



Semina: Ciências Agrárias

ISSN: 1676-546X

semina.agrarias@uel.br

Universidade Estadual de Londrina  
Brasil

de Oliveira Júnior, Carlos Augusto; Silveira Silva, Rodrigo Otávio; Diniz, Amanda Nadia;  
Sadanã Pires, Prhiscylla; Masiero Salvarani, Felipe; Antunes de Assis, Ronnie; Faria  
Lobato, Francisco Carlos

Antimicrobial susceptibility of *Clostridium perfringens* isolated from domestic and wild  
animal species in Brazil

Semina: Ciências Agrárias, vol. 37, núm. 1, enero-febrero, 2016, pp. 257-262

Universidade Estadual de Londrina  
Londrina, Brasil

Available in: <http://www.redalyc.org/articulo.oa?id=445744667049>

- How to cite
- Complete issue
- More information about this article
- Journal's homepage in redalyc.org

redalyc.org

Scientific Information System

Network of Scientific Journals from Latin America, the Caribbean, Spain and Portugal

Non-profit academic project, developed under the open access initiative

# Antimicrobial susceptibility of *Clostridium perfringens* isolated from domestic and wild animal species in Brazil

## Susceptibilidade antimicrobiana de *Clostridium perfringens* isolados de animais domésticos e espécies silvestres no Brasil

Carlos Augusto de Oliveira Júnior<sup>1</sup>; Rodrigo Otávio Silveira Silva<sup>2\*</sup>;  
Amanda Nadia Diniz<sup>3</sup>; Prhiscylla Sadanã Pires<sup>4</sup>; Felipe Masiero Salvarani<sup>5</sup>;  
Ronnie Antunes de Assis<sup>6</sup>; Francisco Carlos Faria Lobato<sup>7</sup>

### Abstract

*Clostridium perfringens* is a microorganism commonly found in the microbiota of humans and animals and a potential cause of enteric, muscle or nervous diseases. The treatment of these diseases is based on antimicrobial therapy and it is extremely important to know the antimicrobial susceptibility profile of the strains present in the region. The aim of this study was to evaluate the antimicrobial susceptibility of *C. perfringens* isolated from domestic and wild animals in Brazil against seven different antimicrobials. Forty-one strains from the stool samples of cattle (n = 12), buffalo (n = 2), goat (n = 3), dogs (n = 12) and wild carnivores (n = 12) were examined. The minimum inhibitory concentration was determined by the agar dilution method using *Brucella* agar supplemented with 5% of sheep blood, 0.1% of vitamin K, 0.1% of hemin and concentrations ranging from 0,25 to 256,0 mg L<sup>-1</sup> of the following antibiotics: erythromycin, florfenicol, metronidazole, oxytetracycline, penicillin, tylosin, and vancomycin. All *C. perfringens* strains were susceptible to florfenicol, metronidazole, penicillin and vancomycin. Two strains (4.9%) were resistant to erythromycin and tylosin, while five (12.2%) were resistant to oxytetracycline, one of which (2.4%) from an ocelot.

**Key words:** Resistance, sensibility antibiotics, buffalo, cattle, dogs, carnivores

### Resumo

*Clostridium perfringens* é um microrganismo comumente encontrado na microbiota de seres humanos e animais e potencial causador de enfermidades entéricas, musculares ou neurológicas. O tratamento das enfermidades é baseado em terapia antimicrobiana, sendo de extrema importância conhecer o perfil de susceptibilidade antimicrobiana das estirpes presentes na região em questão. O presente estudo teve como objetivo avaliar a susceptibilidade de estirpes de *C. perfringens* isolados de animais domésticos

<sup>1</sup> Discente de Doutorado em Ciência Animal, Escola de Veterinária da Universidade Federal de Minas Gerais, EV/UFMG, Belo Horizonte, MG, Brasil. E-mail: carlos.dirgel@hotmail.com

<sup>2</sup> Estagiário Pós-Doutorado (CNPq), EV/UFMG, Belo Horizonte, MG, Brasil. E-mail: rodrigo.otaviosilva@gmail.com

<sup>3</sup> Discente Mestrado em Ciência Animal, EV/UFMG, Belo Horizonte, MG, Brasil. E-mail: amanda.ndiniz@gmail.com

<sup>4</sup> Prof<sup>a</sup>, Centro Universitário de Belo Horizonte, Centro Universitário UniBH, Belo Horizonte, MG, Brasil. E-mail: prisadana.ufmg@hotmail.com

<sup>5</sup> Prof., Adjunto I, Universidade Federal do Pará, UFPA, Castanhal, PA, Brasil. E-mail: felipemasiero@yahoo.com.br

<sup>6</sup> Pesquisador do Laboratório Nacional Agropecuário em Minas Gerais, LANAGRO/MG, Belo Horizonte, MG, Brasil. E-mail: ronnieassis@gmail.com

<sup>7</sup> Prof. Titular da Escola de Veterinária, Universidade Federal de Minas Gerais, EV/UFMG, Belo Horizonte, MG, Brasil. E-mail: lobato.francisco@yahoo.com.br

