Research in Veterinary Science 96 (2014) 251–253

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Fosfomycin susceptibility of canine methicillin-resistant *Staphylococcus pseudintermedius* isolates

Matthew DiCicco [a], Scott Weese [b], Suresh Neethirajan [a,*], Joyce Rousseau [b], Ameet Singh [c]

[a] BioNano Laboratory, School of Engineering, University of Guelph, Guelph N1G 2W1, Canada
[b] Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph N1G 2W1, Canada
[c] Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph N1G 2W1, Canada

ARTICLE INFO

Article history:
Received 9 June 2013
Accepted 1 February 2014

Keywords:
Fosfomycin
Methicillin-resistant
Staphylococcus pseudintermedius
MRSP
fosB

ABSTRACT

The effectiveness of fosfomycin was examined across 31 methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) strains by agar dilution. Prevalence of the fosfomycin-resistance determinant gene, *fosB*, was assessed by PCR analysis. Results found that 84% of isolates were fosfomycin-susceptible. Interestingly, 87% of isolates possessed *fosB*, indicating no association between this putative staphylococci resistance gene and phenotypic resistance. Further evaluation of fosfomycin as a potential treatment of MRSP in dogs is warranted.

Antimicrobial resistance poses a critical challenge in veterinary medicine. Recently, methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has emerged as an increasingly common problem in dogs (Nienhoff et al., 2011; Ruscher et al., 2009). Methicillin-resistant staphylococci are resistant to virtually all beta-lactam antimicrobials (penicillins, cephalosporins, carbapenems) by virtue of the *mecA* gene, which encodes an altered penicillin binding protein. Additionally, MRSP isolates are often resistant to numerous other antimicrobial classes (Wendlandt et al., 2013) providing very few treatment options. Identification of other antimicrobial approaches to the treatment of MRSP, particularly using drugs that are not used for the treatment of critically ill human patients, is important.

Fosfomycin (FOS) is an old antimicrobial that has seen a revival in practice in humans in recent years in both mono- and combination therapy (Michalopoulos et al., 2011). While FOS is more commonly used for treatment of urinary tract infections (Slekovec et al., 2012; Wilson and May, 2013), it has been shown to be effective *in vitro* and *in vivo* for infections of pathogenic canine and feline *Escherichia coli* (Hubka and Boothe, 2011) and other body sites caused by *Staphylococcus aureus* (Miróa et al., 2012), including MRSA (Kono et al., 1994; Lau et al., 1986), and has efficacy as combination therapy against MRSA biofilm (Apisarnthanarak and Mundy, 2007; Tang et al., 2012). Its effective use in the clinical setting has been attributed to its unique antimicrobial mechanism—providing a limited risk of cross-resistance—and ability to penetrate deeply through tissue (Kumon and Ono, 1995; Kusachi et al., 2011; Mikuniya et al., 2007) which are appealing factors. FOS interferes with cell wall synthesis of peptidoglycan and enters FOS-susceptible bacteria by means to two different transport uptake systems; the L-α-glycerophosphate transport system (GlpT) and the hexose–phosphate uptake system (UhpT) (Kahan et al., 1974). Good tissue distribution and biofilm penetration are as well attributed to its low molecular weight and negligible protein binding (Frossard et al., 2000). These properties suggest that FOS might be a viable treatment option for some MRSP infections, but the susceptibility of MRSP to FOS has not been previously reported. This study assessed the effectiveness of FOS against MRSP strains through standard *in vitro* agar dilution experimentation.

A convenience sample of 31 epidemiologically-unrelated MRSP isolates from dogs from Canada ($n = 21$) and the United States ($n = 10$) were studied (Table 1). They had been previously characterized by sequence analysis of the *mec*-associated direct repeat unit (*dru* typing) (Goering et al., 2008). *Dru* types corresponding to the two main international clones ST68 ($n = 17$, 54%) and ST71 ($n = 10$, 32%) accounted for 87% of isolates. FOS susceptibility was determined by agar dilution using Clinical and Laboratory Standards Institute (CLSI) standards (Institute, 2008). Isolates were grown in pure culture on Columbia agar with 5% sheep blood then suspended in phosphate buffered saline (PBS) to achieve a 0.5 McFarland standard ($\sim 10^8$ CFU/ml). Using a Steer replicator

