

Defensin Susceptibility and Colonization in the Mouse Model of AJ100, a Polymyxin B-Resistant, *Brucella abortus* RB51 Isolate

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Abstract Intracellular pathogens selected for increased susceptibility to polycations are commonly attenuated, yet the effect of decreased susceptibility to polycations on pathogenicity has not been researched. The polymyxin-resistant mutant *Brucella abortus* AJ100 was characterized by comparing its susceptibility to the polycationic antibiotic polymyxin B, defensins, and lactoferricin, and its colonization and clearance in the mouse model to the parent strain RB51. MIC (minimum inhibitory concentration) values determined by Etest for AJ100 and RB51 were 1.5 and 0.25 µg/ml, respectively. Though AJ100 is less susceptible to polymyxin B than RB51, it was more susceptible than its parent strain to the cationic defensins melittin, magainin 2, and cecropin P1. In the mouse model, initial colonization of the spleen was lower for AJ100 than RB51, and the rate of clearance from the spleen was faster for AJ100 than RB51. However, initial colonization and clearance rates of AJ100 from the liver were indistinguishable from those of RB51. This study suggests that the susceptibility profile of *Brucella* to polycationic defensins rather than polymyxin B may be indicative of differential survival in the spleen and liver in the mouse and is indicative of spleen and liver residential macrophages' differing ability to inactivate *Brucella*.

Introduction

Brucella is a facultative intracellular Gram-negative bacterium that causes food animal losses due to reproductive failure and is transmitted to humans primarily through contact with infected animals at parturition and through consumption of contaminated, unpasteurized milk and soft cheeses. *Brucella* persists in infected animals, surviving in macrophages even though these cells possess an arsenal of compounds including polycationic antimicrobial peptides of innate immune systems designated “defensins” that aid in destroying engulfed bacteria.

The outer membranes (OM) of Gram-negative bacteria are composed chiefly of lipid A containing LPS and are a target of polycationic compounds such as polymyxin B and defensins [4, 6, 15, 22]. Bacteria with a smooth colony are less susceptible to polymyxin B than their mutants that have a rough colony phenotype, which have reduced binding polycations including polymyxin B [1, 4, 16]. Few studies report the susceptibility of *Brucella* to polymyxin B as minimum inhibition concentration (MIC) values, but the relative susceptibility of *Brucella* strains appears consistent across studies [7, 8, 16]. RB51, a rough *B. abortus* vaccine strain, plates with an efficiency of 15% on medium containing 1.8 U/ml polymyxin B. A mutant of this strain, RB51/HP [10], herein designated “AJ100,” grows in the presence of 1.8 U/ml of polymyxin B. Comparative MIC values have not been reported for either AJ100 or RB51.

Brucella are relatively less sensitive to polymyxin B and other polycationic compounds than *Salmonella*; likely both LPS and outer membranes play a role [4, 5, 7, 15, 16, 23]. Mutants of the facultative intracellular pathogens *Salmonella* and *Brucella* with increased susceptibility to polymyxin B are attenuated and usually exhibit increased susceptibility to other polycationic compounds as well [1,

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3, 5, 9, 12]. The attenuated *B. abortus* 2308 polymyxin B-susceptible mutant strain 65.21 (herein designated “SBvrR”), which has a smooth phenotype, is also more susceptible than the parent strain to the defensin mellitin and the polycation poly-L-lysine [19]. How the decreased sensitivities to polymyxin B and mellitin are linked in SBvrR has not been investigated. Further, it is not known if decreased susceptibility to the defensin mellitin alone would attenuate *Brucella*. Mutants with decreased susceptibility to polymyxin B would be predicted to exhibit decreased susceptibility to other polycations, but this has not been explored.