\* Author for correspondence

e carnívoros silvestres no Brasil frente a sete diferentes antimicrobianos. Foram utilizados 41 isolados originários de bezerros (n = 12), búfalos (n = 2), caprinos (n = 3), cães (n = 12) e espécies de carnívoros silvestres (n = 12). A concentração inibitória mínima foi determinada pelo método de diluição seriada em agar, utilizando-se o agar *Brucella* suplementado com 5% de sangue ovino, 0,1% de vitamina K, 0,1% de hemina e concentrações variando de 0,25 a 256,0 mg L<sup>-1</sup> dos seguintes antimicrobianos: eritromicina, florfenicol, metronidazol, oxitetraciclina, penicilina, tilosina e vancomicina. Todas as estirpes de *C. perfringens* testadas foram sensíveis ao florfenicol, metronidazol, penicilina e vancomicina. Dois isolados (4,9 %) foram resistentes a eritromicina e a tilosina, enquanto que cinco (12,2 %) estirpes foram resistentes a oxitetraciclina, sendo uma delas (2,4 %) proveniente de uma jaguatirica.

**Palavras-chave:** Resistência, sensibilidade, antibióticos, búfalo, bezerros, cães, carnívoros

*Clostridium perfringens* is a Gram-positive, anaerobic bacillus, Classified into five types (A - E) according to the production of four major toxins (alpha, beta, epsilon and iota) and commonly isolated from the environment or gastrointestinal tract of mammals and birds (SILVA et al., 2009; SILVA; LOBATO, 2015). In animals, *C. perfringens* type A causes diarrhea in dogs, pigs and cattle (SILVA; LOBATO, 2015) and also causes gas gangrene in ruminants (GONÇALVES et al., 2006). *C. perfringens* type D causes enterotoxemia in bovine, caprine and ovine species, associated with overfeeding and subsequent hemorrhagic enterocolitis, severe neurological signs and sudden death in some cases (FACURY-FILHO et al., 2009). Despite the importance of *C. perfringens* as a pathogen for humans and animals, the role of this agent in wild carnivores remains unknown. The current understanding of *C. perfringens* pathogenicity is based on reported cases of hemorrhagic enterocolitis and nervous disorders (ZEIRA et al., 2012).

The treatment of *C. perfringens*-associated diseases primarily involves antibiotic therapy (RAMSEY; TENNANT, 2010), and several studies have evaluated the *in vitro* antimicrobial susceptibility of *C. perfringens* to commonly used drugs (JOHANSSON et al., 2004; MARKS; KATHER, 2003; SALVARANI et al., 2012; SILVA et al., 2009; TANSUPHASIRI et al., 2005). However, these studies only assessed isolates from a single animal species and were restricted to strains from domestic animals. Thus, recent reports have highlighted the

need for an empirical analysis of the most effective antimicrobial for treating *C. perfringens* infections in wild animals (ZEIRA et al., 2012).

Furthermore, some studies concerning the antimicrobial susceptibility of *C. perfringens* strains isolated from animals have shown a marked difference in resistance patterns for each country, regardless of the species examined, highlighting the importance of studies using local strains (SALVARANI et al., 2012; SLAVIĆ et al., 2011; SILVA et al., 2009). Despite this known importance, there are no studies concerning the antimicrobial susceptibility of *C. perfringens* isolates from animal species, such as cattle, buffaloes, goats and dogs, in Brazil. Therefore, the aim of this study was to evaluate the *in vitro* antimicrobial susceptibility of *C. perfringens* strains isolated from dogs, goats, cattle, buffaloes and wild carnivores to erythromycin, florfenicol, metronidazole, oxytetracycline, penicillin, tylosin, and vancomycin.

A total of 41 *C. perfringens* strains previously isolated from the stool samples of buffaloes, goats, calves, dogs and wild carnivores were examined in this study (table 1). Of the 41 samples, 38 samples were genotyped as *C. perfringens* type A using multiplex-PCR (VIEIRA et al., 2008), and the remaining three strains, isolated from goats with confirmed enterotoxemia, were genotyped as *C. perfringens* type D. Among the 12 *C. perfringens* strains isolated from wild carnivores, five strains were isolated from crab-eating fox (*Cerdocyon thous*), three strains were isolated from cougar (*Puma concolor*), and a single strain each was isolated

from onçeira (*Leopardus tigrinus*), jaguarundi (*Puma yagouaroundi*), maned-wolf (*Crysocyon brachyurus*) and ocelot (*Leopardus pardalis*). The maned-wolf and the ocelot that the strains were

isolated from were under antibiotic therapy when the stool samples were collected. These animals were sampled at shelters for the rehabilitation of wild animals in Brazil.

**Table 1.** Number and origin, according to animal species and clinical history, of the *Clostridium perfringens* strains evaluated.

