In Vitro Activities of Tulathromycin and Ceftiofur Combined with Other Antimicrobial Agents Using Bovine Pasteurella multocida and Mannheimia haemolytica Isolates*

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The purpose of this study was to determine the activities of two antibacterial agents used in the treatment of bovine respiratory infections—tulathromycin, a macrolide, and ceftiofur, a third-generation cephalosporin—alone, in combination with each other, and in combination with each of seven additional antibiotics (tilmicosin, florfenicol, enrofloxacin, danofloxacin, ampicillin, tetracycline, and penicillin G) against bovine Pasteurella multocida (n = 60) and Mannheimia haemolytica (n = 10) isolates for determination of synergy, antagonism, or indifference. Of 458 organism–drug combinations, 160 combinations of tulathromycin and 209 combinations of ceftiofur with eight antimicrobial drugs were indifferent. One combination was antagonistic (ceftiofur + florfenicol against one isolate of P. multocida). Time–kill studies showed loss of cidality for ceftiofur when combined with florfenicol at 1× the minimal inhibitory concentration. Overall, the in vitro data demonstrated that tulathromycin and ceftiofur, in combination with each other or seven other antimicrobial agents, primarily produce an indifferent response with no occurrences of synergism and rare occurrences of antagonism.

INTRODUCTION

Combination antimicrobial therapy may provide a complementary spectrum that increases the likelihood of activity against multiple pathogens in potentially mixed infections, which in turn may allow for reduced dosages of one or both agents and decrease the probability of induced resistance in the pathogens.1 Antimicrobial combinations are considered synergistic if the effect of the combination is greater than that of either agent alone or greater than the sum of the effects of the individual agents, an-

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agonistic if the combination provides an effect less than that of either agent alone or less than the sum of the effects of the individual agents, and indifferent if the combination provides an effect equal to that of either agent alone. A common in vitro laboratory method to determine whether antimicrobial combinations are synergistic, antagonistic, or indifferent uses fractional inhibitory concentrations (FICs), and the checkerboard technique has been one of the traditional methods to determine FIC indices for synergism, antagonism, or indifference.1,2

Examples of clinically useful synergistic antimicrobial drug combinations in veterinary medicine include penicillin–streptomycin and ampicillin–clavulanic acid for bovine Staphylococcus aureus mastitis, erythromycin–rifampin for Rhodococcus equi pneumonia in foals, minocycline–streptomycin for Brucella canis infections in dogs, and trimethoprim–sulfamethaxazole for coliform meningitis.3 Concurrent use of more than one antimicrobial agent may prove beneficial in the treatment of some diseases, but evidence from controlled clinical investigations to support such use in treating bovine respiratory disease (BRD) is lacking.4–6 Currently, there is no guidance for clinical veterinarians in the field regarding the administration of antibiotic combinations or sequential antimicrobial therapy in the treatment of BRD, especially for longer-acting drugs, even though the latter is used in animals that do not respond successfully to the first course of treatment. With sequential therapy, subsequent medications may be administered before the previous compound has been completely eliminated by the animal. Thus, it is important to know whether any residual drug from the previous dosage regimen will have a positive synergistic effect, a negative antagonistic effect, or no effect on a subsequent medication.

Tulathromycin is a macrolide antimicrobial agent characterized by a 15-membered ring with three amine groups and therefore belongs to the subclass of macrolides termed triamilides. This agent is currently approved for the treatment of BRD associated with Mannheimia haemolytica, Pasteurella multocida, Histophilus somni, and Mycoplasma bovis and for the control of respiratory disease in cattle at high risk of developing BRD associated with M. haemolytica, P. multocida, and H. somni. Ceftiofur is a broad-spectrum third-generation cephalosporin currently approved for treatment of BRD associated with M. haemolytica, P. multocida, and H. somni and for the control of respiratory disease in cattle at high risk of developing BRD associated with the same organisms.