Mice are used extensively as experimental animals to study the pathogenicity and persistence of *Brucella*. They persist in the murine immune system, concentrating in the spleen and liver, and are observed in macrophages despite these cells’ expressing defensins [9]. Following intraperitoneal injection (i.p.), colonization of the spleen and liver of *Brucella* is determined by plate counts over several months. High numbers of 2308, the vaccine challenge strain, are maintained in the spleens of BALB/c mice at 100 days [21], while RB51 is eliminated from BALB/c mice between 4 and 8 weeks [20]. Subcutaneous injection of the smooth polymyxin B-sensitive *B. abortus* 2308 mutant SBvrR was found to be severely attenuated in the mouse model, scarcely replicating, if at all, in mice. *Brucella* were cleared from 50% of the mice by 35 days when given RB51 and by 175 days when given the parent strain 2308 [19].

Here we determine the MIC value for the mutant AJ100 relative to other strains which exhibit susceptibility to polymyxin B, profile the susceptibility of AJ100 to polycationic compounds, and explore the correlation between phenotype regarding susceptibility to polycationic compounds and virulence in the mouse model relative to the parent strain RB51.

Materials and Methods

Brucella Strains

Brucella abortus RB51 (RB51) vaccine strain [18], *B. abortus* RB51/HP (AJ100) [10], *B. abortus* 65.21(SBvrR) [19], and *B. abortus* 2308 (2308), a common *B. abortus* challenge strain, used in this study were obtained from the National Animal Disease Center (ARS/USDA, Ames, IA) culture collection.

Sensitivity to Polycationic Compounds

In vitro activity of polymyxin B was determined by the Etest (AB Biodisk, Piscataway, NJ) containing a preformed

gradient covering a continuous MIC range (0.064–1024 µg/ml) corresponding to 15 twofold dilutions of polymyxin B with a precision of 0.5 dilution. *Brucella* were suspended in saline and the turbidity was adjusted to a 0.5 McFarland standard (5×10^8 colony-forming units [CFU]/ml). The suspension was spread onto Mueller Hinton (MH; Difco Laboratories, Detroit, MI) or pH-adjusted MH agar plates using a cotton swab, and the Etest strips applied. Medium pH values were adjusted prior to autoclaving. Plates were incubated (37°) in ambient air or in an atmosphere of 7.5% CO₂. Results were read after 48 h.

Aqueous solutions of defensins were dropped (1, 2, and 4 µl) onto cultures swabbed as described above to assay strain susceptibility. Stock solutions of the polycationic compounds were prepared as follows: melittin, honeybee venom (10 µg/µl; Sigma Chemical Company, St. Louis, MO), magainin 2, frog skin (1 µg/µl; American Peptide Company [APC], Sunnyvale, CA); lactoferricin, bovine (1 µg/µl; APC); bactenecin, bovine (2 µg/µl; APC); and cecropin P1, porcine (1 µg/µl; APC). Plates were incubated at 37°C and results were recorded after 48 h. A strain showing no inhibition of growth was recorded as resistant (R). If a zone of no growth was noted, the strain was designated susceptible (S). If a zone of nonconfluent growth was noted at the drop point, the strain was designated intermediate (I).

Animal Inoculation

Female 10-week-old BALB/c AnNHsD mice (Harlan Sprague Dawley, Indianapolis, IN) were used. Mice ($n = 25/\text{treatment}$ [trt]) were inoculated intraperitoneally with 0.2 ml of saline or 0.2 ml of saline containing 1×10^7 CFU of *B. abortus* strain AJ100 or strain RB51. Five mice each from the saline control, AJ100, and RB51 groups were euthanized with CO₂/O₂ at 1, 2, 3, 4, and 8 weeks postinoculation (PI). Spleens and livers were weighed and processed for determination of *Brucella* CFU.

Culture Examination of Spleen and Livers

Spleens and livers were homogenized in tissue grinders, serially diluted in saline, and plated on tryptose agar (Difco) containing 5% bovine serum (TSA). After incubation of plates at 37°C with 7.5% CO₂ for 72 h, numbers of CFU were determined for each dilution by standard plate counts. Colonies were confirmed as *Brucella* based on colony morphology, growth characteristics [2], and a *Brucella*-specific polymerase chain reaction (PCR) procedure as described, previously [13].

