools to explore previous hypotheses directly in infected patients.

5. New therapeutic alternatives

5.1. Novel antibiotics

Tigecycline, a glycylcycline compound derived from minocycline, displays broad ranges of MICs and higher MIC90 *in vitro* than doxycycline against *Brucella* sp. [26,27,29,77]. In contrast, a lower MIC90 was reported for tigecycline (0.125 mg/L) compared to tetracycline (0.25 mg/L) for 60 strains of *B. melitensis* [78]. Using the checkerboard method, Aliskan et al. [79] reported a synergistic effect of the combination of tigecycline with either levofloxacin (50% of the 16 strains tested), rifampicin (31.2%), or gentamicin (18.9%). No synergy was observed with tigecycline in combination with streptomycin or cotrimoxazole. Dizbay et al. [80] reported a higher synergistic effect of antibiotic combinations when tigecycline was used compared to doxycycline. It has been proposed to replace doxycycline by tigecycline in current therapeutic protocols of brucellosis [81–83]. Although tigecycline could be as effective as doxycycline in antibiotic combination therapies, there are currently major limitations for its widespread use

in brucellosis patients. At first, tigecycline is tens of times more expensive than doxycycline. Second, it can only be administrated by the parenteral route and thus its use would be restricted to the acute phase of brucellosis and/or in patients hospitalized because of a severe disease. Third, the antibacterial spectrum of tigecycline is much broader than that of doxycycline, including staphylococci (especially methicillin-resistant *S. aureus*), streptococci, enterococci, some anaerobes, and most enterobacterial species (including those secreting extended spectrum beta-lactamases), but not species of the *Proteae* tribe and *Pseudomonas aeruginosa* [83]. Thus, significant alterations of the skin and gut commensal flora would certainly occur in the treated population, with an increased risk of opportunistic infections and development of resistances to the glycylcyclines. Finally, it is not clear if the favorable pharmacokinetic properties of tigecycline in tissues could lead to a significant reduction in duration of treatment or relapse rates. Also, as for tetracyclines, tigecycline is contraindicated in pregnant women and young children.

Among new fluoroquinolones, moxifloxacin did not display higher activity compared to ciprofloxacin *in vitro* [25,29,37] or in animal models [51–54]. The use of a triple combination of doxycycline, rifampicin, and a fluoroquinolone warrants further evaluation, at least for treatment of severe brucellosis cases such as spondylodiscitis, endocarditis, and neurobrucellosis [84–86].

Among the new macrolide compounds, azithromycin displayed *in vitro* bacteriostatic activity against *Brucella* sp. with MIC90 ranging from 0.5 to 8 mg/L [11,26,28,30–32]. A synergistic effect was found when this antibiotic was combined with ciprofloxacin [36]. Azithromycin was also able to control *B. melitensis* infection in the mouse model [54]. However, azithromycin was much less effective than doxycycline to cure brucellosis in Swiss-Webster or BALB/c mice [87]. Although the macrolides are currently not considered suitable for treatment of brucellosis, they could represent a safe alternative in young children and pregnant women. Further evaluation of the *in vitro* and *in vivo* activity of macrolides combined with other antibiotic classes against *Brucella* sp. is warranted.

Medicinal plants have been evaluated for their *in vitro* activity against *B. melitensis.* Using a Mueller Hinton broth dilution method, Motamedi et al. [88] reported that ethanolic and methanolic extracts of six plants displayed anti-*Brucella* activity: *Oliveria decumbens, Salvia sclarea*, *Ferulago abgulata*, *Vitex pseudo-negundo*, *Teucrium pollium*, and *Crocus sativus*. *O. decumbens* was the most effective with similar MICs and MBCs. Al-Mariri and Safi [89] evaluated the activity of essential oils against 16 *Brucella* strains. They found a bacteriostatic effect for essential oils from two medicinal plants: *Thymus syriacus* and *Origanum syriacum*.

