In vitro effect of antibiotics on Mycobacterium avium subspecies paratuberculosis in Herrold’s medium

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ABSTRACT
The isolation of Mycobacterium avium subspecies paratuberculosis (Map) is considered the “Gold Standard” diagnostic test for paratuberculosis. Due to high contamination rates with an observed average of 30% after conventional fecal culture from beef cattle herds, we address this problem by identification of Pseudomonas aeruginosa as the most common contaminant. After antibiotic susceptibility testing, the following antibiotics were efficient to control the growth of Pseudomonas aeruginosa: enrofloxacin, florfenicol and gentamicin. To check the in vitro breakpoint inhibition effect of these antimicrobial agents to Map, clinical isolates of paratuberculosis were tested to examine their growth responses in Herrold’s medium supplemented with these antibiotics at serial dilutions. Inhibition effect on Map was observed for enrofloxacin and gentamicin, florfenicol showed the minimal inhibitory effect to clinical isolates of Map at the proposed dilution ranges.

INTRODUCTION
Paratuberculosis is an insidious, chronic, granulomatous enteritis of ruminants and other animals caused by Mycobacterium avium subsp. paratuberculosis (Map). Clinically affected cattle develop chronic diarrhea and lose weight. The infection spreads mainly by the oral-fecal route, animals are more susceptible to infection shortly after birth and typically do not show outward signs of disease before they are 3-5 years old. In cattle, microbiological culture of the organism from feces, serves as identification of infected animals. This method is considered the reference assay (gold standard), against which other tests are compared. The disadvantages of using fecal culture are prolonged incubation times and contamination is often a problem when culturing fecal specimens. The estimated 33% sensitivity of the fecal culture method is adversely affected by the growth of contaminant bacteria. In a beef herd with paratuberculosis problem a whole herd fecal culture was conducted with overgrowth problem primarily identified as Pseudomonas aeruginosa, the average number of tubes that became contaminated ranged 30%.

The purpose of this communication was to identify antimicrobial agents that minimally growth of Map yet reduce the growth of Pseudomonas aeruginosa.

MATERIALS AND METHODS
Study Herd and sample process
Fecal samples from a beef herd identified as having paratuberculosis were processed as previously described. Briefly, fecal samples (4 g) were decontaminated into 50-ml tubes containing 30 ml sterile, 0,9% hexadecylpyridinium chloride in brain hearth infusion broth (HPC-BHI), vortexed for 30 min and left in an upright position at room temperature for 30 min, after settling the entire supernatant fraction was removed and placed in a 50 ml tube, after overnight decontamination at 37°C samples were centrifuged 1,700 g for 30 min, supernatant were removed and the pellet resuspended in 1 ml antibiotic solution (100 µg/ml nalidixic acid, 100 µg/ml vancomycin, 100 µg/ml nystatin) and incubated overnight at 37°C. Sample suspension (0.2 ml) were inoculated onto 2 tubes of Herrold’s Egg Yolk Medium (HEYM) containing 100 µg/ml nalidixic acid, 100 µg/ml vancomycin, 100 µg/ml nystatin.

Pseudomonas aeruginosa strain and susceptibility testing
A strain of Pseudomonas aeruginosa was isolated in cetrimide agar from contaminated tubes with HEYM cultured with feces from a beef herd with known paratuberculosis. For in vitro qualitative antibiotic sensitivity of Pseudomonas aeruginosa isolate, the disk diffusion method was used with these antibiotics: cefalexin, augmentin, cloxacillin, ceftiofur, ampicillin,
gentamicin, vancomycin, nalidixic ac, enrofloxacin and florfenicol. The zone of bacterial growth inhibition was recorded. For quantitative activity, the minimum inhibitory concentration (MIC) in a tube dilution procedure was used in a series of tubes containing cetrimide agar mixed with serially diluted antibiotic solutions. Solutions of gentamicin and enrofloxacin were performed in sterile distilled water, while florfenicol was dissolved in dimethyl sulfoxide (DMSO). Twofold dilutions of the antibiotics were performed in order to obtain concentration from 1.95 µg/ml to 1000 µg/ml (1.95 µg/ml, 3.9 µg/ml, 7.8 µg/ml, 15.6 µg/ml, 31.2 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500µg/ml, 1000µg/ml), 10 tubes with cetrimide free of antibiotics and with DMSO alone were used as control. The *Pseudomonas aeruginosa* colonies were suspended in sterile distilled water with an equivalent turbidity to the density of McFarland 0.5 barium sulphate. This standard inoculum was applied with a sterile cotton swab. After incubation at 36°C for 2 days, to determine which concentration completely inhibited the growth of *Pseudomonas aeruginosa*, tubes were observed and the MIC was recorded as the first tube in which growth of the organism has been inhibited.