Tulathromycin and ceftiofur were tested in vitro alone, in combination with each other, and in combination with each of seven additional antibiotics using checkerboard broth microdilution plates prepared by a semiautomated method. The objective of this study was to determine the interactions of tulathromycin and ceftiofur with each other and with other antimicrobial agents by generating and analyzing FIC indices for synergism, antagonism, or indifference.1 Examples of clinically useful synergistic antimicrobial drug combinations in veterinary medicine include penicillin–streptomycin and ampicillin–clavulanic acid for bovine Staphylococcus aureus mastitis, erythromycin–rifampin for Rhodococcus equi pneumonia in foals, minocycline–streptomycin for Brucella canis infections in dogs, and trimethoprim–sulfamethaxazole for coliform meningitis.3 Concurrent use of more than one antimicrobial agent may prove beneficial in the treatment of some diseases, but evidence from controlled clinical investigations to support such use in treating bovine respiratory disease (BRD) is lacking.4–6 Currently, there is no guidance for clinical veterinarians in the field regarding the administration of antibiotic combinations or sequential antimicrobial therapy in the treatment of BRD, especially for longer-acting drugs, even though the latter is used in animals that do not respond successfully to the first course of treatment. With sequential therapy, subsequent medications may be administered before the previous compound has been completely eliminated by the animal. Thus, it is important to know whether any residual drug from the previous dosage regimen will have a positive synergistic effect, a negative antagonistic effect, or no effect on a subsequent medication.

Tulathromycin is a macrolide antimicrobial agent characterized by a 15-membered ring with
agar plates containing 5% sheep blood. The quality-control strain *S. aureus* ATCC 913 was originally acquired from the American Type Culture Collection (Manassas, VA).

**Antimicrobial Agents**

The following antibiotic powders were used in the experiments: amoxicillin, potassium clavulanate, florfenicol, tetracycline, tilmicosin, penicillin G, spiramycin, and chloramphenicol (Sigma Chemical, St. Louis, MO); ampicillin (Gibco, Grand Island, NY); danofloxacin, enrofloxacin, and ceftiofur (Riedel-de Haen, Seelze, Germany); and tulathromycin (Pfizer Animal Health).

**Susceptibility and Fractional Inhibitory Concentration Testing**

Mueller–Hinton broth (MHB; Difco Laboratories, Sparks, MD) at pH 7.3 was used for susceptibility testing of all isolates. The minimal inhibitory concentration (MIC) of each drug was determined by broth microdilution according to methods described by the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards).7

Robotics were used to create and inoculate microdilution checkerboard plates. MICs were determined for each drug against each strain, and drug concentrations used for combination testing ranged from below to above the MIC. For each drug combination tested, a 96-well deep-well plate (Beckman Coulter, Fullerton, CA) was filled with MHB containing 1% alamarBlue (Trek Diagnostic Systems, Cleveland, OH) by the Multidrop instrument (Labsystems, Helsinki, Finland). According to the package insert, alamarBlue is a colorimetric redox indicator used to aid visual reading of checkerboard plates; it had no effect on organism growth.8 The MIC for each isolate against each drug was defined as the well that had the lowest concentration of drug that produced no organism growth (i.e., the blue well). A test drug was diluted twofold from column 1 to column 7 of each deep-well mother plate using the Biomek 2000 instrument (Beckman Coulter). Column 8 contained no test drug. Twofold dilutions of the second drug were then added to rows 1 to 7 of each deep-well plate. Row 8 did not contain the second drug. A total of 50 µl was transferred by the Multimiek 96 instrument (Beckman Coulter) from each well of each mother plate to a daughter plate. The Biomek instrument was used to inoculate daughter plates with 50 µl of a 1:100-diluted culture
equal to a 0.5 McFarland standard (approximately $1 \times 10^8$ CFU/ml) from bacterial growth on 18- to 24-hour-old blood agar plates for a final organism concentration of approximately $5 \times 10^7$ CFU/ml. The daughter plates were incubated at 35°C for 18 ± 2 hours.