Statistical Analysis

For statistical comparisons, colonizations of spleen and liver (CFU/g and total CFU/tissue) were analyzed as the logarithm of their value. Due to conversion to logarithm, any colonization data with a value of 0 were analyzed with a value of 1. Statistical differences between treatments were determined by a general linear models procedure, and significant differences were reported when $p < 0.05$. Means for each treatment were compared by use of a least significant difference procedure and are reported as least square mean \pm SE.

Results

Susceptibility to Polymyxin B

Etest MIC values of *B. abortus* strains AJ100 and RB51 differed by sixfold in the presence of 7.5% CO₂. MIC values were 1.5 and 0.25 μ g/ml, respectively (Fig. 1). In general as the pH became more acidic, the *Brucella* strains were less susceptible to polymyxin B. However, RB51 was less susceptible at pH 5 than at pH 6. AJ100 did not grow at pH 5.

Susceptibility to Defensins

Brucella strains were examined for susceptibility to defensins in a drop assay (Table 1). The parent strain of RB51, 2308, was not susceptible to any of the defensins at the concentrations applied. RB51 was susceptible or partially susceptible to two of the defensins, mellitin and cecropin P1, respectively, but only in the presence of CO₂. Among the *Brucella* strains studied, the pattern of susceptibility to the defensins was most similar between

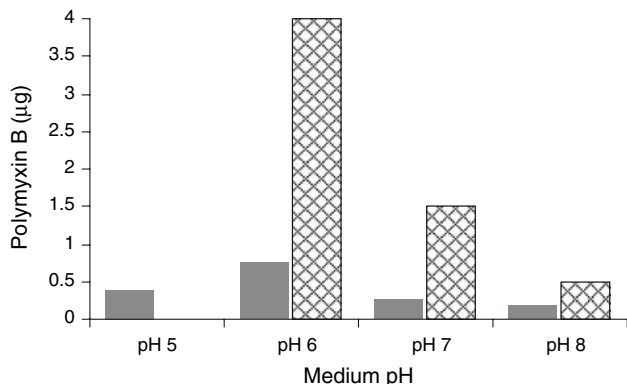


Fig. 1 MIC values of *B. abortus* strains AJ100 (cross-hatch) and RB51 parent strain (black) for polymyxin B at pH values from pH 5 to pH 8.0. Cultures were grown in the presence of CO₂

Table 1 Susceptibility^a of *Brucella abortus* strains to defensins

<i>Brucella</i> strain ^b	Melittin		Magainin 2		Cecropin P1		
	10 μ g	40 μ g	1 μ g	4 μ g	1 μ g	2 μ g	4 μ g
RB51 A	R	R	R	R	R	R	R
RB51 C	S	S	R	R	R	R	I
AJ100 A	S	S	S	S	S	S	S
AJ100 C	S	S	R	R	S	S	S
2308 A	R	R	R	R	R	R	R
2308 C	R	R	R	R	R	R	R
SBvrR A	S	S	R	R	S	S	S
SBvrR C	S	S	R	R	S	S	S

^a R, resistant; S, susceptible; I, intermediate

^b A—grown in ambient air; C—grown in the presence of added CO₂

AJ100 and SBvrR. Both AJ100 and SBvrR were shown to be more susceptible to the defensins mellitin and cecropin P1 than their parent strains, RB51 and 2308, respectively. AJ100 was also sensitive to magainin 2, but only in ambient air. None of the strains used in this study were susceptible to either bactericidin or lactoferricin at 8 and 4 μ g, respectively (data not shown).

Tissue Persistence in a Mouse Model

RB51-infected mice had greater ($p < 0.05$) splenic colonization (CFU/g and total CFU) at 1, 2, 3, and 4 weeks PI than AJ100-inoculated mice (Fig. 2). Liver colonization did not differ ($p > 0.05$) between mice given RB51 versus AJ100. Both RB51 and AJ100 were recovered from spleens and livers of injected mice at 4 weeks but not at 8 weeks.

