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***In vitro* antimicrobial activity of nitrofurantoin against *Escherichia coli* and *Staphylococcus pseudintermedius* isolated from dogs and cats**

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Running title: Nitrofurantoin susceptibility in small animal urinary tract pathogens

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Abstract

Minimum inhibitory concentrations (MIC) of nitrofurantoin were determined by agar dilution in 269 canine and feline isolates of *Escherichia coli* and *Staphylococcus pseudintermedius*, two of the most common bacterial species associated with urinary tract infection (UTI) in small animals. The MIC₉₀ for *E. coli* and *S. pseudintermedius* were 32 and 16 µg/ml, respectively. All isolates, including multidrug-resistant strains of known genetic background, displayed MICs below the drug concentrations reported in canine urine following oral administration of nitrofurantoin. Preliminary data on mutant prevention concentration (MPC) and many years of nitrofurantoin usage in human medicine suggest that emergence of resistant mutants during treatment is not a critical issue for this drug. The study provides species-specific data on nitrofurantoin MIC distribution that can be used for setting dog- and cat-specific breakpoints. Although nitrofurantoin is not an appropriate first-line agent for empirical treatment of canine UTI due to toxicity and poor pharmacokinetic properties, it may be indicated for treatment of UTI caused by multidrug-resistant bacteria, which are otherwise difficult to treat using conventional veterinary antimicrobial agents.

Introduction

Urinary tract infection (UTI) is one of the most commonly diagnosed infectious diseases in small animal practice. The bacterial pathogens most frequently associated with UTI in dogs are *Escherichia coli*, staphylococci, *Proteus*, *Klebsiella*, enterococci and streptococci (Ling et al., 2001). *E. coli* has been reported to account for 33 – 51 % of all cases of canine UTI, while staphylococci, mainly *Staphylococcus pseudintermedius*, are isolated in 12 – 20 % of all cases (Ball et al., 2008; Ling et al., 2001). Beta-lactam antibiotics such as amoxicillin (alone or in combination with clavulanic acid) and cephalosporins are generally regarded as first line agents. However, multidrug-resistant bacteria recently emerged in small animals, namely methicillin-resistant *S. pseudintermedius* (MRSP) (El Zubeir et al., 2007; Kadlec et al. 2010; Loeffler et al., 2007; Perreten et al., 2010; Ruscher et al., 2009) and extended-spectrum beta-lactamase (ESBL)-producing *E. coli* (Carattoli et al., 2005; Ogeer-Gyles et al., 2006; Sanchez et al., 2002; Shaheen et al., 2010; Warren et al., 2001), are per definition resistant to beta-lactams and often additionally resistant to alternative drugs for treatment of UTI.

Nitrofurantoin is a synthetic nitrofuran that reaches very high concentrations in urine. This drug has been used for the treatment of uncomplicated UTIs in humans for more than fifty years (Hooper, 2010). In small animal practice, the use of nitrofurantoin has been gradually abandoned due to higher toxicity and lower pharmacokinetic performance in comparison to beta-lactams, fluoroquinolones and trimethoprim-sulfonamides. The objective of this study was to evaluate the *in vitro* antimicrobial activity of nitrofurantoin against *E. coli* and *S. pseudintermedius* isolates from dogs and cats, including strains previously characterized genetically as ESBL-producing *E. coli* and MRSP. The results were discussed on the basis of the current knowledge of the pharmacokinetic properties of nitrofurantoin in dogs.

Materials and methods

Bacterial strains

A total of 106 *E. coli*, including 6 ESBL- and 9 CMY-producing isolates, and 163 *S. pseudintermedius*, including 13 MRSP, were included in the study. All isolates were collected in the time period 1998 to 2010, with the majority of the strains (88%) isolated from 2005 to 2010. The isolates originated from clinical specimens obtained from infected dogs (n=240) or cats (n=29), except 3 MRSP, 5 ESBL- and 9 CMY-producing *E. coli* which had been isolated from healthy dogs or during necropsy. Thirteen *S. pseudintermedius* isolates (including 2 MRSP) and 39 *E. coli* isolates (including 1 ESBL-producing strain) were isolated from urine. Nine of the 13 MRSP isolates had previously been typed (Perreten et al., 2010) and belonged to seven distinct clonal types (ST54, ST58, ST68, ST69, ST71, ST95 and ST113).