* Corresponding author. Tel.: +1 519 824 4120; fax: +1 519 824 3776.
E-mail address: sneethir@uoguelph.ca (S. Neethirajan).

http://dx.doi.org/10.1016/j.rvsc.2014.02.004
0034-5288/

these suspensions were then inoculated onto plates of Mueller–Hinton agar supplemented with FOS and 25 μg/ml glucose-6-phosphate to improve FOS uptake (Kahan et al., 1974). Results were interpreted as growth (resistant) or no-growth (susceptible) as per EUCAST guidelines (OEGACH, 2009). Twofold dilutions of FOS from 256 to 0.125 μg/ml were tested with EUCAST breakpoints standards for susceptibility (<32 μg/ml) (OEGACH, 2009).

Polymerase chain reaction (PCR) was used to identify a gene that has been associated with plasmid-resistance in staphylococci, *fosB*. PCR primers were *fosB*Fwd, 5′ ACC GGT ACT TTA CAA GAG CGT 3′, and *fosB*Rev, 5′ AAC AGC ACC ATC ACT TCC TT 3′. PCR amplification cycling conditions for *fosB* consisted of 2 min of denaturation at 95 °C, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 57 °C for 15 s and extension at 72 °C for 30 s. One PCR product was sequenced to confirm amplification of *fosB* during assay development (data not presented).

FOS minimum inhibitory concentrations (MICs) ranged from 0.125 to 64 μg/ml with 24 (77%) isolates evaluated as susceptible and an MIC_{50} and MIC_{90} of 0.125 and 64 μg/ml, respectively (Fig. 1). These results indicate that MRSP isolates are often susceptible to FOS *in vitro*, suggesting that this antimicrobial should be studied further in dogs to determine whether it is a viable treatment option for MRSP infections. Its high excretion percentages in urine makes it an ideal candidate for urinary tract and catheter based infections (Cheng et al., 2010), but it may also be effective in infections of other body sites (Gutierrez et al., 2008). Particularly it could be used in skin, soft tissue, and surgical site infections where MRSP predominates; however, care must be taken prior to widespread clinical use. Some of the adverse effects of fosfomycin therapy in veterinary species include the potential for acute renal insufficiency with increased serum levels of blood urea nitrogen and creatinine (Fukata et al., 2008).

While most isolates were susceptible, resistance was detected, even though it is very unlikely that any of these dogs had been exposed to fosfomycin. The prevalence of FOS resistance was higher in *dru* types consistent with ST 71 (7/10, 70%) when compared to those associated with ST68 (0/17, 0%) ($P < 0.001$). The reason for the difference in FOS resistance between the two major genetic lineages is unclear, in large part because the mechanism of resistance is unclear but deserving of further study.

Table 1
The origin location and the sequence type of 31 canine MRSP isolates.

Isolate selected	Anatomical sample site	Origin location	Sequence type	Dru type
A3	Pyoderma	U.S.A	71	9a
A12	Otitis	U.S.A	68	10 h
A14	Nasal swab	U.S.A	68	10 h
A23	Otitis	U.S.A	68	10a
A42	Pyoderma	U.S.A	68	11a
A46	Pyoderma	U.S.A	71	9a
A56	Pyoderma	U.S.A	71	9a
A92	Abscess	U.S.A	71	9a
A132	Nasal swab	U.S.A	68	11a
P147	Nasal swab	U.S.A	n/a	10bm
BH01	Nasal swab	Canada	68	11a
SP77	Pyoderma	Canada	68	11a
SP90	Pyoderma	Canada	71	9a
SP102	Pyoderma	Canada	68	11a
SP104	Pyoderma	Canada	68	10 h
SP105	Pyoderma	Canada	68	10 h
SP106	Pyoderma	Canada	71	9a
SP111	Pyoderma	Canada	n/a	7d
SP112	Pyoderma	Canada	71	9a
SP113	Pyoderma	Canada	71	9a
SP123	Pyoderma	Canada	n/a	7ac
SP132	Pyoderma	Canada	68	10 h
SP135	Pyoderma	Canada	68	10 h
KB14	Pyoderma	Canada	68	11a
KB113	Pyoderma	Canada	68	10 h
KB154	Pyoderma	Canada	71	9a
KB157	Pyoderma	Canada	71	9a
KB184	Pyoderma	Canada	68	10 h
KB280	Pyoderma	Canada	68	11a
KB346	Pyoderma	Canada	68	10 h
KB438	Pyoderma	Canada	n/a	5i