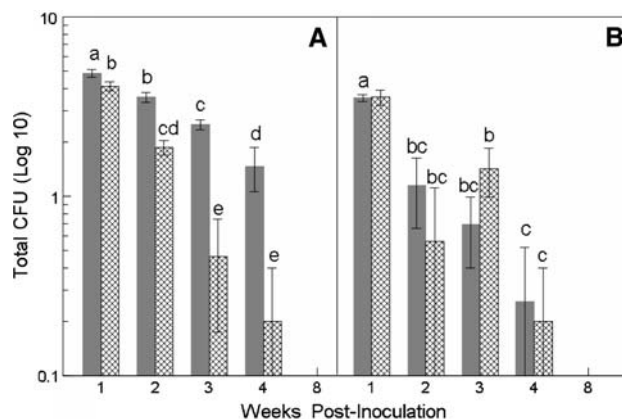


Fig. 2 Colonization of splenic (A) and hepatic (B) tissues (mean log₁₀ total CFU per tissue \pm SE) after intraperitoneal inoculation ($n = 25$ mice/treatment [trt]) with 1×10^7 CFU of *B. abortus* strains RB51 (black columns) or AJ100 (cross-hatched columns). Means with different superscripts differ significantly ($p \leq 0.05$) for that tissue

Table 2 Spleen and liver weights postinoculation (PI)

Weeks PI	Spleen weight (mg)			Liver weight (mg)		
	RB51	AJ100	Control	RB51	AJ100	Control
1	166 ± 38 ^a	168 ± 8 ^a	82 ± 7 ^b	1062 ± 105 ^a	1142 ± 123 ^a	856 ± 61 ^b
2	162 ± 29 ^a	124 ± 4 ^b	87 ± 5 ^c	920 ± 72 ^{ab}	938 ± 98 ^a	838 ± 78 ^b
3	121 ± 17 ^a	100 ± 12 ^b	81 ± 10 ^c	1085 ± 65 ^a	1083 ± 35 ^a	985 ± 32 ^b
4	125 ± 16 ^a	112 ± 10 ^{ab}	99 ± 2 ^b	1024 ± 88 ^a	1006 ± 58 ^a	1010 ± 45 ^a

Note. Weights are recorded as the mean ± SE. Means with different superscripts differ significantly ($p \leq 0.05$) among treatments for that tissue per sampling time

Spleen and liver weights from inoculated and control mice were determined (Table 2). Compared to control mice, spleen weights were greater in mice inoculated with RB51 or AJ100 at 1, 2, 3, and 4 weeks, but not at 8 weeks PI. Compared to AJ100-inoculated mice, RB51-inoculated mice had greater ($p < 0.05$) spleen weights only at 2 and 3 weeks PI. In contrast, liver weights of both RB51 and AJ100 were greater than that of control mice only at week 1 PI. Liver weights between AJ100- and RB51-inoculated mice were significantly different ($p > 0.05$) only at week 2 PI. No statistical difference in weights of spleens or livers among any of the treatment groups was detected at 8 weeks PI.

Discussion

The Etest was used to determine relative MIC values of *Brucella* strains for polymyxin B. It is easily conducted and gave relative results similar to those reported using more time-consuming and complex protocols, such as viability tests with time or dilution end points. Though both 2308 and SBvrR are smooth and less susceptible to polymyxin B than RB51 and AJ100, we found similar-fold differences in polymyxin B susceptibility between the mutants and their parents. The MIC value for SBvrR (16 IU/ml) is eightfold lower than that for the parent strain 2308 (128 IU/ml) in a microtiter plate format growth assay [19], and, by the Etest, the MIC values were fourfold lower, with values of 6 and 24 µg/ml, respectively [11]. The differences observed are likely due not only to protocol differences but also to the higher precision of the Etest. This assay has a precision of 0.5 dilutions, as opposed to twofold dilutions for many other commonly used protocols.

