Project SOP D-JRP17-WP4.SOP2 – AMR testing protocoll including proposals for ECOFFs for emerging *Brucella* sp.

Workpackage 4

Responsible Partner: BfR, FLI

Contributing partners: ANSES, APHA, INIAV, INSA, IZSAM

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AMR TESTING PROTOCOLL INCLUDING PROPOSALS FOR ECOFFS FOR EMERGING *BRUCELLA* SP.

In the course of the project, the microdilution method was established at four laboratories of project partners. A microdilution plate system Micronaut™ (Merlin GmbH, Bornheim-Hersel, Germany), was used. Nine different antibiotic dilutions are applied to their Micronaut-S plate. The antibiotics are chloramphenicol, ciprofloxacin, doxycyclin, gentamycin, levofloxacin, rifampicin, streptomycin, tetracyclin and trimethoprim/sulfamethoxazole. The protocol used is based on the publication of Tscherne et al., Adaptation of Brucella melitensis Antimicrobial Susceptibility Testing to the ISO 20776 Standard and Validation of the Method. Microorganisms 2022, 10, 1470.

1. Purpose

Description of the method of antibiotic susceptibility testing of brucella in the broth microdilution method using commercially obtained Micronaut™ test plates.

2. Abreviations

ATCC	American Type Culture Collection
MIC	Minimal Inhibitory Concentration
EUCAST	European Committee on Antimicrobial Susceptibility Testing
CLSI	Clinical and Laboratory Standards Institute
AST	Antibiotic Susceptibility Testing
COL	Columbia blood agar
CAMHB	Cation adjusted Mueller Hinton broth

3. Reagents, equipment, control strains

- plastic tube 50 ml for MIC plates
- 15 ml-tubes with 5 ml sterile NaCl 0,9% solution (pH 5,5 - 6,5)
- Columbia blood agar plates
- Micronaut-AST IMB gramnegative 1 and gramnegative 2 MIC plates, Merlin
- Columbia agar plates
- 15 ml-tubes with 11 ml CAMHB
- adhesive and **non-perforated** foil for 96-well plates (MICRONAUT MERLIN order no. B3-001-100)
- reference strains: *E. coli* ATCC #25922, *S. aureus* ATCC #29213

4. Quality control

The quality control is based on the specification CLSI (M45-A2 Vol. 30 No. 18) MIC interpretive criteria for susceptibility categories. Reference strains must be tested with each new batch of culture medium in parallel with the test strain under investigation.

5. Procedure

5.1. Cultivation of bacteria

For AST only pure cultures are used. The bacteria are grown on COL medium, 37°C and 5% CO_2. Brucella species are incubated for 48 hours and control strains for 24 hours.

5.2. Inoculation procedure

- isolates are inoculated in 5 ml NaCl 0,9% to match the turbidity equivalent to a 0,5 McFarland turbidity standard
- Prepare a 1:10 dilution of the NaCl- suspension (test strain)
- Keep the reference strains undiluted
- addition of 400 µl *Brucella spp*.- NaCl-suspension volume to 22 ml of CAMHB
- addition of 100 µl *E. coli*- NaCl-suspension volume to 22 ml of CAMHB
- addition of 400 µl *S. aureus*- NaCl-suspension volume to 22 ml of CAMHB

5.3. Inoculation and incubation of MIC plates

- open single packaging of MIC plate (max. up to 30 min before use)
- label test plates with lab identification number and date
- add inoculum to a 50 ml-plastic tub
- fill 100 µl in each cavity of the test plate using an 8-channel-pipette
- cover test plate with a masking foil
- perform purity control of inoculum on pathogen-specific solid culture medium under culture conditions depicted under 5.1
- MIC test plates are incubated in 37°C ambient air for 48 hours. Control strains have to be read after 18 to 20 hours and *Brucella spp*. have to be read first time after 24 hours and second time after 48 hours.

5.4. CFU determination of the inoculum

- Vortex the inoculum for at least 10 s
- Take 10 µl of the inoculum to 10 ml NaCl and vortex for at least 10 s
- Take 100 µl of this mix to the cultivation plate and spread all over the plate.
- You can count the cfu by multiplying the number of bacteria colonies on the plate to 1×10^4. This gives you the cfu/ml. This will give you $(1\text{-}2.5\times10^6)$/ml cfu.
- You should get 20-80 cfu for each bacterium. If you have less than 10 or more than 200 cfu you should repeat the test because you have either to little or too much bacterium from the beginning.