Plates were read visually by observing a reduction (a color change from blue to purple or pink, indicating organism growth) or no reduction (a blue color, indicating no growth of organism as a result of inhibition by drugs). The MIC of each drug could be determined; for wells along the growth–no growth interface, FICs were determined using the following formula:

$$\text{MIC of Drug 1 in Combination} + \text{MIC of Drug 2 in Combination} = \text{MIC of Drug 1 Alone} + \text{MIC of Drug 2 Alone}$$

The average FIC index was calculated from individual FICs by the formula $(\text{FIC}_1 + \text{FIC}_2 + \ldots + \text{FIC}_n)/n$, where n is the total number of individual wells per plate for which FICs were calculated. Synergism was defined as an average FIC index ≤0.5, antagonism as an average FIC index >4.0, and indifference as an average FIC index of 0.51 to 4.0.$^{1,8}$ Ten strains each of $P.\text{multocida}$ and $M.\text{haemolytica}$ from BRD infections were used for each drug combination test. If synergism or antagonism was observed for any drug combination, an additional 50 isolates of $P.\text{multocida}$ or $M.\text{haemolytica}$ were tested to further determine the extent of the synergistic or antagonistic combination.

**It is important to know whether any residual drug from a previous dosage regimen will have a positive synergistic effect, a negative antagonistic effect, or no effect on a subsequent medication.**

Assay reproducibility for synergism was monitored using a combination of amoxicillin and clavulanic acid with $S.\text{aureus}$ ATCC 29213.$^1$ Assay reproducibility for antagonism was monitored using a combination of sparfloxacin and chloramphenicol with $S.\text{aureus}$ ATCC 29213.$^9$

**Time–Kill Assay**

Isolates were streaked on a 5% sheep blood agar plate and incubated at 35°C for 24 hours. Colonies were selected and placed in MHB to equal a 0.5 McFarland standard (approximately $1 \times 10^8$ CFU/ml) and diluted 1:10 in fresh MHB. Diluted cell suspension (0.5 ml) was added to 4.5 ml MHB for a final volume in each tube of 5 ml containing approximately $1 \times 10^6$ CFU/ml cells exposed to ceftiofur alone at $1 \times$ MIC, florfenicol alone at $1 \times$ MIC, and ceftiofur ($1 \times$ MIC) plus florfenicol at $1 \times$, $0.5 \times$, $0.25 \times$, and $0.125 \times$ MIC. A tube containing MHB without drugs and with diluted culture was used as a growth control. A 0-hour time point measurement was obtained by removing an aliquot of broth from each tube, diluting in sterile saline, plating on 5% sheep blood agar plates, and incubating at 35°C for 24 hours. Tubes were then stored in a 35°C incubator, and aliquots were collected at 4, 6, and 24 hours; at each collection time, an aliquot of broth from each tube was diluted in sterile saline, plated on 5% sheep blood agar plates, and incubated at 35°C for 24 hours. CFUs grown on plates were recorded and graphed.
Time–Kill Curves for Cells Exposed to Ceftiofur at 1× MIC for 24 Hr and Then Florfenicol

(Figure 1), and the limit of detection was defined as $2 \log_{10}$ CFU/ml. For analyzing kill curves, synergy was defined as a $\geq 3 \log_{10}$ decrease in bacterial CFU/ml between the drug combination and the most active single drug after 24 hours. Antagonism was defined as a $\geq 3 \log_{10}$ increase in bacterial CFU/ml between the drug combination and the most active single drug after 24 hours.

