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Studies on Time-Kill Kinetics of Different Classes of Antibiotics Against Veterinary Pathogenic Bacteria Including *Pasteurella*, *Actinobacillus* and *Escherichia coli*

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A systematic analysis of the bacteriostatic/bactericidal effect of several antibiotics used in veterinary medicine was carried out by time-kill kinetic analysis using *P. haemolytica*, *P. multocida*, *A. pleuropneumoniae*, and *E. coli*. The antibiotics tested were enrofloxacin, danofloxacin, erythromycin, tilmicosin, penicillin G, ceftiofur and tetracycline. Unexpectedly, the antibiotics well characterized as bacteriostatic agents against human pathogens such as tetracycline and macrolides, showed bactericidal activity against *P. haemolytica* and *A. pleuropneumoniae*. In contrast, tetracycline and erythromycin were bacteriostatic and tilmicosin was bactericidal against *P. multocida*. In addition, *P. multocida* was killed by fluoroquinolones at a slower rate than the other bacteria. Spectrum analysis revealed that ceftiofur and tilmicosin were good substrates of the universal efflux pump, AcrA/B, but penicillin and tetracycline were not. The fluoroquinolones were modest substrates for AcrA/B.

Several antibiotics are available for therapeutic treatment of bacterial infections, particularly respiratory disease, in livestock. These include fluoroquinolones, macrolides, β-lactams and tetracyclines, whose mechanisms of action, bactericidal/bacteriostatic activity, resistance mechanisms, *etc.* are well characterized against human pathogens, especially Gram-positive bacteria and *E. coli*[1,2]. Respiratory infection in livestock is caused primarily by unique Gram-negative bacteria including *Pasteurella haemolytica* (bovine shipping fever), *Pasteurella multocida* (bovine shipping fever and swine/poultry respiratory disease) and *Actinobacillus pleuropneumoniae* (swine respiratory disease). It is known that these Gram-negative bacteria are more susceptible to various antibiotics and detergents than the typical enteric Gram-negatives, such as *E. coli* and *Salmonella* species[1]. A 4th international conference focusing on the HAP group of pathogenic bacteria (*Haemophilus-Actinobacillus-Pasteurella*) was held recently in Acapulco, Mexico[7]. In antibiotic therapy, bactericidal potency of antibiotics as well as their kill rate are important factors in evaluating their ability to control disease in conjunction with their pharmacokinetics. These factors are analyzed routinely for novel antibiotics using human pathogens but information on veterinary pathogens is lacking. The purpose of this paper is to analyze bactericidal potency and kill rate against the three livestock respiratory pathogens described above and *E. coli* as a control, using seven antibiotics commonly used in veterinary medicine in the treatment of respiratory disease. In addition, antibacterial spectrum and activity of these antibiotics were determined against eight aerobic animal pathogens and several genetically defined mutants. These results will serve as baseline information for the future evaluation of novel antibiotics in animal health. Surprisingly our data revealed that antibiotics well characterized as bacteriostatic agents, such as tetracycline and the macrolides, showed bactericidal activity against *P. haemolytica* and *A. pleuropnemoniae*. On the other hand, tetracycline and erythromycin were bacteriostatic and tilmicosin was bactericidal against *P. multocida*. Spectrum analysis showed that ceftiofur and tilmicosin (and erythromycin as reported[13]) were good substrates of the universal efflux pump, AcrA/B system, which contributes to the higher *E. coli* MICs, but penicillin and tetracycline were poor substrates. The fluoroquinolones showed moderately increased activity against the mutant strain which indicates that they are

modest substrates for the AcrA/B system. The MIC results of the *S. typhimurium* deep rough mutant compared to those of *E. coli* and *S. choleraesuis* indicate that poor outer membrane penetration significantly contributes to higher MICs of macrolides against enteric bacteria in addition to the AcrA/B universal efflux system.