MIC testing

MIC values were determined by agar dilution in accordance with the Clinical and Laboratory Standards Institute (CLSI 2008). The range of concentrations tested was between 2 and 128 µg/ml. The stock solution was prepared dissolving 250 mg nitrofurantoin (Sigma-Aldrich, Germany) in 5 ml of dimethylformamide (DMF) to yield a final concentration of 50 mg/ml. Mueller Hinton agar plates (Oxoid, UK) containing nitrofurantoin were stored at 4°C in dark plastic bags, and used within 9 days after preparation. The following reference strains were used for quality control: *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

MPC testing

Mutant prevention concentrations (MPCs) of nitrofurantoin were measured in two isolates of each species. MPC testing was performed according to previous studies (Blondeau, 2009; Hansen and Blondeau, 2005). Briefly, for each strain the bacterial content of three 5% blood agar plates displaying confluent growth was transferred to 100 ml of Mueller-Hinton broth (Oxoid, UK) using a sterile swab. Following overnight incubation, 50 ml of overnight culture were centrifuged at 5000 x *g* for 30 min, and the pellet was resuspended in 2 ml of broth to reach a cellular density of at least 10¹⁰ CFU/ml. Mueller-Hinton plates containing nitrofurantoin were inoculated using 100µl of this bacterial suspension and incubated at 37°C. The lowest drug concentration preventing growth was recorded as the MPC. Bacterial colonies and smears on plates containing nitrofurantoin concentrations higher than the MIC of the wild-type strain were subcultured onto agar plates containing the same drug concentration and subjected to MIC determination.

Results and discussion

The MICs of nitrofurantoin ranged between 8 and 32 µg/ml in *E. coli* and between 4 and 16 µg/ml in *S. pseudintermedius* (Table 1). The MIC₉₀ was 32 µg/ml for *E. coli* and 16 µg/ml for *S. pseudintermedius*. The MIC₅₀ values for the two species were 16 and 8 µg/ml, respectively. As breakpoints for nitrofurantoin resistance have not yet been established for animal *E. coli* and *S. pseudintermedius*, only human breakpoints (S≤32 µg/ml, R≥128 µg/ml) (CLSI 2010) are available as references for interpretation of the MIC results. According to such breakpoints none of the isolates tested was resistant to nitrofurantoin. All isolates, including the multidrug-resistant strains of known genetic background, displayed MICs lower than the concentration of nitrofurantoin achieved in urine following oral administration to dogs. Conklin et al. (1969)

reported that four hours after oral administration (4-5 mg/kg body weight) nitrofurantoin concentrations in urine are above 60 µg/ml for the macrocrystalline formulation and above 100 µg/ml for the microcrystalline formulation. For both formulations, a slight increase in drug concentration was observed following repeated administration (Conklin et al., 1969). No data are currently available about the urinary excretion profile within four hours after administration. In an absorption and disposition study of nitrofurantoin in dogs performed by Niazi et al. (1983), oral administration of a 100 mg tablet resulted in a peak plasma level ranging from 1.5 to 4.0 µg/ml, and an elimination half-life of 32 to 87 minutes, which means that the drug is rapidly distributed and eliminated from plasma. If one assumes that the urinary excretion rate changes according to the plasma levels, these data indicate that urinary concentrations are high shortly after administration but rapidly decrease in parallel with the decrease of the plasma levels. More detailed information on the excretion profiles and on the antibacterial action of the drug (i.e. concentration- vs. time-dependent) are needed in order to optimize dosage regimens in small animals.

There are several disadvantages associated with the clinical use of nitrofurantoin in small animals. The drug is a known gastrointestinal irritant and may cause nausea and emesis as side-effects in both dogs and cats. Due to the short plasma half-life (Niazi et al., 1983), nitrofurantoin requires frequent administration (i.e. three to four times daily). Furthermore, it cannot be used for treatment of UTI associated with *Proteus* and *Pseudomonas* as these organisms are generally resistant to this drug (Rohrich et al., 1983). Although better alternatives than nitrofurantoin exist as first-line agents for treatment of canine UTI, the results of this study combined with the current knowledge of the pharmacokinetics in dogs suggest that this drug may be indicated for treatment of UTI caused by ESBL-producing *E. coli* and MRSP. The recent spread of these