**Drug-Exposure Assays**

Isolates were streaked on a 5% sheep blood agar plate and incubated at 35°C for 24 hours. Colonies were selected and placed in MHB to equal a 0.5 McFarland standard (approximately $1 \times 10^8$ CFU/ml). A final concentration of approximately $1 \times 10^7$ CFU/ml cells was exposed to $1 \times$ MIC ceftiofur (0.002 µg/ml), $10 \times$ MIC ceftiofur (0.2 µg/ml), $1 \times$ MIC florfenicol (4 µg/ml), and $10 \times$ MIC florfenicol (40 µg/ml) for 24 hours at 35°C. Cells were then placed in centrifuge tubes and centrifuged at 5,000 rpm for 10 minutes. The supernatant was poured off, and MHB was added to the tubes, which were then vortexed and spun again at 5,000 rpm for 10 minutes to remove and wash away antibiotic. Cells were then resuspended in fresh MHB and vortexed. Ceftiofur-exposed cells were added to tubes containing florfenicol at 0×, 0.5×, 1×, 2×, and 20× MIC (Figures 2 and 3),
and florfenicol-exposed cells were added to tubes containing ceftiofur at 0×, 0.5×, 1×, 2×, and 20× MIC.

A 0-hour time point measurement was obtained by removing an aliquot of broth from each tube, diluting in sterile saline, plating on 5% sheep blood agar plates, and incubating at 35°C for 24 hours. Tubes were stored in a 35°C incubator, and aliquots were collected at 1, 2, 3, and 6 days; at each collection time, an aliquot of broth from each tube was diluted in sterile saline, plated on 5% sheep blood agar plates, and incubated at 35°C for 24 hours. CFUs grown on plates were recorded and graphed, and the limit of detection was defined as 2 log_{10} CFU/ml. After plotting the number of recovered CFUs at each time point, a ≥3 log_{10} decrease in bacterial CFU/ml between the drug and control curves on each day was determined.

## RESULTS

### Susceptibility Results

MIC ranges and the MIC at which 50% and 90% of isolates are inhibited (MIC_{50} and MIC_{90}, respectively) were determined for this study, and susceptibilities were determined based on veterinary-specific or CLSI-approved human interpretive criteria (Table 1). The MIC_{90} for tulathromycin was 4 µg/ml for both *P. multocida* and *M. haemolytica*. The MIC_{90} for ceftiofur was <0.06 µg/ml for both organisms.

### Fractional Inhibitory Concentrations

The combined activities of tulathromycin, ceftiofur, and other antimicrobial agents from the in vitro checkerboard interactions against bovine isolates of *P. multocida* and *M. haemolytica* are summarized in Tables 2 through 4. Average minimum and maximum FICS (average Σ FIC index range) and interpretations for the activities of tulathromycin in combination with eight of eight antimicrobials showed indifference for 160 organism–drug combinations; synergism or antagonism was not observed (Table 2). Minimum and maximum FICS and interpretations for the activities of ceftiofur in combination with seven of eight antimicrobials showed indifference for 209 organism–drug combinations; synergism was not observed.
Ceftiofur combined with florfenicol was antagonistic with one isolate of *P. multocida* (FIC = 6.91; Table 3). Additional drug combinations tested included tilmicosin with enrofloxacin, tilmicosin with florfenicol, florfenicol with enrofloxacin, and tetracycline with penicillin G. For these 80 organism–drug combinations, all showed indifference (Table 4). Inherent assay variability appeared to be kept at a minimum, as determined from the results for the control plates for synergism (100% synergism with amoxicillin–clavulanic acid plates using *S. aureus* ATCC 29213 in four separate runs) and the control plates for antagonism (100% antagonism with sparfloxacin–chloramphenicol plates using *S. aureus* ATCC 29213 in four separate runs; Table 5). These control plates, which were made during each drug combination experiment, were included to ensure the accuracy of the assay.

### Time–Kill Assay

A single isolate of *P. multocida* that exhibited antagonism when tested with ceftiofur + florfenicol in the checkerboard assay (FIC = 6.91) was retested in the checkerboard assay to confirm antagonism and then further tested in a time–kill assay (Figure 1). Growth curves for this isolate at the organism’s MIC was bactericidal with ceftiofur (MIC = 0.002 µg/ml) and bacteriostatic with florfenicol (MIC = 4 µg/ml; Figure 1). Cidality of ceftiofur at 1× MIC was suppressed when combined with florfenicol at 1× MIC. As the concentration of florfenicol was lowered to 0.125× MIC, cidality of ceftiofur at 1× MIC was restored at 6 and 24 hours.
Drug-Exposure Assay

