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Effect of Antibiotics Contained in Two *Brucella* Selective Media on Growth of *Brucella abortus*, *B. melitensis*, and *B. ovis*

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The MIC and the highest concentration enabling bacterial growth (CEG) of the antibiotics contained in two selective media were determined for *Brucella abortus*, *B. melitensis*, and *B. ovis*. The nalidixic acid and bacitracin contained in Farrell's selective medium were responsible for the inhibitory effects observed.

Most brucellae can be isolated in common basal media (1), but the use of nonselective media cannot be recommended for the primary isolation of brucellae because of the high number of overgrowing contaminants usually present in clinical veterinary samples. Accordingly, selective media are necessary for the isolation of brucellae. Several selective media containing antibiotics and/or bacteriostatic dyes, either in combination or alone added to different basal media, have been described for *Brucella abortus* (3, 5, 7, 8) and *B. ovis* (2, 10). Farrell's selective medium, developed for the isolation of *B. abortus* from milk (4), is also recommended for the isolation of *B. melitensis* (1), but its efficacy for this purpose has never been rigorously proved. In fact, the sensitivity of this medium for the isolation of *B. melitensis* from naturally infected sheep and goats has been significantly lower than that obtained with modified Thayer-Martin's medium (6). The aim of the present work was to determine the susceptibilities of *B. abortus*, *B. melitensis*, and *B. ovis* to the antibiotics contained in both Farrell's and modified Thayer-Martin's selective media.

The *Brucella* strains used in the different experiments either were isolated and typed in our laboratory by standard procedures (1) or were kindly supplied and typed by J. M. Verger (Laboratoire de Pathologie Infectieuse et Immunologie, Nouzilly, France). All strains were kept freeze-dried until use. The strains used for the determination of the antibiotic susceptibility were 70 *B. abortus* strains (38 strains of biovar 1, 4 strains of biovar 2, 25 strains of biovar 3, and 3 strains of biovar 9), 140 *B. melitensis* strains (71 strains of biovar 1 and 69 strains of biovar 3), and 121 *B. ovis* strains. The antibiotics tested (Table 1) were nalidixic acid, bacitracin, colistin methanesulfonate, cycloheximide, polymyxin B sulfate, vancomycin, nystatin, and nitrofurantoin (all purchased from Sigma Chemical Co., St. Louis, Mo.). The working antibiotic dilutions were obtained for each antibiotic from a stock solution containing 5,120 μg (or IU) per ml. Dilutions were prepared to obtain plates containing final concentrations ranging from 1 to 512 μg (or IU) per ml of culture medium. In a preliminary experiment it was determined that the Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% yeast extract (Difco) and 5% sterile bovine serum (Seromed; Biochrom KG, Berlin, Germany) was suitable for the growth of all strains tested. This medium was autoclaved and cooled to 56°C, and the serum was added (MH-YS medium). Fifty milliliters of the different antibiotic solutions were then added to 450 ml of MH-YS medium. After pouring, the media were incubated for 24 h at 37°C to check for contamination and were then stored at 4°C for a maximum of 4 days before use. After reconstitution from lyophilized stocks and after checking once for purity and dissociation (1), selected colonies of each strain were transferred to MH-YS slants, and the slants were incubated at 37°C in 10% CO_2 for 24 h. The organisms were then suspended in sterile phosphate-buffered saline, and the solution was spectrophotometrically (Philips PV8025 UV/VIS; Philips Analytical, Cambridge, United Kingdom) adjusted to contain about 10^9 CFU/ml (A_{600} of 0.165 under our conditions). Suspensions of each strain were inoculated (1-μl inoculating loops) in duplicate onto MH-YS medium without antibiotic and then into the media containing the different antibiotics from the lowest to the highest concentration. To determine the MICs, the plates were incubated at 37°C in 10% CO_2 for 5 days and were examined daily. The two parameters determined for each *Brucella* species were the MIC and the highest antibiotic concentration enabling the growth (CEG) for all strains tested. The results are presented in Table 1. The MICs obtained were significantly higher than the corresponding antibiotic concentrations contained in both selective media. However, nalidixic acid and bacitracin at the concentrations used in Farrell's medium showed inhibitory effects for some *Brucella* strains. The remaining antibiotics showed MICs and CEGs significantly higher than the corresponding concentrations contained in both Farrell's and Thayer-Martin's selective media. When testing *B. abortus*, the CEG of nalidixic acid was lower (2 μg/ml) than the corresponding concentration contained in Farrell's medium (5 μg/ml). When nalidixic acid was used at 5 μg/ml, the antibiotic was inhibitory for four (2.7%) of the *B. abortus* strains tested. However, when testing *B. melitensis* and *B. ovis* the CEG was 8 μg/ml, and accordingly, none of the strains of either species was inhibited by the nalidixic acid concentration contained in Farrell's medium (5 μg/ml). The bacitracin used in Farrell's medium (25 IU/ml) inhibited the growth of 37.2% of *B. ovis* strains tested (CEG = 4 IU/ml), but it did not show inhibitory effects for *B. melitensis* or *B. abortus* (CEG = 128 IU/ml in both cases).