Materials and Methods

Bacterial Strains

The following bacterial strains were used in the antibacterial spectrum assay: *Bordetella bronchiseptica* ATCC19395, *E. coli* ATCC25922, *E. coli* W4680 (isogenic parent strain of *ΔacrA/B*[13]), *E. coli* WZM120 (*ΔacrA/B* mutant[13]), *P. haemolytica* ATCC14003, *P. multocida* ATCC15743, *Staphylococcus aureus* ATCC29213, *Salmonella choleraesuis* ATCC19430, *Salmonella typhimurium* LT2 SGSC230 (*pyrE*$^+$, *rfaF511*, RD2 LPS deep rough mutant[17]), *A. pleuropneumoniae* ATCC27088, and *Haemophilus somnus* ATCC43625.

The following strains were used for time-kill kinetic analysis: *P. haemolytica* 59B0046 (bovine lung origin), *P. multocida* 59A0067 (turkey origin), *A. pleuropneumoniae* 44A0030 (swine lung origin) and *E. coli* 51A0150 (poultry lung origin). All strains used in this study are available upon request.

Media and Antibiotics

Media used for these studies included Mueller Hinton broth and agar (Difco). For *A. pleuropneumoniae* strains, Mueller Hinton broth supplemented with β-nicotinamide adenine dinucleotide (15 μg/ml, purchased from Sigma) was used.

The following antibiotics were used in this study: enrofloxacin, danofloxacin, erythromycin, tilmicosin, penicillin G, ceftiofur and tetracycline. These antibiotics were commercially purchased from Sigma or supplied from Pfizer's in-house collection.

Antibacterial Assay

Performed by broth microdilution method as previously described[8]. Experiments were carried out in duplicate and independently twice on different days. MICs were reproducible.

Time-kill Kinetics

Time-kill kinetics were determined according to guidelines from NCCLS and ASM[9,10,14]. Detailed experimental methods were described previously[9,15]. Analysis was carried out at the concentration of 4 times and 8 times the MIC for each antibiotic. The lower limit of sensitivity for colony counts was 300 CFU/ml[16].

Results

Antibacterial Spectrum Assay

Results of the antibacterial spectrum assay are presented in Table 1. The two fluoroquinolones, enrofloxacin and danofloxacin showed identical antibacterial spectrum against the animal pathogens tested. Danofloxacin and enrofloxacin showed 4 and 8 times improved potency against AcrA/B deletion *E. coli* mutant strain (WZM120) compared to its isogenic parent strain W4680.

Two commonly used macrolides, erythromycin and tilmicosin showed similar spectrum except that erythromycin was more potent against *H. somnus*. Neither macrolide was effective against *E. coli* or *S. choleraesuis*. *S. typhimurium* LT2 RD2 strain showed significantly increased susceptibility to both macrolides compared to *E. coli* and *S. choleraesuis*. This *Salmonella* strain carries a deep rough mutation, which lacks O-antigen, outer core and part of the inner core of LPS[17]. *P. haemolytica*, *P. multocida* and *A. pleuropneumoniae* showed similar susceptibility against both macrolides to the LT2 deep rough mutant. Both macrolides showed 16~32 fold improvement in potency against *E. coli* WZM120 (*ΔacrA/B*) strain compared to the isogenic parent strain W4680.

Of the two β-lactams, penicillin G and ceftiofur, the latter showed superior activity against all the bacteria tested except *B. bronchiseptica*. A significant improvement in the MIC of ceftiofur was observed against *E. coli* WZM120 (*ΔacrA/B*) compared to the isogenic parent strain W4680 (32 fold).

Tetracycline showed broad antibacterial activity ranging from 0.125 μg/ml to 2.0 μg/ml against all bacteria tested. Overall, the fluoroquinolones were the most potent broad spectrum agents, followed by ceftiofur, penicillin G, tetracycline and the macrolides, which showed modest antibacterial spectrum.