This single isolate was further tested in an in vitro drug-exposure assay to represent a treatment setting in which an animal is given one antibiotic followed by another antibiotic rather than given both agents concurrently. Cells at approximately $1 \times 10^7$ CFU/ml were exposed to ceftiofur at 1x and 10x MIC for 24 hours before being exposed to florfenicol for 6 days. Because ceftiofur is a cidal drug, this higher cell concentration was chosen to ensure recovery of cells after 24 hours of pre-exposure to ceftiofur. Cells exposed to ceftiofur at 1x MIC for 24 hours, in which approximately $1 \times 10^4$ CFU/ml were recovered and then exposed to florfenicol, showed inhibition at 1x and higher florfenicol concentrations through day 6 and did not show resistance (Figure 2). Cells exposed to ceftiofur at 10x MIC for 24 hours, in which approximately $1 \times 10^3$ CFU/ml were recovered and then exposed to florfenicol, showed inhibition at all florfenicol concentrations through day 6 and did not show resistance (Figure 3). Thus, exposure of cells first to ceftiofur for 24 hours and then to florfenicol for 6 days did not show evidence of any antagonistic effect. Cells exposed to florfenicol for 24 hours followed by ceftiofur for 6 days showed similar growth inhibition at all concentrations of ceftiofur (data not shown).

DISCUSSION

Tulathromycin is a relatively new antimicrobial agent belonging to the macrolide class and is primarily active against gram-negative pathogens associated with BRD. Ceftiofur is a broad-spectrum third-generation cephalosporin that is also active against gram-negative pathogens associated with BRD. Studies have found instances of improved efficacies of certain antibiotics when they are combined with antibiotics of other classes and used for the treatment of specific diseases. None of those studies involved BRD. An extended literature search has found that to date there are no journal articles related to studies of the activities of tulathromycin or ceftiofur in combination with each other or with other drugs. Thus, it is important to know if the drug remaining from the previous dosage regimen will have a positive synergistic effect, a negative antagonistic effect, or no effect on the medication subsequently administered. These studies with tulathromycin and ceftiofur in combination with each other and with other antimicrobial agents were performed to determine whether synergism, antagonism, or indifference would be the primary response when the combinations were tested against isolates of bovine \textit{P. multocida} and \textit{M. haemolytica}. Combinations that showed an effect other than indifference were further evaluated by time–kill and sequential drug-exposure studies.

The results from this study predominantly showed indifference when tulathromycin or ceftiofur was combined with each other or any of seven other antimicrobial agents and tested against isolates of bovine \textit{P. multocida} and \textit{M. haemolytica}. In the evaluation of the activities of 458 organism–drug combinations, 449 combinations (98%) were indifferent. Of the four determinations of synergism (0.9%), all resulted from synergy controls using amoxicillin + clavulanic acid. Of the five determinations of antagonism (1.1%), four resulted from antagonism controls using sparfloxacin + chloramphenicol and one from ceftiofur + florfenicol. For the isolate exhibiting antagonism in the ceftiofur + florfenicol combination, the MIC of ceftiofur increased by three twofold dilutions (0.002 µg/ml to 0.016 µg/ml in the presence of 1x MIC florfenicol) and the florfenicol MIC (4 µg/ml) remained unchanged in any concentration of ceftiofur. A time–kill study showed the antagonistic effect of florfenicol, which was measured to be bacteriostatic in this study, on
however, they did conclude that all combinations that showed synergism based on FIC also showed synergism by time–kill assays. In this study, the one isolated result of antagonism was reproduced in the checkerboard format and also determined in time–kill studies at 24 hours. Problems have been associated with the checkerboard microdilution method itself, such as variability in the inoculum as a potential source of error as well as the selection of inappropriate antibiotic concentrations.

The antimicrobial drugs chosen to be tested in combination with ceftiofur and tulathromycin in this study are commonly known to be used for the treatment of BRD-infected cattle in the clinical setting.

