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Antimicrobial susceptibility of *Clostridium difficile* isolated from animals and humans in Brazil

Sensibilidade antimicrobiana de *Clostridium difficile* isoladas de animais e humanos no Brasil

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ABSTRACT

*The objective of this study was to evaluate antimicrobial susceptibility in **Clostridium difficile** strains isolated from animals and humans in Brazil. The 54 **C. difficile** strains used were isolated from stool samples from piglets (n=16), dogs (n=13), humans (n=13), foals (n=8) calves (n=2), an ocelot (n=1) and a maned wolf (n=1). Antimicrobial susceptibility was determined using the serial plate agar dilution method for penicillin, florfenicol, oxytetracycline, erythromycin, vancomycin, metronidazole and tylosin. The **C. difficile** strains assessed were susceptible to metronidazole and vancomycin. Florfenicol resistance was rarely observed; 52 (96.4%) strains were sensitive to this antimicrobial. Five (9.3%), five (9.3%), 14 (25.9%) and 20 (37.0%) strains were resistant to oxytetracycline, penicillin, tylosin and erythromycin respectively.*

Key words: *nosocomial diarrhea, pseudomembranous colitis, resistance.*

RESUMO

*O objetivo do presente trabalho foi avaliar a sensibilidade antimicrobiana de estirpes de **Clostridium difficile** isoladas de animais e humanos no Brasil. Foram utilizados 54 estirpes de **C. difficile** isoladas de fezes de leitões (n=16), cães (n=13), seres humanos (n=13), potros (n=8), bezerros (n=2), jaguatirica (n=1) e um lobo-guará (n=1). A sensibilidade antimicrobiana foi determinada pelo método de diluição seriada em ágar para penicilina, florfenicol, oxitetraciclina, eritromicina, vancomicina, metronidazol e tilosina. Todos os isolados foram sensíveis ao metronidazol e á vancomicina. Resistência ao florfenicol foi rara, sendo que 52 (96,4%) das estirpes foram sensíveis a esse antimicrobiano. Cinco (9,3%), cinco (9,3%), 14 (25,9%) e 20 (37,0%) foram resistentes a oxitetraciclina, penicilina, tilosina e eritromicina, respectivamente.*

Palavras-chave: *diarreia nosocomial, colite pseudomembranosa, resistência.*

INTRODUCTION

Clostridium difficile is a Gram-positive, spore-forming bacillus responsible for most human antibiotic-associated diarrhea cases. The main risk factors associated to ***C. difficile*** infection (CDI) in humans are age older than 65, previous antibiotic therapy and prolonged hospitalization. An increase of the occurrence of community-acquired CDI, mainly in pregnant or puerperal women and children, has drawn attention to possible changes in the disease epidemiology (SILVA JÚNIOR, 2012).

In veterinary medicine, it produces diarrhea and colitis in several species and primarily affects domestic animals (SILVA et al., 2013a). However recently, its effects on wild species have been reported (BOJESEN et al., 2006; SILVA et al., 2013b). Similar to the human disease, ***C. difficile*** infections (CDIs) in animals are commonly associated with antimicrobial treatments (BÅVERUD, 2004; SONGER et al., 2009; HOPMAN et al., 2011; SILVA et al., 2012).

Recent studies have shown that isolates from human cases of pseudomembranous colitis caused by ***C. difficile*** are genetically similar to strains isolated from domestic animals, which suggest that this disease could be zoonotic (JHUNG et al., 2008; NORMAN et al., 2011). Although ***C. difficile*** is a major cause of enteric disorders in humans and animals as well as a possible zoonotic agent, few studies have evaluated the antimicrobial susceptibility

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of ***C. difficile*** strains in Brazil. Most current reports are not robust and are limited to human isolates. Thus, the objective of this study was to evaluate the antimicrobial susceptibility of ***C. difficile*** strains isolated from animals and humans in Brazil.

MATERIAL AND METHODS

Fifty-four ***C. difficile*** strains isolated from piglets (n=16), dogs (n=13), humans (n=13), foals (n=8), calves (n=2), an ocelot (n=1) and a maned wolf (n=1) were used in this study. Table 1 summarizes the species and strains included in this study as well as their clinical history. These isolates belong to the ***Clostridium*** strains library of the Veterinary School of Federal University of Minas Gerais (UFMG) and were isolated in previous published works (between 2009 and 2013) or ongoing projects (SILVA et al., 2011; SILVA et al., 2013b, Silva et al., 2013c; SILVA et al., 2013d). After isolation and confirmation of identity by a previous described multiplex PCR (SILVA et al., 2011), all strains were lyophilized and kept at -20°C until reconstitution for this work.