Time-kill Kinetic Study

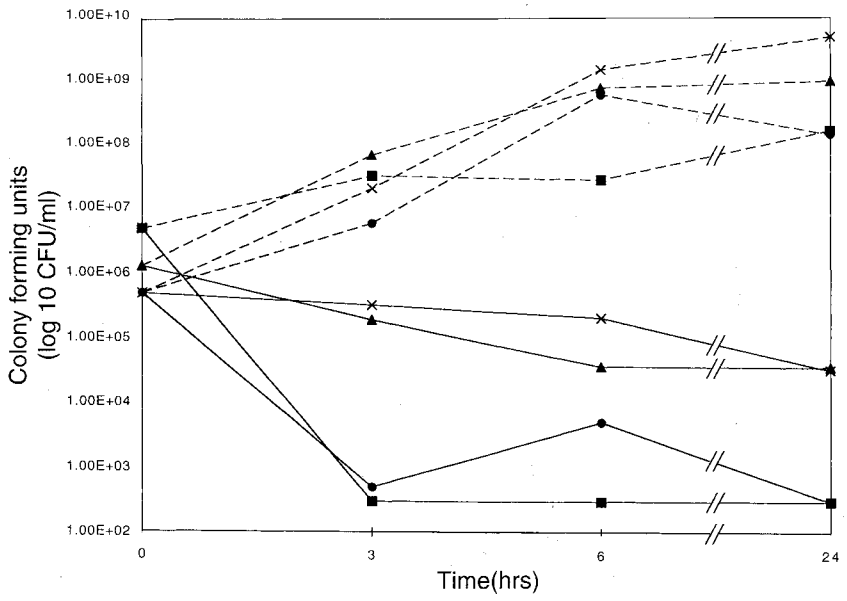
Results of time-kill kinetic studies at 8× MIC of various antibiotics are presented in Fig. 1 to Fig. 7. The time-kill kinetics of 4× MIC were similar or identical to those at the 8× MIC level with slightly less potency (data not shown). As stated in the Materials and Methods, the lower limit was set at 300 CFU/ml and this

Table 1. Antibacterial spectrum.

Bacteria	Antibiotics MIC (μg/ml)						
	Enrofloxacin	Danofloxacin	Erythromycin	Tilmicosin	Penicillin G	Ceftiofur	Tetracycline
B. bronchiseptica ATCC19395	0.5	1.0	16	64	>64	>64	0.25
E. coli ATCC25922	0.008	0.015	64	64	64	0.5	0.5
E. coli W4680	0.03	0.03	32	32	32	0.5	1.0
E. coli WZM120 (*ΔacrA/B*)	0.004	0.008	1.0	2.0	16	0.015	0.25
P. haemolytica ATCC14003	0.015	0.015	4.0	2.0	0.06	0.008	0.25
P. multocida ATCC15743	0.004	0.004	2.0	1.0	0.06	0.002	0.125
S. aureus ATCC29213	0.06	0.125	0.25	0.5	4.0	1.0	0.25
S. choleraesuis ATCC19430	0.008	0.008	64	64	4.0	0.5	0.5
S. typhimurium LT2 SGSC230	0.008	0.03	4.0	4.0	8.0	0.5	2.0
A. pleuropneumoniae ATCC27088	0.008	0.008	2.0	4.0	0.25	0.004	0.125
H. somnus ATCC43625	0.03	0.06	0.5	8.0	0.06	0.001	0.25

Fig. 1. Erythromycin time-kill kinetics.

---■--- *P. haemolytica* 0×, —■— *P. haemolytica* 8×, ---▲--- *P. multocida* 0×, —▲— *P. multocida* 8×, ---●--- *A. pleuropneumoniae* 0×, —●— *A. pleuropneumoniae* 8×, ---×--- *E. coli* 0×, —×— *E. coli* 8×.



threshold was used as the lowest colony count as shown in Fig.1 to Fig. 7.

P. haemolytica colony counts were reduced by at least 3 logs during the initial 3 hours exposure to all the antibiotics tested at 8× MIC including tetracycline and macrolides which have been characterized as bacterio-

Fig. 2. Tilmicosin time-kill kinetics.

---■--- *P. haemolytica* 0×, —■— *P. haemolytica* 8×, ---▲--- *P. multocida* 0×, —▲— *P. multocida* 8×, ---●--- *A. pleuropneumoniae* 0×, —●— *A. pleuropneumoniae* 8×, --- × --- *E. coli* 0×, — × — *E. coli* 8×.