Robotic instruments used in the experiments described here and elsewhere alleviated the laborious and time-consuming constraints associated with checkerboard assays. In our experiments, the results for each synergistic or antagonistic combination were successfully reproduced upon retesting, whereas combinations that showed indifference were not further evaluated. Assay variability appeared minimal as determined by amoxicillin–clavulanic acid synergy and sparfloxacin–chloramphenicol antagonism control combinations (Table 5).

There is no gold standard for testing antimicrobial drug combinations. Numerous definitions have been assigned for synergism, antagonism, and indifference. Cappelletty and Rybak defined synergy as having an FIC index ≤0.25, whereas antagonism has been defined as an FIC index >1, ≥1, ≥2, or >4. From these different values, a result may be defined as indifferent or antagonistic depending on the

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**TABLE 4. Results of Checkerboard Testing of the Activities of Other Drug Combinations against Organisms Used in This Study***

<table>
<thead>
<tr>
<th>Drug Combination</th>
<th>Average Σ FIC Index Range</th>
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<tbody>
<tr>
<td></td>
<td>P. multocida (n = 10)</td>
</tr>
<tr>
<td>Tilmicosin–Enrofloxacin</td>
<td>1.04–1.47</td>
</tr>
<tr>
<td>Tilmicosin–Florfenicol</td>
<td>0.58–0.94</td>
</tr>
<tr>
<td>Florfenicol–Enrofloxacin</td>
<td>1.34–1.78</td>
</tr>
<tr>
<td>Tetracycline–Penicillin G</td>
<td>1.1–1.77</td>
</tr>
</tbody>
</table>

*Results for fractional inhibitory concentration (FIC) indices for 80 drug–isolate combinations tested in this study. FIC indices of ≤0.5 are synergistic, FIC indices of >0.5–4.0 are indifferent, and FIC indices >4.0 are antagonistic.

**TABLE 5. Results of Quality-Control Checkerboard Testing for Synergism and Antagonism Using S. aureus ATCC 29213***

<table>
<thead>
<tr>
<th>Drug Combination</th>
<th>Average Σ FIC Index Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin–Clavulanic acid</td>
<td>0.34–0.38†</td>
</tr>
<tr>
<td>Sparfloxacin–Chloramphenicol</td>
<td>6.47–6.49‡</td>
</tr>
</tbody>
</table>

*Results for fractional inhibitory concentration (FIC) indices for eight drug–isolate combinations tested in this study. FIC indices of ≤0.5 are synergistic, FIC indices of >0.5–4.0 are indifferent, and FIC indices >4.0 are antagonistic.

†Drug combination resulted in synergism for each of four tests.
‡Drug combination resulted in antagonism for each of four tests.

the cidality of ceftiofur (Figure 1). However, a sequential drug-exposure study in which the isolate was exposed to MIC and supra-MIC levels of ceftiofur followed by exposure to florfenicol showed no antagonistic effects (Figures 2 and 3). Because only one of 60 isolates of *P. multocida* displayed an antagonistic reaction with this combination, we feel that the result is a rare occurrence and would not be seen in a clinical setting.

Differences in the results of checkerboard assays for the detection of in vitro interactions between antimicrobial agents have been demonstrated. Mackay et al. tested the same checkerboard on 3 separate days to test reproducibility and found no statistically significant differences;
CONCLUSION

The combination of tulathromycin or ceftiofur with a particular antibiotic used in treating cattle with BRD infections caused by *P. multocida* or *M. haemolytica* predominantly results in indifference and rarely results in antagonism. These results should not be interpreted as supporting the concurrent administration of antimicrobial medication for treatment of BRD. However, these results do indicate that antimicrobials administered sequentially after tulathromycin or ceftiofur should not be adversely affected by concentrations remaining in the animal from the previous regimen. In vivo animal studies are needed to establish clinical correlation with these in vitro results.

REFERENCES


14. Sweeney MT, Zurenko GE. In vitro activities of linezolid combined with other antimicrobial agents against staphylococci, enterococci, pneumococci, and selected gram-negative organisms. *Antimicrob Agents...*