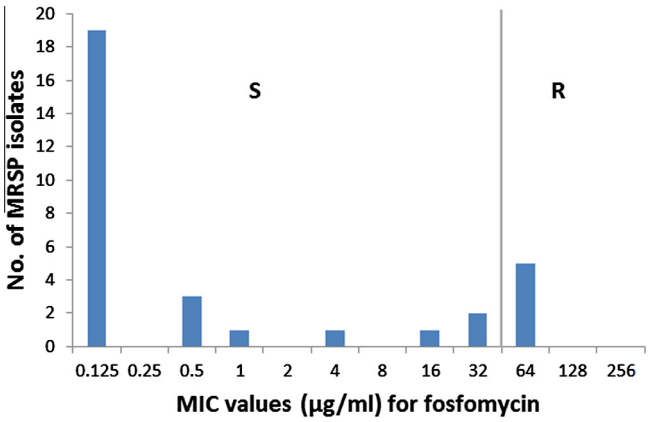

Fig. 1. Distribution of fosfomycin minimum inhibitory concentration (MIC) of 31 methicillin-resistant *S. pseudintermedius* isolates by agar dilution. Vertical line indicates EUCAST breakpoint. S = susceptible, R = resistant.

One potential mechanism of fosfomycin resistance is the presence of *fosB*, a gene the encodes enzyme-based modification of fosfomycin after cellular uptake. *fosB* was identified in 27 of 31 isolates (87%) with 20 of 24 (83%) FOS-susceptible isolates and 7 of 7 (100%) FOS-resistant isolates expressing the gene. There was no association between the presence of *fosB* and FOS-resistance (Fisher's exact $P = 0.55$). Reasons for this are unclear, and include the potential that *fosB* does not actually confer resistance, that *fosB* is not constitutively expressed or that some of these strains had a non-functional gene. Study of gene expression would be required to determine whether there is variable expression, or no expression, of this gene. Sequence analysis of the entire gene could also be performed to attempt to identify any potentially relevant gene alterations.

It is important to consider issues pertaining to interpretation of *in vitro* testing. Veterinary breakpoints are lacking and while there is a clear, highly susceptible wild-type population and a resistant population, some isolates that fall within the susceptible range have substantially higher MICs than others. Scrutiny of breakpoints is needed to ensure that these isolates are truly likely to be susceptible *in vivo*; in particular the isolate with a FOS MIC of 16 μg/ml that is grouped with the resistant population, quite apart from the wild type group (Fig. 1). Additionally, systemic use of this antimicrobial in mono-therapy in humans has resulted in the selection of mutant strains showing high-level chromosomal and plasmid-based resistance (Reeves, 1994; Thauvin et al., 1988), rising concerns about monotherapy in animals. An apparent effect of strain was also noted with all resistant isolates belonging to ST71-associated *dru* types, and no resistance among *dru* types associated ST68, the other major international clone (Perreten et al., 2010). The potential efficacy of FOS may therefore be regional and best in areas where ST71 does not predominant (e.g. North America) (Perreten et al., 2010).

Despite these potential concerns, recent studies that have highlighted the clinical efficacy of FOS in combination with other antimicrobials for the treatment of nosocomial infections for various Gram-positive and Gram-negative bacteria (Anderson et al., 2013; Michalopoulos et al., 2010; Miróa et al., 2012), suggest that evaluation of FOS-containing drug combinations for MRSP infections in dogs might be warranted.

The high prevalence of *fosB* seen here was surprising given the low prevalence of FOS-resistance. The lack of an association between FOS-resistance and the presence of *fosB* could be the result of repression of or a non-functional *fosB* gene. Whether a functional *fosB* gene or different mechanism was the cause of the FOS-resistant population is unclear but indicates the need for further study of FOS resistance in MRSP, particularly since all FOS-resistant isolates possessed *fosB*. Additionally, it can reasonably be assumed that it is extremely unlikely animals from which these isolates originated had been treated with FOS based on anecdotal understanding of the exceedingly rare use of this drug in dogs, raising questions about why this gene was so commonly present.