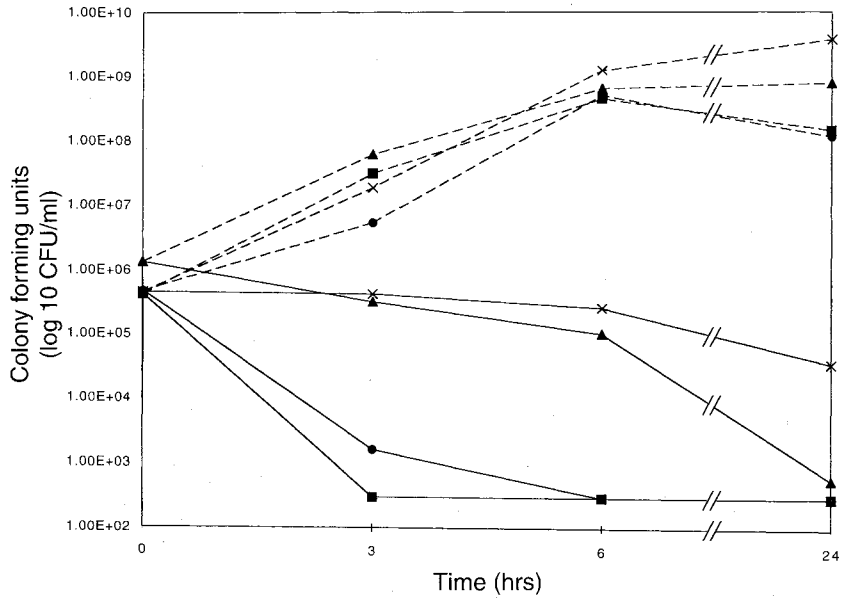
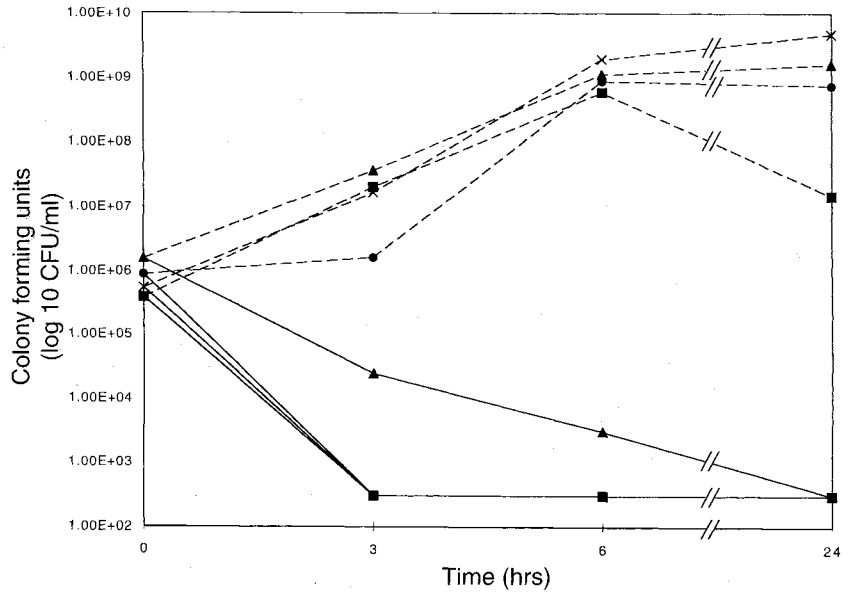


Fig. 3. Enrofloxacin time-kill kinetics.

---■--- *P. haemolytica* 0×, —■— *P. haemolytica* 8×, ---▲--- *P. multocida* 0×, —▲— *P. multocida* 8×, ---●--- *A. pleuropneumoniae* 0×, —●— *A. pleuropneumoniae* 8×, --- × --- *E. coli* 0×, — × — *E. coli* 8×.



static agents (Fig. 1~7). After 24 hours exposure to all the antibiotics tested, no regrowth of *P. haemolytica* was observed. The time-kill kinetic profile at 4×MIC (data not shown) of all antibiotics tested is identical to that of 8×MIC against *P. haemolytica*.

Bactericidal agents, such as the fluoroquinolones and

Fig. 4. Danofloxacin time-kill kinetics.