Growth of *Brucella* slightly increases the pH of many bacterial culture media though absorption of CO₂ acidifies it. As the medium acidifies, there is a concurrent reduction in antimicrobial activity of cations due to a reduction in its charge. However, in the case of AJ100 susceptibility to maganin was less when cultured in the presence of CO₂ than in ambient air. Screening of mutants for change in

polycationic susceptibility will need to be carried out in the presence of CO₂ even if the strains studied are CO₂ independent or a class of mutants is likely to be missed.

The target or locus resulting in increased sensitivity to mellitin either does not cause susceptibility to polymyxin B or has the opposite effect. Characterization of the *Brucella* BvrR/BvrS mutants has begun and the effect of several loci and likely mechanisms are emerging [14, 15]. As BvrR/BvrS compose a two-component system which enables bacteria to quickly and specifically respond to environmental changes by globally modulating and coordinating expression of many gene products affecting different functional pathways, many loci may be involved, especially given that BvrR/BvrS may regulate other two-component systems. Identification of loci involved in the disparate susceptibility of AJ100 to the polycationic compounds polymyxin B and mellitin via microarray and other studies could identify specific loci for further study.

Attenuated intracellular bacterial pathogens have been identified by screening for increased susceptibility to polymyxin B. This predicts that increased resistance to polycations would increase pathogenicity. However, this was not found to be the case for AJ100. The observed discordance between susceptibility of AJ100 to polymyxin B and mellitin that we observed relative to SBvrR was resolved by determination of the relative pathogenicity of AJ100 to colonize and persist in a murine model compared to the parent strain. Increased susceptibility of AJ100 to the defensins mellitin, maganin, and cecropin correlated directly with attenuation in the spleen.

Attenuation of AJ100 relative to RB51 was observed in the spleen (Fig. 2) but not the liver. This attenuation may be dependent on differences in polycationic content between spleen and liver resident macrophages, as it has been shown that defensin expression by the innate immune system affects tissue colonization. Transgenic mice expressing a human intestinal defensin were protected from infection by *Salmonella typhimurium* [17], which otherwise is quite deadly to mice. As the defensins used in this study were isolated from higher organisms, unlike polymyxin B, which comes from bacteria, the profiles of

sensitivity to defensins may be a better predictor of attenuation for intracellular bacteria than susceptibility to polymyxin B.

In summary, AJ100 is sixfold more resistant to polymyxin B than the parental RB51 strain as determined by the Etest. Though AJ100 was less susceptible to this polycationic antibiotic than its parent strain, it was more susceptible to the cationic defensins, mellitin, maganin, and cecropin. This profile correlated with attenuation of AJ100 in the mouse model. AJ100 did not colonize mice spleens to the same extent as RB51 and was cleared from the spleen faster. On the other hand, livers were colonized and cleared similarly by these two strains. The profile of susceptibility to defensins may predict attenuation better than susceptibility to polymyxin B.