The isolates that were tested were a population of convenience; however, they were from various surveillance studies and no potential biases, particularly with respect to FOS resistance, are readily identifiable. And although the relatively small sample size should be considered, the results indicate that FOS might be able to play a role in the treatment of MRSP infections. Further study into the mechanisms of FOS-resistance and the discrepancy in *fosB* prevalence and FOS-resistance is needed. The emergence of resistance seen in these strains without previous exposure raises concerns regarding resistance determinants to other species when used in mono-therapy. However, successful clinical trials with combinational therapy against Gram-positive cocci show potential for a new treatment for MRSP based infections and ultimately indicate the need for proper *in vivo* study of this drug.

Acknowledgements

The authors sincerely thank the Natural Sciences and Engineering Research Council of Canada for funding this study.

References

Anderson, G., Kenney, T., MacLeod, D., 2013. Eradication of *Pseudomonas aeruginosa* biofilms on cultured airway cells by a fosfomycin/tobramycin antibiotic combination. Pathogens and Disease 67, 39–45.

Apisarnthanarak, A., Mundy, L.M., 2007. Successful treatment of disseminated methicillin-resistant *Staphylococcus aureus* with fosfomycin, cefoperzone/sulbactam and rifampin followed by fusidic acid and rifampin. International Journal of Infectious Disease 11, 283–284.

Cheng, A.C., Hajkowicz, K.M., Grayson, M.L., 2010. A clinical review of antibacterial, antifungal, and antiviral drugs. In: Grayson, M.L., Crowe, S., McCarthy, J., Mills, J., Mouton, J., Norrby, R., Paterson, D., Pfaller, M. (Eds.), Kucers' the Use of Antibiotics. Edward Arnold, London, UK, pp. 2011–2023.

Frossard, M., Joukhadar, C., Erovic, B.M., Dittrich, P., Mrass, P.E., Van Houte, M., Burgmann, H., Georgopoulos, A., Muller, M., 2000. Distribution and antimicrobial activity of fosfomycin in the interstitial fluid of human soft tissues. Antimicrobial Agents and Chemotherapy 44, 2728–2732.

Fukata, T., Imai, N., Shibata, S., 2008. Acute renal insufficiency in cats after fosfomycin administration. Veterinary Record 163, 337–338.

Goering, R.V., Morrison, D., Al-Doori, Z., Edwards, G.F., Gemmell, C.G., 2008. Usefulness of mec-associated direct repeat unit (dru) typing in the epidemiological analysis of highly clonal methicillin-resistant *Staphylococcus aureus* in Scotland. Clinical microbiology and infection: The official publication of the European Society of Clinical Microbiology and Infectious Diseases 14, 964–969.

Gutierrez, O.L., Ocampo, C.L., Aguilera, J.R., Luna, J., Sumano, L.H., 2008. Pharmacokinetics of disodium-fosfomycin in mongrel dogs. Research in Veterinary Science 85, 156–161.

Hubka, P., Boothe, D.M., 2011. In vitro susceptibility of canine and feline *Escherichia coli* to fosfomycin. Veterinary Microbiology 149, 277–282.

Institute, C.L.S. 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. In M31-A (Wayne, PA).

Kahan, F.M., Kahan, J.S., Cassidy, P.J., Kropp, H., 1974. The mechanism of action of fosfomycin (phosphonomycin). Annals of the New York Academy of Sciences 235, 364–386.

Kono, K., Takeda, S., Tatara, I., Arakawa, K., Tanaka, H., Miyake, S., Minamikawa, H., Hoshino, H., Sato, M., Hattori, F., 1994. Combined therapy with arbekacin and fosfomycin for methicillin-resistant *Staphylococcus aureus* infections. The Japanese Journal of Antibiotics 47, 798–803.

Kumon, H., Ono, N., 1995. Combination effect of fosfoymcin and ofloxacin against *Pseudomonas aeruginosa* growing in a biofilm. Antimicrobial Agents and Chemotherapy 39, 1038–1044.

Kusachi, S., Nagao, J., Yoshihisa, S., Watanabe, M., 2011. Antibiotic time-lag combination therapy with fosfomycin for postoperative intra-abdominal abcesses. Journal of Infection Chemotherapy 17, 91–96.