---■--- *P. haemolytica* 0×, —■— *P. haemolytica* 8×, ---▲--- *P. multocida* 0×, —▲— *P. multocida* 8×, ---●--- *A. pleuropneumoniae* 0×, —●— *A. pleuropneumoniae* 8×, ---×--- *E. coli* 0×, —×— *E. coli* 8×.

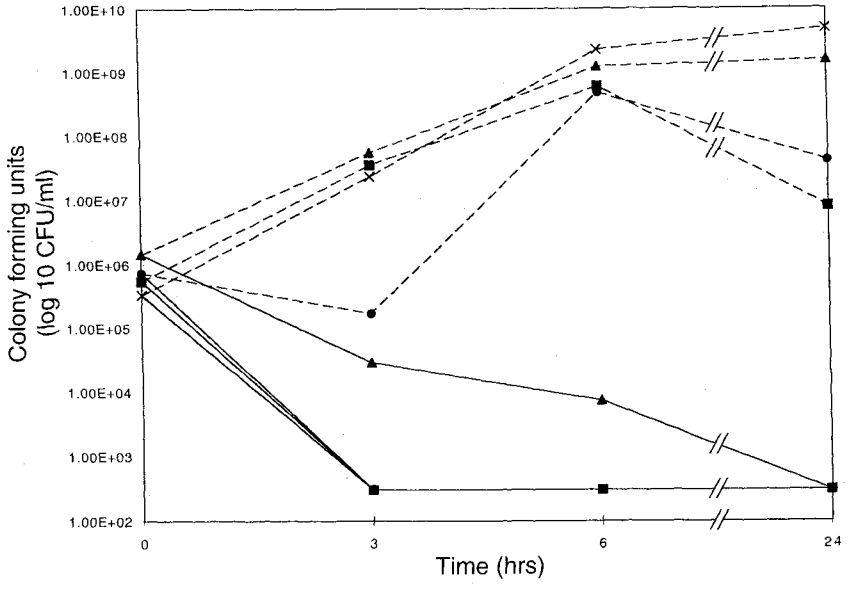
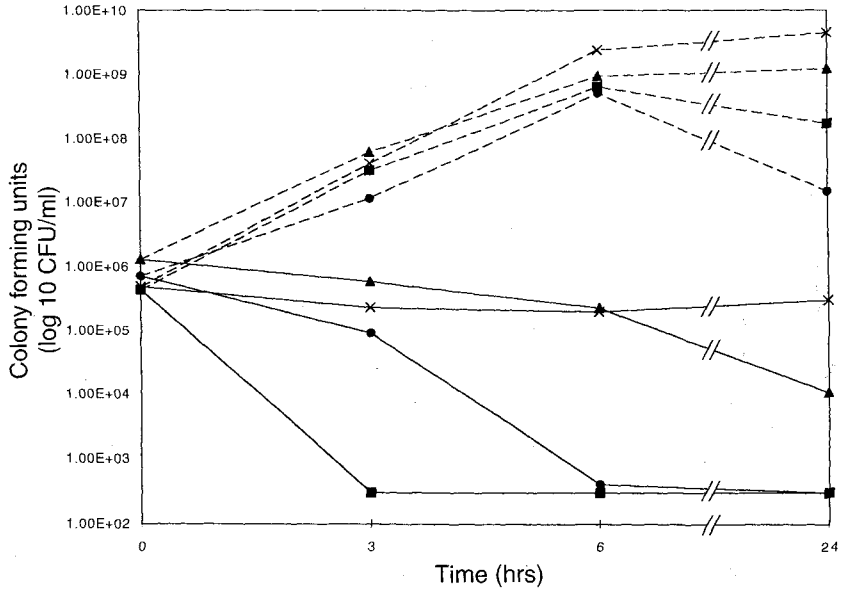


Fig. 5. Tetracycline time-kill kinetics.

---■--- *P. haemolytica* 0×, —■— *P. haemolytica* 8×, ---▲--- *P. multocida* 0×, —▲— *P. multocida* 0×, ---●--- *A. pleuropneumoniae* 0×, —●— *A. pleuropneumoniae* 8×, ---×--- *E. coli* 0×, —×— *E. coli* 8×.