multidrug-resistant bacteria in dogs and cats represents a serious therapeutic challenge for small animal veterinary practitioners since such bacteria are often resistant to all the conventional veterinary antimicrobial products available for treatment of UTI in small animal medicine (Carattoli et al., 2005; El Zubeir et al., 2007; Loeffler et al., 2007; Ogeer-Gyles et al., 2006; Perreten et al., 2010; Ruscher et al., 2009; Sanchez et al., 2002; Shaheen et al., 2010; Warren et al., 2001). As a consequence, some veterinarians have started to use critically-important antimicrobial drugs such as the carbapenems. Usage of these last-resort antimicrobials is an unwanted practice in veterinary medicine due to public health concerns (Boothe, 2006). This new trend in antimicrobial usage emphasizes the need for alternative treatment options and nitrofurantoin represents an ideal alternative to carbapenems in the treatment of canine or feline UTI associated with multidrug-resistant bacteria. The clinical efficacy of nitrofurantoin has been recently documented in a case of lower UTI caused by MRSP and *Enterococcus faecalis* in a cat with pre-existing and recurrent urethral obstruction (Pomba et al., 2010).

The MPC values were 128 µg/ml for both *E. coli* strains and 64 µg/ml for both *S. pseudintermedius* strains. The MIC was recorded for all presumptive mutants isolated from MPC plates and only one *E. coli* strain had a higher MIC (128 µg/ml) compared with the respective wild type strain (16 µg/ml). The clinical relevance of the MPC data is uncertain since the apparent bacterial growth on MPC plates containing drug concentrations above MIC was not due to the emergence of a subpopulation of resistant mutants but appeared to be an artefact determined by the high density of the inoculum. Resistance to nitrofurantoin in *E. coli* is known to occur as a result of step-wise mutations (Breeze and Obaseiki-Ebor, 1983; Sandegren et al., 2008). Assuming that the drug concentrations achievable in canine urine during therapy

approximate 60 to 100 µg/ml depending on the specific formulation (Conklin et al., 1969), nitrofurantoin would not be effective against mutants with high MICs such as that obtained from the MPC test in this study (MIC=128 µg/ml). However, it has been shown that the growth rate of first-step mutants is impaired at concentrations well below 100 µg/ml, and that second-step mutants have an impaired growth rate at 100 µg/ml (Sandegren et al., 2008). Based on these data, emergence of resistant mutants during therapy does not seem to be a critical issue in the treatment of UTI with nitrofurantoin. It should be noted that after many years of use in human medicine, nitrofurantoin resistance is still uncommon in *E. coli* isolated from human UTI (Garau, 2008; Kashanian et al., 2008; Schito et al., 2009; Zhanel et al., 2005).

In conclusion, the use of nitrofurantoin for treatment of UTI in dogs is a matter of weighing the risks of toxicity against the benefits of clinical efficacy. Due to its high toxicity and poor pharmacokinetic performance, nitrofurantoin is not a suitable first-line agent for empirical treatment of UTI in small animals. However, the results of this study indicate that the use of nitrofurantoin may be indicated for treatment of UTI caused by MDR *E. coli* or MRSP, which are otherwise difficult to treat using conventional veterinary antimicrobial products. Standard dosage regimens seem sufficient to kill susceptible strains and probably also prevent selection of resistant mutants during therapy. The MIC distribution data generated by this study may be used for setting species-specific epidemiological cut-off values and clinical breakpoints for nitrofurantoin susceptibility testing in UTI isolates from dogs and cats. Setting of UTI-specific clinical breakpoints will require additional data on the PK/PD properties of nitrofurantoin in these animal species and clinical outcome data from perspective clinical studies.

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- 251

251 **Table 1.** Nitrofurantoin minimum inhibitory concentrations (MIC) in *Escherichia coli* and
 252 *Staphylococcus pseudintermedius* isolates from small animals.

Species	No. isolates	MIC (µg/ml)						
		2	4	8	16	32	64	128
<i>E. coli</i>	106	0 (0%)	0 (0%)	4 (3,77%)	90 (84,91%)	12 (11,32%)	0 (0%)	0 (0%)
<i>S. pseudintermedius</i>	163	0 (0%)	1 (0,61%)	122 (74,85%)	40 (24,54 %)	0 (0%)	0 (0%)	0 (0%)