5.5. Interpretation

- purity control check (→ if purity control is impure repeat MIC testing using pure culture)
- cfu determination check
- wipe off possible condensate from the base of MIC test plate
- cautiously remove masking foil
- assess growth control (if growth control is uncolonized repeat procedure)
- assess growth in each well of the MIC test plate (growth = turbidity or bud)
- determine MIC according to the "microdilution reading guide"
- repeat the reading by a second person**.** If those two people have different results, discuss and report just one result. Make a comment in the data entry mask.
- assess reference strains first and compare MICs as outlined in appendix 2 and 3 of this document, if valid

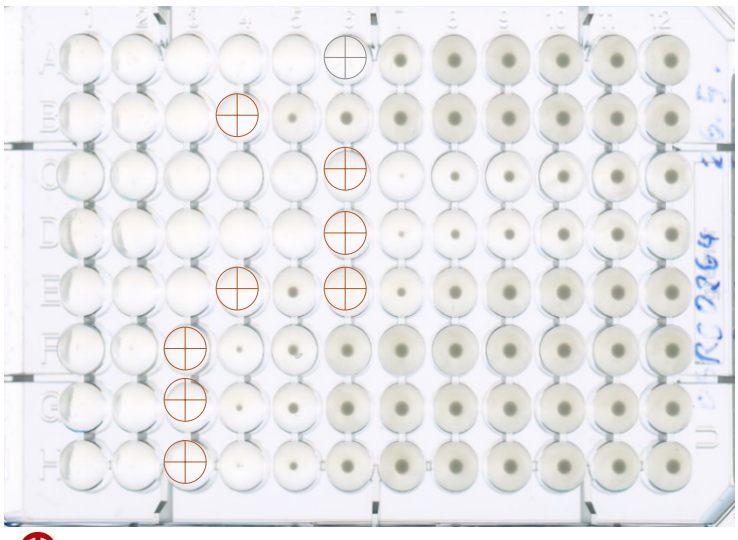
6. Additional information

6.1. Layout_Gram-negative MIC plate (used during the project)

	1	2	3	4	5	6	7	8	9	10	11	12
GEN	8	4	2	1	0.5	0.25	0.125	0.0625	0.031	0.016	0.008	0.004
STR	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.031	0.016	0.008
DOX	8	4	2	1	0.5	0.25	0.125	0.0625	0.031	0.016	0.008	0.004
TET	8	4	2	1	0.5	0.25	0.125	0.0625	0.031	0.016	0.008	0.004
CMP/ RAM	CMP 8	CMP 4	CMP 2	CMP 1	CMP 0.5	RAM 8	RAM 4	RAM 2	RAM 1	RAM 0.5	RAM 0.25	RAM 0.125
T/S	4/76	2/38	1/19	0.5/ 9.5	0.25/ 4.76	0.125/ 2.375	0.0625 / 1.187	0.031/ 0.594	0.016/ 0.297	0.0078/ 0.148	0.0039/ 0.074	0.00195/ 0.037
CIP	4	2	1	0.5	0.25	0.125	0.0625	0.031	0.016	0.008	0.004	0.002
LEV	4	2	1	0.5	0.25	0.125	0.0625	0.031	0.016	0.008	0.004	**GC**

CMP: Chloramphenicol
CIP: Ciprofloxacin
DOX: Doxycyclin
GEN: Gentamycin
LEV: Levofloxacin
RAM: Rifampicin:
STR: Streptomycin
TET: Tetracyclin
T/S: Trimethoprim/Sulfamethoxazole
GC: Growth Control

6.2. Example for an MIC plate with a Brucella isolate from frog



⊕ value to be noted

6.3. Quality control MIC for control strains (EUCAST)

Antimicrobial agent	E. coli		S. aureus	
	Target	Range	Target	Range
GEN	0.5	0.25-1	0.25-0.5	0.125-1
STR	na	na	na	na
DOX	na	na	0.25	0.125-0.5
TET	na	na	0.25-0.5	0.125-1
CMP	4	2-8	4-8	2-16
RAM	na	na	0.008	0.004-0.016
T/S	≤0.5	na	≤0.5	na
CIP	0,008	0.004-0.016	0.25	0.125-0.5
LEV	0.016-0.03	0.008-0.06	0.125-0.25	0.06-0.5

ECOFFs for emerging *Brucella* sp.

Some of the project partner labs were also involved in the "Adaptation of *Brucella melitensis* Antimicrobial Susceptibility Testing to the ISO 20776 Standard and Validation of the Method." (Tscherne et al.;2022). The number of studies and samples necessary to make a sound statement on ECOFFs exceeds the possibilities given in this project. For this reason, the results obtained were only evaluated in terms of their suitability for phenotyping.