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Pharmacodynamic interactions of amikacin with selected β-lactams and fluoroquinolones against canine *Escherichia coli* isolates

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ABSTRACT

Knowledge of *in vitro* antimicrobial interactions can serve as a guide for clinical application of combination antimicrobial regimens. The aim of the present study was to determine the pharmacodynamic interactions of amikacin with either amoxicillin/clavulanic acid, ceftazidime, enrofloxacin or marbofloxacin against clinical canine *Escherichia coli* isolates. Bactericidal activity of individual antimicrobials was assessed by use of static kill curves. Interactions between amikacin and each of the β-lactams or fluoroquinolones were subsequently analyzed by employing the fractional maximal effect method. Amikacin, compared with all other agents, displayed the most rapid and extensive bacterial killing, the lowest level (with respect to MIC) at which half the maximal effect was observed and the most linear concentration-effect relationship. The combinations of amikacin with amoxicillin/clavulanic acid or ceftazidime were completely synergistic in four and three out of the five investigated isolates, respectively, with additivity being sporadically observed. On the other hand, the combinations of amikacin with enrofloxacin or marbofloxacin yielded a mosaic of interaction types with no discernible pattern or differentiation between fluoroquinolone-susceptible and resistant isolates; synergy was only infrequently observed, mainly at increased fluoroquinolone concentrations. In conclusion, the combinations of amikacin with the two β-lactams were found to be more promising, in terms of synergy achievement, compared with the respective combinations with the two fluoroquinolones.

1. Introduction

Combination antimicrobial therapy is an acknowledged strategy for the treatment of severe bacterial infections in human and veterinary medicine. Compared to monotherapy, the concurrent administration of two or more antimicrobials, typically acting *via* different molecular mechanisms, broadens the spectrum of activity and offers the potential of achieving a synergistic bacterial killing (Chan et al., 2006; Vidaillac et al., 2009; Patil et al., 2015). Furthermore, antimicrobial combinations can be useful in the enhancement of biofilm disruption, as well as in the suppression of antimicrobial resistance emergence, thus helping preserve last resort agents (Safarika et al., 2015; Wu et al., 2015; Ahmad et al., 2016). In small animal medicine, antimicrobial combinations find use in the treatment of bacteraemia, infective endocarditis, certain types of central nervous system, skin, urinary, respiratory, intestinal, intra-abdominal and other infections (Weese et al., 2011; Greene, 2012; Song et al., 2015; Lappin et al., 2017).

Escherichia coli is a gram-negative, facultative aerobic, nonspore-forming rod, member of the commensal intestinal microflora of dogs and other mammals, that is also often implicated in the pathogenesis of intestinal and extra-intestinal infections, including those of soft tissues and of urinary, respiratory and reproductive tracts (Marks et al., 2011; Weese et al., 2011; Vingopoulou et al., 2014; Rzewuska et al., 2015; Morrissey et al., 2016; Lappin et al., 2017), some of which can require combination antimicrobial therapy (Greene, 2012).

Beta lactams and fluoroquinolones are among the most used antimicrobials for the treatment of infections in dogs caused by strains of *E. coli* (Weese et al., 2011; Greene, 2012; Lappin et al., 2017). Use of aminoglycosides, despite their frequently excellent potency, has been historically associated with irregular occurrence of serious adverse effects, such as nephrotoxicity, cochleovestibular toxicity or neuromuscular blockade and thus, has been reserved for cases in which the implicated bacteria were resistant to safer alternatives. However, since the discovery that extended-interval aminoglycoside dosing significantly

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reduces the risk of toxicity, these agents have cautiously regained their place in the chemotherapeutic arsenal against many aerobic infections, typically those caused by gram-negative bacteria or staphylococci (Papich and Riviere, 2009; Papich, 2012). Amikacin is one of the most potent aminoglycosides used in human and veterinary medicine, displaying a broad spectrum of activity and a relative resilience against most aminoglycoside-inactivating enzymes (Sarno et al., 2003; Veyssier and Bryskier, 2005; KuKanich and Coetzee, 2008).

The present study aimed to explore the potential of *in vitro* synergy between amikacin and an aminopenicillin/β-lactamase inhibitor combination (amoxicillin/clavulanic acid), a third-generation cephalosporin (ceftazidime) and two second-generation fluoroquinolones (enrofloxacin and marbofloxacin), against canine *E. coli* isolates, by using the fractional maximal effect (FME) method. In this method, tested concentrations for each drug in combination are meticulously calculated on the basis of a non-linear concentration-effect relationship, interactions are measured at different drug concentration ratios and their qualitative characterization (*i.e.* synergistic, additive, indifferent or antagonistic) is based on the difference between the observed combined antimicrobial effect and the expected, theoretical effect, if the two drugs acted additively (Li et al., 1993; Desbiolles et al., 2001; Nguyen et al., 2009; Buyck et al., 2015).

2. Materials and methods

2.1. Bacterial isolates

Five (n = 5) *E. coli* isolates, recovered from dogs suffering from naturally occurring infections, were used in this study. After culture and identification by conventional biochemical tests and the automated VITEK®2 system (bioMerieux, Marcy-l'Étoile, France), all isolates were subcultured in 15% glycerol-supplemented Brain Heart Infusion Broth and stored at − 85 °C, pending further analysis.