To detect the possible effects of these two antibiotics on the number of CFU, 28 *B. abortus* and 31 *B. melitensis* strains used in the experiment described above were tested in (i) the commercial Farrell's medium (3), that was made up with *Brucella* medium base (Oxoid, Unipath Ltd., Basingstoke, England) as the basal medium according to the manufacturer. Once they were autoclaved and cooled to 56°C, 50 ml of sterile bovine serum (Seromed) and the freeze-dried commercial selective supplement (Oxoid) were added. This antibiotic supplement contains (per milliliter of medium) nalidixic acid (5 μg), bac-

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TABLE 1. MIC and CEG[a] of the antibiotics contained in Farrell's and modified Thayer-Martin's *Brucella* selective media

Antibiotic	*B. abortus*		*B. melitensis*		*B. ovis*	
	MIC	CEG	MIC	CEG	MIC	CEG
Nalidixic acid (5)[b]	32	2	64	8	64	8
Bacitracin (25)[b]	>512	128	512	128	64	4
Colistin methanesulfonate (7.5)[c]	>512	512	>512	512	512	128
Cycloheximide (100)[b]	>512	512	>512	512	512	256
Polymyxin B sulfate (5)[b]	256	64	>512	512	512	128
Vancomycin (20)[b], (3)[c]	>512	512	512	256	512	128
Nystatin (100)[b], (12.5)[c]	>512	512	512	256	512	256
Nitrofurantoin (10)[c]	512	128	>512	128	512	128

[a] Both expressed in μg or IU/ml.
[b] The values in parentheses indicate the concentration of the corresponding antibiotic in Farrell's medium.
[c] The values in parentheses indicate the concentration of the corresponding antibiotic in modified Thayer-Martin's medium.