**Map strains and susceptibility testing**

The study involved 2 clinical strains of *Map* that were isolated from fecal specimens submitted to the laboratory for cultural examination. The strains were identified on the basis of phenotypic characteristics. The quantitative drug susceptibility tests were performed on those antibiotics with sensitivity effect against *Pseudomonas aeruginosa*. Each of two strains were suspended in distilled water, this suspension was inoculated in two sets of 10 tubes with HEYM containing mycobactin J (2mg/L) and serial dilution of one of these three antibiotics: enrofloxacin, gentamicin and florfenicol from 1.95 µg/ml to 1000 µg/ml were diluted serially as described in *Pseudomonas aeruginosa* with a set of 10 tubes free of antibiotics and with DMSO as control. Each of 10 tubes was inoculated with 200 µl of standard inoculum similar to *Pseudomonas aeruginosa*. The inoculated slants were incubated at 37°C and for bacterial growth the surface were observed visually for 1.5 month. The MICs of inoculated tubes containing several sequential dilutions of each drug was the lowest concentration of the antimicrobial agent that inhibited the colonies growing on the drug-free control media.

**Statistical methods**

Fisher’s exact test was used to analyze the interaction between antibiotics and the *in vitro* growing effect to *Map* and *Pseudomonas aeruginosa*.

**RESULTS**

In the qualitative antibiotic sensitivity against *Pseudomonas aeruginosa* the next antibiotics demonstrated a zone of inhibition around the discs: enrofloxacin, gentamicin and florfenicol. To these antibiotics, the growth end points MIC for *Map* were read when organisms reached good macroscopic growth in tubes with antibiotics and control tubes after 1.5 months of incubation.

The presence of *Pseudomonas aeruginosa* was confirmed by the appearance of colonies with pigmentation in cetrimide agar (a selective medium for *Pseudomonas aeruginosa*), and the observation of Gram stained smears. The identification of *Map* colonies were based on the observation of acid-fast organisms from smears stained with Ziehl-Neelsen method and phenotypic characteristics as time required to develop a visible colony with typically morphology resembling *Map*.

MICs were as follow: gentamicin 15.62 (µg/ml), enrofloxacin 7.8 (µg/ml) and florfenicol 1000 (µg/m). The breakpoints for *Pseudomonas aeruginosa* were read after 2 days of incubation with the next MIC results: gentamicin 31.25 (µg/ml), enrofloxacin 7.8 (µg/ml) and florfenicol 250 (µg/ml). These results are summarized in Table 1. No statistical significant interactions was found between *Pseudomonas aeruginosa* and *Map* with gentamicin, enrofloxacin and florfenicol. Enrofloxacin present the breakpoint for *Pseudomonas aeruginosa* and *Map* at the same concentration (7.8 µg/ml), gentamicin have a MIC for *Map* (15.62 µg/ml) below to the end point of *Pseudomonas aeruginosa* (31.25 µg/ml), florfenicol
shows activity against *Pseudomonas aeruginosa* at 250 µg/ml, for *Map* MIC limits could not be established because it was beyond the proposed range.