2.2. Antimicrobial susceptibility testing

Amikacin and ceftazidime reference standards were obtained from European Pharmacopoeia (Strasbourg, France), whereas amoxicillin, clavulanic acid (as potassium clavulanate), enrofloxacin and marbofloxacin were from Sigma-Aldrich Co. (St. Louis, MO, USA). Minimal Inhibitory Concentrations (MICs) of all drugs against the investigated isolates were determined using the macrodilution (tube) broth method (CLSI, 2013), in cation-adjusted Mueller-Hinton broth (MHB). Amoxicillin and clavulanic acid were combined at the CLSI-recommended 2:1 ratio. Susceptibility testing was performed in triplicate, using *E. coli* ATCC 25922 as a quality control strain. Interpretation of the results was performed according to established interpretative criteria (CLSI, 2013, 2015, 2017).

Phenotypic assessment of β-lactamase production and genotypic detection of: i) genes encoding for β-lactamases, ii) mutations in the quinolone resistance-determining region (QRDR) in the *gyrA* gene, and iii) plasmid-mediated quinolone resistance (PMQR) genes, namely *aac (6′)-Ib-cr* (also conferring resistance to aminoglycosides), *qnrA*, *qnrB*, *qnrS*, *qepA*, *oqxA* and *oqxB*, were performed as previously described (Vingopoulou et al., 2014). Efflux pump activity was also investigated by measuring marbofloxacin and enrofloxacin MICs in the presence of 80 μg/mL of the efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PAβN). A fourfold (or greater) reduction in the respective MIC values indicated a significant efflux pump overexpression (Vingopoulou et al., 2014).

2.3. Individual kill curves

After determination of MICs, a series of antimicrobial solutions in MHB containing doubling concentrations were prepared for each antimicrobial-isolate combination, before bacterial inoculation at 5×10^5 CFU/mL (final drug levels ranged from ¼ to 16 × MIC). Growth and sterility control tubes were also used. All tubes were incubated at 35 °C and viable counts were determined at 0, 2, 5, 8 and 24 h on Tryptic Soy Agar plates. The limit of quantification was set at 50 CFU/mL (*ca.* 1.70 logCFU/mL) and experimentations were also performed in triplicate.

The Area Between the Bactericidal and the Control curve from time zero to 24 h ($ABBC_{24}$), calculated by the trapezoidal method using log-transformed bacterial density data (logCFU/mL) was employed to quantify the antimicrobial effect. The sigmoidal E_{max} model was used to perform a non-linear regression of the antimicrobial effect ($ABBC_{24}$ value) on drug concentration (C): $E_{(C)} = E_{max} \times C^n / (EC_{50}{}^n + C^n)$, where $E_{(C)}$ is the antimicrobial effect at concentration C, E_{max} is the maximal effect, EC_{50} is the concentration that yields 50% of E_{max} and n is the Hill coefficient, describing the steepness of the middle portion of the concentration-effect sigmoidal curve. Each EC_{50} was further normalized by the respective MIC into a hybrid parameter, namely EC_{50}/MIC, as previously shown (Delis et al., 2010). This new parameter mirrors the fraction of MIC at which half the maximal effect is obtained and is used to quantify and compare the sub-MIC activities of antimicrobials.

2.4. Combination kill curves – fractional maximal effect (FME) method

Following analysis of the individual kill curves, concentrations that yielded 10, 30, 50, 70 and 90% of E_{max}, named 0.1FME, 0.3FME, 0.5FME, 0.7FME and 0.9FME, respectively, were calculated by reverse implementation of the sigmoidal E_{max} equation. Subsequently, kill curves containing amikacin and one of the other antimicrobials ("drug B": amoxicillin/clavulanic acid, ceftazidime, enrofloxacin or marbofloxacin) were constructed for each *E. coli* isolate, in triplicate. The two drugs were examined at ordered levels, so that the sum of their FMEs always equaled 1.0 (*i.e.* $0.1FME_{amikacin} + 0.9FME_{drug\ B}$, $0.3FME_{amikacin} + 0.7FME_{drug\ B}$, $0.5FME_{amikacin} + 0.5FME_{drug\ B}$, $0.7FME_{amikacin} + 0.3FME_{drug\ B}$ and $0.9FME_{amikacin} + 0.1FME_{drug\ B}$) and kill curves were analyzed as previously, $ABBC_{24}$ providing the magnitude of the antimicrobial effect. This "observed" effect (E_{obs}) was then compared with the "expected" effect of the combination, supposing the activity of the two drugs were independent and strictly additive (E_{add}), and with the effect of the most active of the two drugs (E_{best}, as derived from the individual kill curves), before synergy, additivity, indifference or antagonism were explored (Fig. 1).

2.5. Statistical analysis

The Friedman test and *post hoc* analysis based on Wilcoxon signed-rank tests with application of Bonferroni adjustment were used for multiple comparisons of pharmacodynamic parameters across antimicrobials. Wilcoxon one sample signed-rank tests were used to compare E_{obs} with E_{add} and E_{best} in the combination kill curves. Descriptive and inferential statistics were obtained by the combined use of Microsoft Excel 2013 (Microsoft Corp., Redmond, WA, USA) and IBM® SPSS® Statistics 24.0 software (IBM Corp., Armonk, NY, USA). Level of significance was set at 5%, two-tailed.

3. Results

A relative variability in the susceptibilities of the canine *E. coli* isolates (n = 5) to the investigated antimicrobials was observed (Table 1). All isolates were susceptible to amikacin and ceftazidime, whereas resistance was observed in some isolates against amoxicillin/clavulanic acid (D3Ec and D4Ec), enrofloxacin and marbofloxacin (D4Ec and D5Ec for both fluoroquinolones). Implicated resistance mechanisms are also presented in Table 1.

Amikacin displayed the more rapid and extensive bacterial killing, as mirrored by the significantly higher E_{max} value (183.49 ± 7.79