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References

- Allen CA, Adams LG, Ficht TA (1998) Transposon-derived *Brucella abortus* rough mutants are attenuated and exhibit reduced intracellular survival. *Infect Immun* 66:1008–1016
- Alton GG, Jones LM, Angus RD, Verger JM (1988) Techniques for the Brucellosis laboratory. Institut National de la Recherche Agronomique, Paris
- Bader MW, Navarre WW, Shiau W, Nikaido H, Frye JG, McClelland M, Fang FC, Miller SI (2003) Regulation of *Salmonella typhimurium* virulence gene expression by cationic antimicrobial peptides. *Mol Microbiol* 50:219–230
- Freer E, Moreno E, Moriyon I, Pizarro-Cerda J, Weintraub A, Gorvel JP (1996) *Brucella-Salmonella* lipopolysaccharide chimeras are less permeable to hydrophobic probes and more sensitive to cationic peptides and EDTA than are their native *Brucella* sp. counterparts. *J Bacteriol* 178:5867–5876
- Groisman EA, Parra-Lopez C, Salcedo M, Lipps CJ, Heffron F (1992) Resistance to host antimicrobial peptides is necessary for *Salmonella* virulence. *Proc Natl Acad Sci USA* 89:11939–11943
- Guo L, Lim KB, Poduje CM, et al. (1998) Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell* 95:189–198
- Guzman-Verri C, Manterola L, Sola-Landa A, Parra A, Cloeckert A, Garin J, Gorvel JP, Moriyon I, Moreno E, Lopez-Goni I (2002) The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the *Rhizobiaceae*. *Proc Natl Acad Sci USA* 99:12375–12380
- Halling SM (1996) The effects of magainin 2, cecropin, mastoparan and mellitin on *Brucella abortus*. *Vet Microbiol* 51:187–192
- Hancock REW, Scott MG (2000) The role of antimicrobial peptides in animal defenses. *Proc Natl Acad Sci USA* 97:8856–8861
- Jensen AE, Ewalt DR, Cheville NF, Thoen CO, Payeur JB (1996) Determination of stability of *Brucella abortus* RB51 by use of genomic fingerprint, oxidative metabolism, and colonial morphology and differentiation of strain RB51 from *B. abortus* isolates from bison and elk. *J Clin Microbiol* 34:628–633
- Jensen AE, Halling SM (2007) Effect of polymyxin B and environmental conditions on isolation of *Brucella* species and the vaccine strain RB51. *Comp Immunol Microbiol Infect Dis* (Submitted)
- Lapaque N, Moriyon I, Moreno E, Gorvel J-P (2005) *Brucella* lipopolysaccharide acts as a virulence factor. *Curr Opin Microbiol* 8:60–66
- Lee I-K, Olsen SC, Bolin CA (2001) Effects of exogenous recombinant interleukin-12 on immune responses and protection against *Brucella abortus* in a murine model. *Can J Vet Res* 65:223–228
- Manterola L, Guzmán-Verri C, Chaves-Olarte E, Barquero-Clave E, de Minguel MJ, Moriyón I, Grillo MJ, López-Goni I, Moreno E (2007) BvrR/BvrS-controlled outer membrane proteins Omp3a and Omp3b are not essential for *Brucella abortus* virulence. *Infect Immun* 75:4867–4874
- Manterola L, Moriyon I, Moreno E, Sola-Landa A, Weiss DS, Koch MHJ, Howe J, Brandenburg K, Lopez-Goni I (2005) The lipopolysaccharide of *Brucella abortus* BvrS/BvrR mutants contains lipid A modifications and has higher affinity for bactericidal cationic peptides. *J Bacteriol* 187:5631–5639
- Martinez de Tejada G, Pizarro-Cerda J, Moreno E, Moriyon I (1995) The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides. *Infect Immun* 63:3054–3061
- Salzman NH, Ghosh D, Huttner KM, Paterson Y, Bevins CL (2003) Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* 422:522–526
- Schurig GG, Roop II RM, Bagchi T, Boyle S, Buhman D, Sriranganathan N (1991) Biological properties of RB51; a stable rough strain of *Brucella abortus*. *Vet Microbiol* 28:171–188
- Sola-Landa A, Pizarro-Cerda J, Grillo M-J, Moreno E, Moriyon I, Blasco J-M, Gorvel J-P, Lopez-Goni I (1998) A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. *Mol Microbiol* 29:125–138
- Stevens MG, Olsen SC, Pugh GW Jr, Brees D (1995) Comparison of immune responses and resistance to brucellosis in mice vaccinated with *Brucella abortus* 19 or RB51. *Infect Immun* 63:264–270
- Tatum FM, Morfitt DC, Halling SM (1993) Construction of a *Brucella abortus* RecA mutant and its survival in mice. *Microb Path* 14:177–185
- Vaara J (1992) Agents that increase the permeability of the outer membrane. *Microbiol Rev* 56:395–411
- Zhou Z, Ribeiro AA, Lin S, Cotter RJ, Miller SI, Raetz CRH (2001) Lipid A modifications in polymyxin-resistant *Salmonella typhimurium*. *J Biol Chem* 276:43111–43121