### Table 1. Susceptibilities of *Map* and *Pseudomonas aeruginosa* (*Ps. aeruginosa*) to florfenicol, gentamicin and enrofloxacin.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Florfenicol (Ps. aeruginosa)</th>
<th>Gentamicin (Ps. aeruginosa)</th>
<th>Enrofloxacin (Ps. aeruginosa)</th>
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(*) MIC breakpoints to the corresponding tube with antibiotic concentration expressed in (µg/ml)

(*) Growing in the presence of antibiotics

### DISCUSSION
The bacteriologic culture of feces in bovine paratuberculosis remains the reference assay, but some problems in culturing *Map* are the slow growth of the organism, the degree and the nature of contamination. In this case we have identified *Pseudomonas aeruginosa* as the most common contaminant. The isolation of *Pseudomonas aeruginosa* as contaminant seems not to be casual, other reports describes an unusual level of contamination caused also by *Pseudomonas aeruginosa* in HEYM tubes inoculated with fecal samples. We used BHI in the decontamination process for these studies, previous work suggests that BHI may not be a required component in the decontamination procedures, the exclusion of BHI from the isolation protocol, may result in increased *Map* detection rate.

After having performed qualitative antibiotic sensitivity, enrofloxacin, gentamicin and florfenicol were found to have activity against *Pseudomonas aeruginosa*. In the MIC results, gentamicin and enrofloxacin demonstrated discernible growth end points for *Pseudomonas aeruginosa*, 31.25 µg/ml and 7.8 µg/ml respectively but similar MIC breakpoints were observed for *Map*. This *in vitro* antimicrobial susceptibility of *Map*, excludes the use of these antibiotics in processing methods as initial decontaminant or be incorporated in HEYM as selective agents. Florfenicol, an analogue of chloramphenicol, possess an antimicrobial spectrum similar to that of chloramphenicol, and has been reported to have superior *in vitro* bactericidal activity compared with chloramphenicol. In addition this last antibiotic serves as a frame of reference for *Map*, because it has already been incorporated as a component in selective media for *Map* isolation. Florfenicol was effective controlling the growth of *Pseudomonas aeruginosa* at 250 µg/ml, the MIC for *Map* could not be determined as it was near or above their tested dilution schedule (up to 1000 µg/ml). In previous reports a slight depression of the *Map* growth at 200 µg/ml using chloramphenicol as selective agent was reported during the first 8 weeks of incubations in the number and size of colonies with recuperation after 12 weeks. To evaluate if florfenicol is harmful to *Map*, upper full range relevant MIC limits should be performed *in vitro* with these and other isolates of *Map*. This experience was designed to be done with more statistical relevant number of clinical isolates but in a previous pilot work not all isolates grew well in subcultures. Discrepancy in capacity of growth between field strains, mainly the speed of growth and the erratic behavior prevented the inclusions of a higher number of field isolates. The harmful effect of any agent for a selective isolation of mycobacteria can be evaluated as a frequency of isolation from clinical isolates or the time required for appearance and number of clearly visible colonies. In this case tubes with antibiotics were read when organisms reached macroscopic growth equal to that of control tubes, this happened after 6 weeks of incubation apparently and no inhibitory effect of florfenicol was observed to *Map*. To establish any harmful effect of
florfenicol for Map further studies should be performed at higher MIC concentration with clinical samples and to evaluate the speed of growth and number of colonies in comparison with control medium.

CONCLUSION
Due to the in vitro inhibitory growing effect of gentamicin and enrofloxacin observed for Map, these antibiotics can not be used to prevent and control the overgrown problem caused by Pseudomonas aeruginosa. Florfenicol should further be evaluated to assure that it is harmless at the recommended concentration to stop the growing of Pseudomonas aeruginosa.

REFERENCES

Note from the editor: This paper was presented only as an abstract at the 9ICP. It was not presented as a poster or an oral presentation. Therefore, the 9ICP delegates had no possibility of discussing the study.