β-lactams effectively killed *A. pleuropneumoniae* during the initial 6 hours exposure at 8×MIC. After 24 hours, no regrowth was observed. The kill rate of the fluoroquinolones was superior to those of the β-lactams after the initial 3 hours exposure. Bacteriostatic agents such as tilmicosin and tetracycline killed *A. pleuro-*

Fig. 6. Penicillin G time-kill kinetics.

---■--- *P. haemolytica* 0×, —■— *P. haemolytica* 8×, ---▲--- *P. multocida* 0×, —▲— *P. multocida* 8×, ---●--- *A. pleuropneumoniae* 0×, —●— *A. pleuropneumoniae* 8×, ---×--- *E. coli* 0×, —×— *E. coli* 8×.

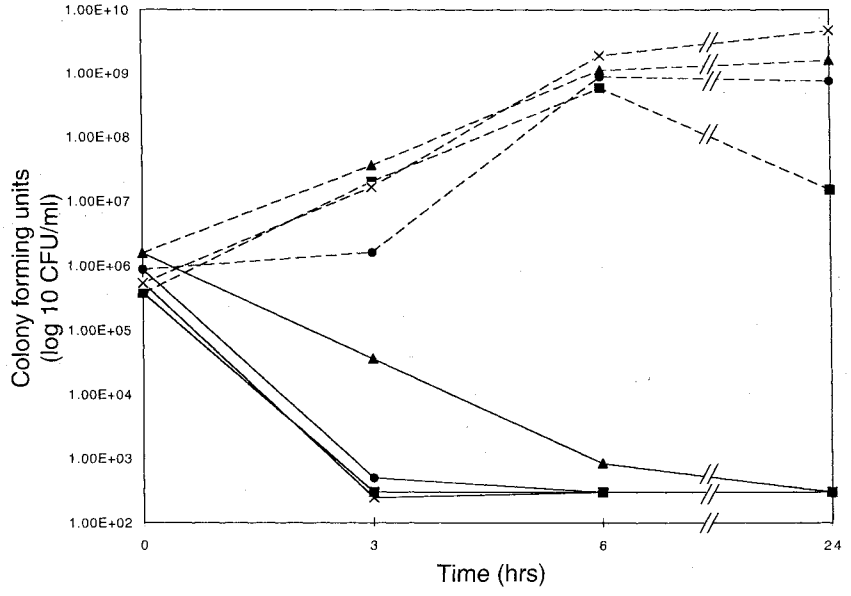
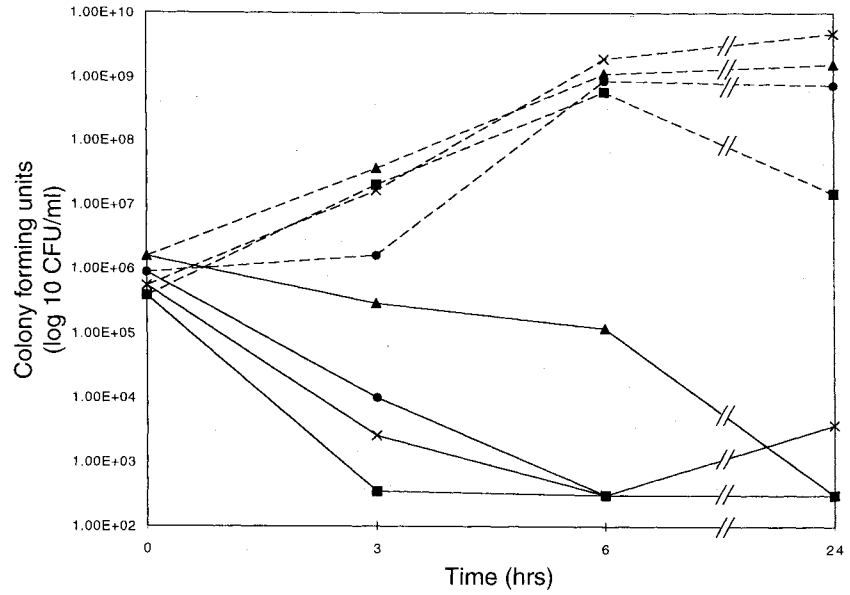


Fig. 7. Ceftiofur time-kill kinetics.

