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1	In vitro antimicrobial activity of nitrofurantoin against Escherichia coli and Staphylococcus
2	pseudintermedius isolated from dogs and cats
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15	

15 Abstract

Minimum inhibitory concentrations (MIC) of nitrofurantoin were determined by agar dilution in 16 269 canine and feline isolates of *Escherichia coli* and *Staphylococcus pseudintermedius*, two of 17 18 the most common bacterial species associated with urinary tract infection (UTI) in small animals. 19 The MIC90 for *E. coli* and *S. pseudintermedius* were 32 and 16 µg/ml, respectively. All isolates, 20 including multidrug-resistant strains of known genetic background, displayed MICs below the 21 drug concentrations reported in canine urine following oral administration of nitrofurantoin. 22 Preliminary data on mutant prevention concentration (MPC) and many years of nitrofurantoin 23 usage in human medicine suggest that emergence of resistant mutants during treatment is not a 24 critical issue for this drug. The study provides species-specific data on nitrofurantoin MIC 25 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 26 toxicity and poor pharmacokinetic properties, it may be indicated for treatment of UTI caused by 27 multidrug-resistant bacteria, which are otherwise difficult to treat using conventional veterinary 28 29 antimicrobial agents.

30 Introduction

31 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 32 33 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 34 35 staphylococci, mainly *Staphylococcus pseudintermedius*, are isolated in 12 - 20 % of all cases 36 (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. 37 38 However, multidrug-resistant bacteria recently emerged in small animals, namely methicillinresistant S. pseudintermedius (MRSP) (El Zubeir et al., 2007; Kadlec et al. 2010; Loeffler et al., 39 40 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 41 et al., 2010; Warren et al., 2001), are per definition resistant to beta-lactams and often 42 additionally resistant to alternative drugs for treatment of UTI. 43 Nitrofurantoin is a synthetic nitrofuran that reaches very high concentrations in urine. This drug 44 45 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 46 47 due to higher toxicity and lower pharmacokinetic performance in comparison to beta-lactams, 48 fluoroquinolones and trimethoprim-sulfonamides. The objective of this study was to evaluate the 49 in vitro antimicrobial activity of nitrofurantoin against E. coli and S. pseudintermedius isolates 50 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 51 52 pharmacokinetic properties of nitrofurantoin in dogs.

53 Materials and methods

54 Bacterial strains

55 A total of 106 *E. coli*, including 6 ESBL- and 9 CMY-producing isolates, and 163 *S*.

56 *pseudintermedius*, including 13 MRSP, were included in the study. All isolates were collected in

57 the time period 1998 to 2010, with the majority of the strains (88%) isolated from 2005 to 2010.

58 The isolates originated from clinical specimens obtained from infected dogs (n=240) or cats

59 (n=29), except 3 MRSP, 5 ESBL- and 9 CMY-producing *E. coli* which had been isolated from

60 healthy dogs or during necropsy. Thirteen *S. pseudintermedius* isolates (including 2 MRSP) and

61 39 *E. coli* isolates (including 1 ESBL-producing strain) were isolated from urine. Nine of the 13

62 MRSP isolates had previously been typed (Perreten et al., 2010) and belonged to seven distinct

63 clonal types (ST54, ST58, ST68, ST69, ST71, ST95 and ST113).

64 MIC testing

MIC values were determined by agar dilution in accordance with the Clinical and Laboratory 65 66 Standards Institute (CLSI 2008). The range of concentrations tested was between 2 and 128 67 µg/ml. The stock solution was prepared dissolving 250 mg nitrofurantoin (Sigma-Aldrich, 68 Germany) in 5 ml of dimethylformamide (DMF) to yield a final concentration of 50 mg/ml. 69 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 70 71 for quality control: Staphylococcus aureus ATCC 29213, Enterococcus faecalis ATCC 29212, 72 Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853.

73 MPC testing

74 Mutant prevention concentrations (MPCs) of nitrofurantoin were measured in two isolates of 75 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 76 plates displaying confluent growth was transferred to 100 ml of Mueller-Hinton broth (Oxoid, 77 78 UK) using a sterile swab. Following overnight incubation, 50 ml of overnight culture were 79 centrifugated 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 80 inoculated using 100µl of this bacterial suspension and incubated at 37°C. The lowest drug 81 82 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 83 subcultured onto agar plates containing the same drug concentration and subjected to MIC 84 determination. 85

86

87 Results and discussion

The MICs of nitrofurantoin ranged between 8 and 32 µg/ml in E. coli and between 4 and 16 88 89 µg/ml in S. pseudintermedius (Table 1). The MIC₉₀ was 32 µg/ml for E. coli and 16 µg/ml for S. 90 *pseudintermedius*. The MIC₅₀ values for the two species were 16 and 8 μ g/ml, respectively. As 91 breakpoints for nitrofurantoin resistance have not yet been established for animal E. coli and S. 92 pseudintermedius, only human breakpoints (S≤32 µg/ml, R≥128 µg/ml) (CLSI 2010) are 93 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 94 95 strains of known genetic background, displayed MICs lower than the concentration of 96 nitrofurantoin achieved in urine following oral administration to dogs. Conklin et al. (1969)

97 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 98 99 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 100 101 currently available about the urinary excretion profile within four hours after administration. In 102 an absorption and disposition study of nitrofurantoin in dogs performed by Niazi et al. (1983), 103 oral administration of a 100 mg tablet resulted in a peak plasma level ranging from 1.5 to 4.0 μ g/ml, and an elimination half-life of 32 to 87 minutes, which means that the drug is rapidly 104 105 distributed and eliminated from plasma. If one assumes that the urinary excretion rate changes 106 according to the plasma levels, these data indicate that urinary concentrations are high shortly 107 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. 108 concentration- vs. time-dependent) are needed in order to optimize dosage regimens in small 109 110 animals.