itracin (25 IU), cycloheximide (100 μg), polymyxin B sulfate (5 IU), vancomycin (20 μg), and nystatin (100 IU). (ii) The strains were also tested in modified Thayer-Martin's medium, which was made up with GC medium base (Biolife Italiana s.r.l., Milano, Italy) according to the instructions of the manufacturer, and were dissolved in a volume of 500 ml of distilled water. Once it was autoclaved and cooled to 56°C, 500 ml of a sterile 2% hemoglobin (Difco) solution was added. After homogenization, the following antibiotics (all from Sigma) were added (per milliliter): colistin methanesulfonate (7.5 μg), vancomycin (3 μg), nitrofurantoin (10 μg), and nystatin, but we used 100 IU instead of 12.5 IU of nystatin, a modification that improves the sensitivity of the medium (6). (iii) MH-YS medium was prepared as described above, but it contained nalidixic acid (5 μg/ml) or bacitracin (25 IU/ml). (iv) As a control MH-YS medium without antibiotics was used. Twenty-four-hour growth slants were collected and spectrophotometrically adjusted as described above. Then duplicate plates of each selective medium were inoculated with 0.1 ml from the 10^{-6} dilution from the suspension of each strain. Once spread, the plates were dried for a few minutes in a laminar flow hood and were incubated at 37°C in 10% (vol/vol) CO_2 for 7 days to determine the numbers of CFU. For analyzing the CFU data, a two-way (strain and medium) factorial analysis of variance was applied to each species by using the General Linear Models procedure of the Statistical Analysis System package (9). This analysis of variance showed a significant difference among strains ($P = 0.0001$) and media ($P = 0.0001$) for both *Brucella* species. Moreover, a statistically significant interaction effect ($P = 0.0001$) between strain and medium was also obtained for both species. The mean CFU values obtained with the 28 *B. abortus* (Table 2) and 31 *B. melitensis* (Table 3) strains tested in the different media were compared by the Duncan's multiple range test. Nalidixic acid and bacitracin significantly reduced the numbers of *B. abortus* (Table 2) and *B. melitensis* (Table 3) CFU with respect to those in the control medium (at least $P < 0.05$). Moreover, Farrell's medium significantly ($P < 0.01$) reduced the numbers of CFU of both *B. abortus* and *B. melitensis* when compared with those in the control medium. Modified Thayer-Martin's medium did not affect the numbers of *B. melitensis* but significantly ($P < 0.05$) reduced the numbers of *B. abortus* CFU (Tables 2 and 3).

The selective medium most commonly used for the primary isolation of *B. melitensis* and *B. abortus* from contaminated sources is Farrell's medium (1). However, the results presented in Tables 2 and 3 demonstrate that this medium has inhibitory effects for both species. When testing the individual effects of the antibiotics contained in both media (Table 1), only nalidixic acid and bacitracin showed some inhibitory effect. Bacitracin was clearly responsible for the inhibitory effect of Farrell's medium for *B. ovis* (Table 1). Moreover, even though the CEG of bacitracin for *B. melitensis* was significantly higher than its concentration in Farrell's medium, bacitracin significantly reduced the numbers of *B. melitensis* CFU (Table 3). On the other hand, the nalidixic acid CEG for *B. melitensis* was too close to its concentration in Farrell's medium (Table 1), accounting for the significant decrease in the numbers of *B. melitensis* CFU produced (Table 3). Interestingly, the nalidixic acid CEG for *B. abortus* was lower than the corresponding concentration in Farrell's medium, and 4 of the 70 *B. abortus* strains tested were inhibited when nalidixic acid was used at its concentration in Farrell's medium. Moreover, this concentration significantly reduced the numbers of *B. abortus* CFU (Table 2). Therefore, the effects of both antibiotics either alone or in association is responsible for the inhibitory effects of Farrell's medium for all *Brucella* species tested and explain the reported (6) higher degree of sensitivity of modified Thayer-Martin's medium for the isolation of *B. melitensis*. Accordingly, a theoretically ideal antibiotic supplement for the selective isolation of brucellae should not include both antibiotics.

TABLE 2. Effect of culture medium on the mean numbers of CFU obtained with the 28 *B. abortus* strains tested

Medium	CFU (Mean ± SD)[a]
Farrell	56.86 ± 21.45[b]
MH-YS, nalidixic acid (5 μg/ml)	58.68 ± 15.50[b,c]
Modified Thayer-Martin	63.16 ± 19.74[c]
MH-YS, bacitracin (25 IU/ml)	63.27 ± 13.39[c]
MH-YS	68.79 ± 23.67[d]

[a] Mean values with the same letter are not significantly different (at least $P > 0.05$).

TABLE 3. Effect of culture medium on the mean numbers of CFU obtained with the 31 *B. melitensis* strains tested

Medium	CFU (mean ± SD)[a]
Farrell	74.48 ± 40.66[b]
MH-YS, bacitracin (25 IU/ml)	82.21 ± 35.79[c]
MH-YS, nalidixic acid (5 μg/ml)	85.69 ± 45.37[c]
Modified Thayer-Martin	99.50 ± 48.25[d]
MH-YS	99.42 ± 52.44[d]

[a] Mean values with the same letter are not significantly different (at least $P > 0.05$).

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