5.2. Intracellular delivery of antibiotics

The aminoglycosides are able to penetrate eukaryotic cells, albeit very slowly, but concentrate in the acidic lysosomal compartment because of their weak base nature [43]. At acidic pH, these antibiotics are partially inactivated because of their protonation. Although intracellular pharmacokinetic studies were mostly conducted using uninfected eukaryotic cells, it is tempting to extrapolate these data to *Brucella*-infected cells. *In vitro* studies have been performed to evaluate the influence of increased uptake of aminoglycosides within eukaryotic

cells on their activity against intracellular *Brucella.* These antibiotics (especially streptomycin and gentamicin) were either included in liposomes or attached to nanoparticles. Phagocytosis of liposomal or nanoparticle formulations of aminoglycosides by *Brucella*-infected macrophages resulted in higher intracellular activity compared to free aminoglycosides against *B. melitensis*, *B. abortus*, or *B. canis* [90–94]. These formulations of the aminoglycosides were also significantly more effective in animal models [90,94,95]. The targeted delivery of aminoglycosides could be a promising therapeutic alternative, both increasing their intracellular activity and reducing their side effects by reducing their concentration in kidneys and the cochleovestibular system. However, there are currently technical limitations in the preparation of liposomal or nanoparticle forms of antibiotics and safety concerns, especially for nanoparticles that limit their use in humans [96].

5.3. Peptide nucleic acids

Peptide nucleic acids (PNAs) are artificially synthesized polymers similar to DNA or RNA that can be used as antisense therapies. They show high specificity in binding to complementary DNAs, resistance to nucleases and proteases, and a high stability over a wide pH range. They readily cross the bacterial cell membranes when coupled with a cell-penetrating peptide. Rajasekaran et al. [97] reported growth inhibition of *B. suis* by PNAs, both in cell-free medium and in murine macrophages. In tryptic soy broth, the inhibitory PNAs were those targeting the genes *kdtA* (coding for a transferase affecting lipid A), *tsf* (elongation factor *Ts*), *polA* (DNA polymerase I), and *rpoB* (subunit B of RNA polymerase). In contrast, in J774A.1 murine macrophages, the inhibitory PNAs targeted the genes *asd* (coding for an aspartate-semialdehyde dehydrogenase involved in diaminopimelic acid synthesis), *gyrA* (subunit A of DNA gyrase), *dnaG* (protein primase that initiates DNA replication), and *polA*. The PNAs were thus able to penetrate the eukaryotic and bacterial membranes, and could represent new therapeutic alternatives for intracellular pathogens such as *Brucella* sp.

5.4. Enhancement of the host response

Multiplication within phagocytic cells is a major virulence factor of *Brucella* species. The host response to *Brucella* infection could be strengthened by restoring the ability of phagocytic cells to control intracellular multiplication of these bacteria and eradicate them via the phagolysosomal pathway, especially using cytokines. Jiang and Baldwin [98] reported the *in vitro* inhibition of *B. abortus* multiplication in BALB/c J774A.1 murine macrophages by gamma interferon (IFN-γ) or to a lesser extent interleukin-2 (IL-2, 100 U/mL). In contrast, IL-1α, IL-4, IL-6, tumor necrosis factor alpha (TNF-α), and granulocyte macrophage–colony-stimulating factor (GM–CSF) had no significant effect on intracellular growth of *B. abortus*. The protective role of IFN-γ was also emphasized by Murphy et al. [99], using IFN-γ gene knockout mice infected with *B. abortus.* When adsorbed to albumin nanoparticles, IFN-γ was even more effective to control *B. abortus* infection in RAW 264.7 macrophages and BALB/c mice [100]. Fahel et al. [101] recently reported in a mouse model of *B. abortus* infection that a higher host resistance to infection was associated with an increased expression of interleukin-12 (IL-12), gamma interferon (IFN-γ), and inducible nitric oxide synthase (iNOS) during the course of

infection. This protective Th1 immune response was negatively regulated by 5-Lipooxygenase (5-LO), an enzyme required for the production of the lipid mediators leukotrienes and lipoxins. The use of interferon-gamma has never been reported so far in brucellosis patients.

6. Conclusion

Brucellosis remains a prevalent disease in the world, a major concern in public health and an economic burden in agriculture. Although effective vaccines are available for the livestock, treatment of brucellosis remains challenging in both animals and humans. Recommendations for treatment of common clinical forms of human brucellosis have been addressed, especially by the WHO. However, treatment optimization is still needed for severe forms of the disease and in young children and pregnant women. Moreover, current treatment recommendations could be challenged by the emergence of acquired resistances to first-line drugs in *Brucella* species, although this fear needs to be confirmed with certainty in the clinical situation. Alternative therapeutic options are needed to reduce the human and economic costs associated with this disease. This could be achieved through the development of new molecules but also by an optimized use of currently available antibiotics. However, controlling *Brucella* infection in the livestock remains a priority.

Author details

Max Maurin[1,2*]

Address all correspondence to: MMaurin@chu-grenoble.fr

1 Bacteriology Laboratory, Biology and Pathology Institute, Grenoble University Hospital, France

2 LAPM, UJF/CNRS, Joseph Fourier-Grenoble 1 University, France

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