The minimum inhibitory concentration (MIC) was determined using the serial agar dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011) and The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2011). The following antimicrobials were tested: penicillin, florfenicol, oxytetracycline, erythromycin, vancomycin, metronidazole and tylosin. For an assay control, the reference specimen ***Bacterioides fragilis*** (ATCC 25285) was used. For the antimicrobials, the following concentrations were tested: 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0, 128.0 and 256.0µg ml^{-1}. Tests were performed on Brucella agar (Difco Laboratories, USA) supplemented with 5% horse blood, hemin and vitamin K (CLSI, 2011).

RESULTS

The MIC results for the 54 ***C. difficile*** strains analyzed are in table 2. The strains were susceptible to metronidazole and vancomycin. Susceptibility to florfenicol and oxytetracycline was observed in 96.3% and 79.6% of the strains respectively. Five (9.3%), five (9.3%), 14 (25.9%) and 20 (37.0%) strains were resistant to oxytetracycline, penicillin, tylosin and erythromycin respectively.

DISCUSSION

The ***C. difficile*** strains assessed were susceptible to metronidazole and vancomycin, which are the antimicrobials commonly used to treat CDI

Table 1 - Number of ***Clostridium difficile*** strains used to evaluate antimicrobial susceptibility in each animal species and their clinical history. Tabelas para CR têm no máximo três linhas contínuas. As demais devem ser deletadas ou substituídas por linha tracejadas

Species	Clinical history	Number of strains	Total
Dogs	Diarrheic (other causes)	5	13
	Apparently healthy	8	
Piglets	Confirmed CDI	8	16
	Diarrheic (other causes)	4	
	Apparently healthy	4	
Foals	Confirmed CDI	4	8
	Diarrheic (other causes)	2	
	Apparently healthy	2	
Calf	Diarrheic (other causes)	2	2
Ocelot	Confirmed CDI	1	1
Maned wolf	Diarrheic (other causes)	1	1
Human	Confirmed CDI	13	13
Total			54

Table 2 - The MIC in μg ml^{-1} and antimicrobial susceptibility classification for 54 ***Clostridium difficile*** strains isolated from human and animal feces.

Antimicrobial	----------Number of strains and MIC (μg ml^{-1})----------												-------Classification (%)------		
	0.25	0.5	1	2	4	8	16	32	64	128	256	>256	S^1	MS^2	R^3
Metronidazole	20	30	3	1									100	0	0
Vancomycin	28	19	7										100	0	0
Florfenicol			10	42		2							96.3	3.7	0
Oxytetracycline	34	7				2	6					5	79.6	11.1	9.3
Penicillin	3	24	22	5									50.0	40.7	9.3
Tylosin	7	29	1	1	1		1			3		11	72.2	1.9	25.9
Erythromycin	13	10	7	4			2			2	4	12	63.0	0	37.0

Legend**:** S^1: susceptible, MS^2: moderately susceptible. R^3: resistant (R). Classification based on the CLSI (2011) and EUCAST (2011).

in humans, horses and dogs (BÅVERUD, 2002; MARKS & KATHER, 2003; BÅVERUD, 2004; SPIGAGLIA et al., 2011; SILVA et al., 2013c). These results are similar to previous studies for several domestic animal species (BÅVERUD et al., 2003; MARKS & KATHER, 2003; POST & SONGER, 2004; FRY et al., 2012). Currently, metronidazole-resistant strains have only been isolated from horses in the USA (JANG et al., 1997; MAGDESIAN et al., 2006). Similar to observations in animals, metronidazole-resistant ***C. difficile*** strains are rarely isolated from humans (SHAH et al., 2010; SPIGAGLIA et al., 2011).

Vancomycin-resistant ***C. difficile*** isolates are also extremely rare for both animal and human strains. In addition, vancomycin is considered clinically more effective and generates fewer relapse cases following CDI treatment in humans (SHAH et al., 2010); therefore, it is considered the best option by many clinicians. In addition to this study, only one other study in Brazil has evaluated antimicrobial susceptibility in six ***C. difficile*** strains isolated from humans with CDI; similar to this study, the isolates were also susceptible to metronidazole and vancomycin (BALASSIANO et al., 2009).

None of the strains were resistant to florfenicol, whereas five isolates (9.3%) (two pig isolates, two human isolates and one ocelot isolate) were resistant to oxytetracycline. In general, a large variation in the MIC values were observed for tetracycline, what has also been reported in studies with ***C. difficile*** strains isolated from pigs and humans (DELMÉE & AVESANI, 1988; POST & SONGER, 2004; SHAH et al., 2010). However, almost all isolates from the earlier studies were susceptible to this antimicrobial. Resistance to this class of antimicrobials is commonly associated with the *tet* genes, especially *tetM* (HUANG et al., 2009). The high susceptibility of ***C. difficile*** to tetracyclines differs from other ***Clostridium*** species, especially for ***C. perfringens***, which is a species commonly resistant to tetracycline (SLAVIĆ et al., 2011).