111 There are several disadvantages associated with the clinical use of nitrofurantoin in small 112 animals. The drug is a known gastrointestinal irritant and may cause nausea and emesis as side-113 effects in both dogs and cats. Due to the short plasma half-life (Niazi et al., 1983), nitrofurantoin 114 requires frequent administration (i.e. three to four times daily). Furthermore, it cannot be used for 115 treatment of UTI associated with *Proteus* and *Pseudomonas* as these organisms are generally 116 resistant to this drug (Rohrich et al., 1983). Although better alternatives than nitrofurantoin exist 117 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 118 treatment of UTI caused by ESBL-producing E. coli and MRSP. The recent spread of these 119

120 multidrug-resistant bacteria in dogs and cats represents a serious therapeutic challenge for small 121 animal veterinary practitioners since such bacteria are often resistant to all the conventional 122 veterinary antimicrobial products available for treatment of UTI in small animal medicine 123 (Carattoli et al., 2005; El Zubeir et al., 2007; Loeffler et al., 2007; Ogeer-Gyles et al., 2006; 124 Perreten et al., 2010; Ruscher et al., 2009; Sanchez et al., 2002; Shaheen et al., 2010; Warren et 125 al., 2001). As a consequence, some veterinarians have started to use critically-important 126 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 127 128 new trend in antimicrobial usage emphasizes the need for alternative treatment options and 129 nitrofurantoin represents an ideal alternative to carbapenems in the treatment of canine or feline 130 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 131 with pre-existing and recurrent urethral obstruction (Pomba et al., 2010). 132

133

The MPC values were 128 µg/ml for both *E. coli* strains and 64 µg/ml for both *S.* 134 pseudintermedius strains. The MIC was recorded for all presumptive mutants isolated from MPC 135 136 plates and only one *E. coli* strain had a higher MIC (128 µg/ml) compared with the respective 137 wild type strain (16 µg/ml). The clinical relevance of the MPC data is uncertain since the 138 apparent bacterial growth on MPC plates containing drug concentrations above MIC was not due 139 to the emergence of a subpopulation of resistant mutants but appeared to be an artefact 140 determined by the high density of the inoculum. Resistance to nitrofurantoin in E. coli is known 141 to occur as a result of step-wise mutations (Breeze and Obaseiki-Ebor, 1983; Sandegren et al., 142 2008). Assuming that the drug concentrations achievable in canine urine during therapy

143 approximate 60 to 100 µg/ml depending on the specific formulation (Conklin et al., 1969), 144 nitrofurantoin would not be effective against mutants with high MICs such as that obtained from the MPC test in this study (MIC=128 μ g/ml). However, it has been shown that the growth rate of 145 146 first-step mutants is impaired at concentrations well below 100 µg/ml, and that second-step 147 mutants have an impaired growth rate at 100 µg/ml (Sandegren et al., 2008). Based on these 148 data, emergence of resistant mutants during therapy does not seem to be a critical issue in the 149 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, 150 151 2008; Kashanian et al., 2008; Schito et al., 2009; Zhanel et al., 2005). 152 In conclusion, the use of nitrofurantoin for treatment of UTI in dogs is a matter of weighing the 153 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 154 155 treatment of UTI in small animals. However, the results of this study indicate that the use of 156 nitrofurantoin may be indicated for treatment of UTI caused by MDR E. coli or MRSP, which 157 are otherwise difficult to treat using conventional veterinary antimicrobial products. Standard 158 dosage regimens seem sufficient to kill susceptible strains and probably also prevent selection of 159 resistant mutants during therapy. The MIC distribution data generated by this study may be used 160 for setting species-specific epidemiological cut-off values and clinical breakpoints for 161 nitrofurantoin susceptibility testing in UTI isolates from dogs and cats. Setting of UTI-specific 162 clinical breakpoints will require additional data on the PK/PD properties of nitrofurantoin in 163 these animal species and clinical outcome data from perspective clinical studies.

164

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251

251 **Table 1**. Nitrofurantoin minimum inhibitory concentrations (MIC) in *Escherichia coli* and

252 *Staphylococcus pseudintermedius* isolates from small animals.

E. coli 10 S. 16 pseudintermedius	olates 06	2 0 (0%) 0 (0%)	4 0 (0%) 1 (0,61%)	8 4 (3,77%) 122 (74,85%)	16 90 (84,91%) 40 (24,54 %)	32 12 (11,32%) 0 (0%)	64 0 (0%) 0 (0%)	128 0 (0% 0 (0%
E. coli 10 S. 16 pseudintermedius	53	0 (0%)	0 (0%)	4 (3,77%) 122 (74,85%)	90 (84,91%) 40 (24,54 %)	12 (11,32%)	0 (0%)	0 (0%
S. 16 pseudintermedius	53	0 (0%)	1 (0,61%)	122 (74,85%)	40 (24,54 %)	0 (0%)	0 (0%)	0 (0%
pseudintermedius				5	2	5		
				5	2			