---■--- *P. haemolytica* 0×, —■— *P. haemolytica* 8×, ---▲--- *P. multocida* 0×, —▲— *P. multocida* 8×, ---●--- *A. pleuropneumoniae* 0×, —●— *A. pleuropneumoniae* 8×, ---×--- *E. coli* 0×, —×— *E. coli* 8×.



pneumoniae following exposure for 24 hours. In the initial 3 hours, tetracycline reduced colony counts by less than one $\log_{10}$ but did suppress bacterial growth. Extensive killing was observed in the subsequent 3 hours at 8 × MIC (three $\log_{10}$ reduction of viable colony counts between 3 to 6 hours). In the erythromycin study *A.*

pleuropneumoniae regrew after 6 hours, however, this population was killed in the subsequent 18 hours exposure to drug (Fig.1). We did not observe any regrowth of *A. pleuropneumoniae* with the other antibiotics. Erythromycin was bacteriostatic at 4 × MIC (data not shown). Tilmicosin and tetracycline were bactericidal at 4 × MIC (data not shown) but their initial kill rates were much slower than those of 8 × MIC and a longer exposure time (24 hours) was needed to kill all detectable bacteria.

At 8 × MIC, *E. coli* was effectively killed by the fluoroquinolones (which are bactericidal agents), but not by macrolides and tetracycline (which are bacteriostatic agents). The latter did however suppress growth. Effective killing of *E. coli* by the β-lactams (penicillin G and ceftiofur) was observed in the initial 6 hours, but bacterial regrowth was observed with ceftiofur after 24 hours exposure (Fig. 7).

P. multocida was killed gradually over 24 hours by the fluoroquinolones and β-lactams, however the kill kinetics of penicillin G (Fig. 6) during the initial 6 hours was superior to that of the fluoroquinolones (Figs. 3 and 4) and ceftiofur (Fig. 7). Tilmicosin killed *P. multocida* gradually over 24 hours, however erythromycin and tetracycline did not show effective bactericidal activity against *P. multocida*.

Discussion

Time-kill Kinetic Analysis

Based on their mechanism of action, protein synthesis inhibitors such as macrolides and tetracyclines are considered to be bacteriostatic agents, which suppress bacterial growth. Bacteria exposed to those antibiotics at the MIC or higher can grow again after antibiotic elimination. DNA replication inhibitors (fluoroquinolones) and cell wall inhibitors (β-lactams) are considered to be bactericidal agents, which kill bacteria. Bacterial cells exposed to those antibiotics at the MIC or higher can no longer grow even after the elimination of antibiotics. These analyses have been well documented in *S. aureus* and *E. coli*[1,2]. Our analyses, however, with animal respiratory pathogens revealed interesting differences.

In the present study, not only bactericidal agents but also bacteriostatic agents, such as tetracycline and macrolides, unexpectedly showed bactericidal activity against *P. haemolytica* and *A. pleuropneumoniae*. All the antibiotics effectively killed *P. haemolytica* during the initial 3 hours exposure at both 4 × and 8 × MIC. At present, it is unknown why *P. haemolytica* was so susceptible to killing by various antibiotics during this short term exposure. However, it should be noted that the field isolates of *P. haemolytica* tested contain lysogenic phage. The SOS induction by fluoroquinolones triggers the release of lysogenic phage and forces *P. haemolytica* into a lytic cycle[5]. One hypothesis is that phage induction may occur not only following fluoroquinolone treatment but also following exposure to other antibiotic classes. Further studies will be needed to answer this question.

All antibiotics tested showed bactericidal activity against *A. pleuropneumoniae* at 8 × MIC. In contrast to *P. haemolytica*, at 4 × MIC against *A. pleuropneumoniae* erythromycin was bacteriostatic, and tilmicosin and tetracycline were bactericidal with slower kill rates (data not shown).