The five penicillin-resistant strains were isolated from three foals, all of which had a confirmed CDI diagnosis, and two piglets. Interestingly, in two of the foals, the diarrhea caused by ***C. difficile*** began after penicillin G was administered under suspicion of pneumonia (SILVA et al., 2012). This result is consistent with observations by BÅVERUD (2002), who reported that beta-lactams are commonly associated with CDI in foals and adult horses. In contrast to reports in other countries (HUANG et al., 2009, SHAH et al., 2010), none of the human isolates were resistant to penicillin in this study, and only one of the 13 strains tested (7.7%) was moderately susceptible to this compound.

In this study, most strains were resistant to the macrolides erythromycin and tylosin. Detection of ***C. difficile*** resistance to these compounds was previously reported in strains from various sources, including equine and human isolates (DELMÉE & AVESANI, 1988; BÅVERUD et al., 2003; SPIGAGLIA et al., 2011). In this study, of the 20 (37%) erythromycin-resistant strains, ten (18.5%) were isolated from piglets, four (7.4%) from dogs, three (5.6%) from humans, two (3.7%) from horses and one (1.9%) from a calf. Of these isolates, 16 (80%) had an MIC greater than or equal to 256.0μg mL^{-1}. In humans, certain macrolides are listed as antimicrobials commonly involved in CDI cases (ZILBERBERG & SHORR, 2013). Unfortunately, few studies have examined strains from animals, but erythromycin has been reported as a major cause of colitis in mares (BÅVERUD, 2002). Interestingly, the erythromycin-

resistant ***C. difficile*** strains isolated from humans vary greatly between countries, with reports ranging from 87% of isolates with resistance in England to 0% in Switzerland (HUANG et al., 2009).

The striking bimodal behavior of these isolates for erythromycin resistance suggests a genetic factor that encodes resistance. In fact, studies have shown that the resistance of ***Clostridium*** strains to this antimicrobial is primarily related to the *erythromycin ribosomal methylase* (*erm*) genes, which encode a methylase that inhibits erythromycin activity (SLAVIĆ et al., 2011). Studies that have examined antimicrobial-resistance genes are rare for ***C. difficile*** strains isolated from animals. However, FRY et al. (2012) recently reported a high correlation between erythromycin resistance and the *ermB* gene in ***C. difficile*** strains isolated from pigs, which confirms this hypothesis. Notably, however, not all strains with high levels of erythromycin resistance encode the *erm* genes, suggesting that there are other unknown mechanisms involved in resistance (HUANG et al., 2009).

Certain studies have reported high susceptibility of ***C. difficile*** strains to tylosin, which suggests that this antimicrobial could be used in pig feed to decrease or eliminate ***C. difficile*** in animal feces (POST & SONGER, 2004; SONGER & ANDERSON, 2006). In contrast, 14 strains (25.9%) in this study were resistant to tylosin; eight were isolated from piglets and three were isolated from dogs, suggesting that this antimicrobial would not be effective for CDI prevention, control or treatment in the species evaluated herein.

Interestingly, tylosin is an antimicrobial commonly used in Brazilian pig production, and all the strains included in this study were from farms that reported using tylosin at some stage in the animal life cycle. In contrast, oxytetracycline is a compound that has been banned from animal feed in Brazil since 1998 (BRASIL, 1998). Two oxytetracycline-resistant strains were isolated from two different pigs on the same farm; both pigs had CDI. On the investigated farm, the owner reported using oxytetracycline in animal feed despite the ban.

Six strains (11.1%) were had high MIC values for three different antimicrobials (tylosin, erythromycin and oxytetracycline); five of the strains were isolated from pigs and one from a human. One of these strains was isolated from a piglet with CDI and was also resistant to penicillin. Antibiotic therapy plays a central role in CDI development, and the risk increases considerably when ***C. difficile*** is also resistant to the antimicrobial used (OWENS et al., 2008). Considering that CDIs may be zoonotic, this study indicates that antimicrobials must be used rationally, especially in pig production. Moreover, further studies are needed aiming alternative methods to prevent, control and treat CDIs in domestic animals, what would further reduce the need for antimicrobials in these species.

The results from these antimicrobial susceptibility tests should be interpreted with caution because *in vitro* results do not always reflect the *in vivo* behavior of the evaluated drug. Furthermore, studies have shown that the majority of ***C. difficile*** isolates from drug-induced CDI cases in humans were susceptible *in vitro* to the drug used (DZINK & BARTLETT, 1980), which suggests that successful treatment depends on both ***C. difficile*** susceptibility to antimicrobials and other factors related to the microbiota (BÅVERUD, 2002). In any case, because it is not routinely performed, assessing antimicrobial susceptibility of ***C. difficile*** strains isolated from humans and animals, including the two strains isolated from wild animals, may be useful in treating enteric disorders caused by this agent. This is the first study to evaluate antimicrobial susceptibility in ***C. difficile*** strains isolated from animals in Brazil and showed the necessity of control measures of CDI in both humans and animals to avoid the spread of multi resistant strains. Additional studies should also include the ribotyping and evaluation of resistance genes for the strains used herein.

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