Lau, W.Y., Teoh-Chan, C.H., Fan, S.T., 1986. In vitro and in vivo study of fosfoymcin in methicillin-resistant *Staphylococcus aureus*. The Journal of Hygiene 96, 416–423.

Michalopoulos, A., Virtzili, S., Rafailidis, P., Chalevelakis, G., Damala, M., Falagas, M.E., 2010. Intravenous fosfomycin for the treatment of nosocomial infections caused by carbapenem-resistant *Klebsiella pneumoniae* in critically ill patients: a prospective evaluation. Clinical Microbiology and Infection: The Official publication of the European Society of Clinical Microbiology and Infectious Diseases 16, 184–186.

Michalopoulos, A.S., Livaditis, I.G., Gougoutas, V., 2011. The revival of fosfomycin. International Journal of Infectious Diseases 15, e732–e739.

Mikuniya, T., Kato, Y., Ida, T., 2007. Treatment of *Pseudomonas aeruginosa* biofilms with a combination of fluoroquinolones and fosfomycin in a rat urinary tract infection model. Journal of Infection and Chemotherapy 13, 285–290.

Miróa, J., Entenza, J., Rio, A., Velasco, M., 2012. High-dose daptomycin plus fosfomycin is safe and effective in treating methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* endocarditis. Antimicrobial Agents and Chemotherapy 56, 4511–4515.

Nienhoff, U., Kadlec, K., Chaberny, I.F., Verspohl, J., Gerlach, G.F., Kreienbrock, L., Schwarz, S., Simon, D., Nolte, I., 2011. Methicillin-resistant *Staphylococcus pseudintermedius* among dogs admitted to a small animal hospital. Veterinary Microbiology 150, 191–197.

OEGACH 2009. EUCAST breakpoints (www.oegach.at, Austrian Society for Antimicrobial Chemotherapy).

Perreten, V., Kadlec, K., Schwarz, S., Gronlund Andersson, U., Finn, M., Greko, C., Moodley, A., Kania, S.A., Frank, L.A., Bemis, D.A., Franco, A., Iurescia, M., Battisti, A., Duim, B., Wagenaar, J.A., van Duijkeren, E., Weese, J.S., Fitzgerald, J.R., Rossano, A., Guardabassi, L., 2010. Clonal spread of methicillin-resistant *Staphylococcus pseudintermedius* in Europe and North America: an international multicentre study. The Journal of Antimicrobial Chemotherapy 65, 1145–1154.

Reeves, D.S., 1994. Fosfomycin trometamol. Journal of Antimicrobial Chemotherapy 34, 853–858.

Ruscher, C., Lubke-Becker, A., Wleklinski, C.G., Soba, A., Wieler, L.H., Walther, B., 2009. Prevalence of methicillin-resistant *Staphylococcus pseudintermedius* isolated from clinical samples of companion animals and equidaes. Veterinary Microbiology 136, 197–201.

Slekovec, C., Leroy, J., Vernaz-Hegi, N., Faller, J.P., Sekri, D., Hoen, B., Talon, D., Bertrand, X., 2012. Impact of a region wide antimicrobial stewardship guideline on urinary tract infection prescription patterns. International Journal of Clinical Pharmacy 34, 325–329.

Tang, H., Chen, C., Cheng, K., 2012. In vitro efficacy of fosfomycin-containing regimens against methicillin-resistant *Staphylococcus aureus* in biofilms. Journal of Antimicrobial Chemotherapy 67, 944–950.

Thauvin, C., Lemeland, J.F., Humbert, G., Fillastre, J.P., 1988. Efficacy of pefloxacin–fosfomycin in experimental endocarditis caused by methicillin-resistant *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 32, 919–921.

Wendlandt, S., Fessler, A.T., Monecke, S., Ehricht, R., Schwarz, S., Kadlec, K., 2013. The diversity of antimicrobial resistance genes among staphylococci of animal origin. International Journal of Medical Microbiology IJMM 303, 338–349.

Wilson, D.T., May, D.B., 2013. Potential role of fosfomycin in the treatment of community-acquired lower urinary tract infections caused by extended-spectrum beta-lactamase-producing Escherichia coli. American Journal of Therapeutics 20, 685–690.