The time-kill kinetic profile of *P. multocida* was significantly different from those of *P. haemolytica* and *A. pleuropneumoniae*. Fluoroquinolones were bactericidal against *P. multocida*, however their initial kill rates were significantly slower than observed for *P. haemolytica*, *A. pleuropneumoniae* and *E. coli*. *P. multocida* was gradually killed by fluoroquinolones at both 4 × and 8 × MIC. Tetracycline and erythromycin were bacteriostatic, and tilmicosin was bactericidal at both 4 × and 8 × MIC. These results suggest that there exists a significant biological diversity among *Pasteurella* and *Actinobacillus*, particularly based on drug interaction, in spite of their close taxonomic relationship. It is particularly interesting that significant differences exist in bactericidal profiles between *P. haemolytica* and *P. multocida*, even though they are in the same genus.

Macrolide Penetration Through the Outer Membrane of Gram-negative Bacteria

Macrolides are known to be more effective against Gram-positive than Gram-negative bacteria[2]. Their ineffectiveness against Gram-negative bacteria is due to the outer membrane permeability barrier. The molecular size of macrolides is close to or larger than the upper limit of porin channels and therefore they cannot easily penetrate the outer membrane, reducing their potency against Gram-negatives[4,6]. In our spectrum assay, tilmicosin and erythromycin showed the same results as outlined above. Both macrolides were very effective against *S. aureus* (MIC 0.5 μg/ml and ≤0.25 μg/ml, respectively) but were ineffective against *E. coli* and *S. choleraesuis* (MICs 64 μg/ml). Both macrolides, however, showed improved activity against the deep rough mutant *S. typhimurium* LT2 strain (4.0 μg/ml each). This improvement is due to incomplete LPS, which disrupts

the outer membrane allowing macrolide antibiotics to enter more easily than across the wild type outer membrane. *P. haemolytica*, *P. multocida* and *A. pleuropneumoniae* had similar MICs for both macrolides to *S. typhimurium* LT2 strain. These results suggest that the outer membrane structure of these bacteria may differ from that of the well characterized *E. coli* and *Salmonella* species. CONRAD *et al.* recently reported that the LPS of *P. multocida* P-1581 is homogeneous and predominantly low molecular weight (estimated MW 5,700 daltons), and there was no smooth-form LPS by overloaded SDS-PAGE analysis[3]. This result strongly supports the conclusion of our present study.

Contribution of Universal Efflux Pump, AcrA/B, to Antibiotic Susceptibility

A universal efflux pump was recently identified in *Pseudomonas aeruginosa* (MexA/B)[11] as well as in *E. coli* (AcrA/B)[13]. This universal efflux pump can exclude many classes of antibiotics from cells and is widely recognized as an important factor in the intrinsic antibiotic resistance of *P. aeruginosa* and enteric bacteria. Previously we reported that hygromycin A is also a good substrate for the AcrA/B efflux pump of *E. coli* and this efflux mechanism significantly contributed to the reduced potency of hygromycin A against enteric bacteria[8]. In the present study, we analyzed seven antibiotics for activity against an isogenic pair of *ΔacrA/B* and wild-type *E. coli*. The *ΔacrA/B* mutant lacks the universal efflux pump AcrA/B due to a deletion mutation, which significantly increases the potency of substrate antibiotics compared to its isogenic parent strain[13]. Erythromycin (which is a 14-membered ring macrolide) showed 32 times improved potency against this mutant and this finding was well documented in the original study[13]. Tilmicosin, which is a 16-membered ring macrolide, showed similar increased potency against this mutant. This indicates that AcrA/B can equally recoginize both the 14-membered ring erythromycin and 16-membered ring tilmicosin as substrates in spite of different size and structure of the macrolide ring. This efflux mechanism, in conjunction with outer membrane penetration discussed above, may be the major contributing factors to intrinsic macrolide resistance of enteric bacteria. Ceftiofur also showed a 32 times increase in potency against the deletion mutant but penicillin G did not. It is interesting to note that the structural difference of β-lactams appears to affect substrate specificity of the AcrA/B efflux pump, as ceftiofur, a cephalosporin, is a good substrate but penicillin G is not. Both fluoroquinolones showed a moderate increase in potency (4~8 fold) against the mutant strain, which indicates that neither fluoroquinolone is a good substrate for the AcrA/B system. At the present time, it is not known whether a universal efflux pump such as AcrA/B or MexA/B exists among the HAP bacteria.

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