Species	Clinical History	Number of strains	Total
Cattle	Diarrheic	12	12
Buffaloes	Diarrheic	2	2
Dogs	Diarrheic	2	12
	Apparently healthy	10	
Goats	Confirmed enterotoxemia by <i>C. perfringens</i> type D	3	3
Wild carnivores	Apparently healthy	11	12
	Diarrheic	1	
Total			41

The minimum inhibitory concentration (MIC) was determined using agar dilution method, as recommended (Clinical and Laboratory Standards Institute, CLSI, 2011). The following antimicrobials were tested: penicillin, florfenicol, oxytetracycline, erythromycin, vancomycin, metronidazole and tylosin. The reference specimen *Bacterioides fragilis* (ATCC 25285) was used as a control. 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 256.0 µg mL<sup>-1</sup>.

The tests were performed on Brucella agar (Difco Laboratories, USA) supplemented with 5 % sheep blood, 10 µL of hemin and 10 µL of vitamin K (CLSI, 2011).

The results of the MIC analysis for the 41 *C. perfringens* strains are summarized in table 2. All strains were susceptible to florfenicol, metronidazole, penicillin and vancomycin. Two strains (4.9 %) were resistant to erythromycin and tylosin, whereas five strains (12.2 %) were resistant to oxytetracycline.

**Table 2.** Distribution of the minimal inhibitory concentrations (µg mL<sup>-1</sup>) and classification in susceptible, moderately susceptible and resistant for Forty-one *Clostridium perfringens* strains isolated from cattle, buffaloes, dogs, goats and wild carnivores.

Antimicrobial	Number of strains and MIC (µg mL <sup>-1</sup> )												Classification (%)		
	0.25	0.5	1	2	4	8	16	32	64	128	256	>256	S <sup>1</sup>	MS <sup>2</sup>	R <sup>3</sup>
Florfenicol			1	40									100	0	0
Metronidazole	25	16											100	0	0
Penicillin	41												100	0	0
Vancomycin	11	29	1										100	0	0
Erythromycin		2	18	19								2	95.1	0	4.9
Tylosin	20	19										2	95.1	0	4.9
Oxytetracycline		10	14	8			4	1				4	78.0	9.8	12.2

S<sup>1</sup>: Susceptible, MS<sup>2</sup>: moderately susceptible. R<sup>3</sup>: resistant (R). Classification based on the recommendations of the CLSI (2011).

In the present study, all isolates were susceptible to penicillin, consistent with previous studies of strains isolated from various domestic species (GHARAIBEH et al., 2010; SLAVIĆ et al., 2011; SILVA et al., 2009). The beta-lactams are commonly used for the treatment of *C. perfringens*-associated diseases, and several studies have suggested that these antibiotics exhibit satisfactory results both *in vitro* and *in vivo* (MOODY et al., 1990). Although penicillin-resistant *C. perfringens* strains have previously been reported (TANSUPHASIRI et al., 2005), this event is rare and has not been identified in studies of *C. perfringens* strains isolated from foals, piglets and broiler chickens in Brazil (SALVARANI et al., 2012; SILVA et al., 2009, 2013a).

Similar to previous reports, strains resistant to vancomycin and florfenicol were not observed in this study (GHOLAMIANDEHKORDI et al., 2009; JOHANSSON et al., 2004; SILVA et al., 2013a). Commonly used against infections with multi-resistant microorganisms, reports of *C. perfringens* resistance to vancomycin are rare (TANSUPHASIRI et al., 2005). Moreover, in contrast to these results, recent studies have reported florfenicol-resistant *C. perfringens* isolates from cattle, chickens and pigs (GHARAIBEH et al., 2010; SALVARANI et al., 2012; SLAVIĆ et al., 2011).

Consistent with the results obtained in the present study, other authors have reported that metronidazole, a drug commonly used to treat diarrhea in dogs and cattle, shows high efficacy *in vitro* against *Clostridium* (MARKS, KATHER, 2003). Notably, *C. perfringens* strains resistant to this antimicrobial have also been increasingly documented (MARKS; KATHER, 2003; SLAVIĆ et al., 2011). Weese et al. (2001) reported a case of recurrent diarrhea from metronidazole-resistant *C. perfringens*, suggesting that clinicians must be aware of potential antimicrobial resistance when using metronidazole to treat diseases caused by this bacterium.

Two strains (4.9 %), isolated from healthy dogs, were resistant to macrolides (erythromycin and tylosin). Although studies with poultry (GHOLAMIANDEHKORDI et al., 2009; JOHANSSON et al., 2004) and pigs (SALVARANI et al., 2012) have reported a high number of *C. perfringens* strains susceptible to these antibiotics, resistance to these compounds is not rare (GHARAIBEH et al., 2010; SLAVIĆ et al., 2011). Importantly, two out of 12 samples isolated from dogs (16.7 %) were resistant to erythromycin and tylosin, representing a higher rate than the 1.6 % resistant strains previously reported (MARKS, KATHER, 2003) in a study with *C. perfringens* strains isolated from dogs in Canada, but lower than the 17 and 58 % of strains resistant to tylosin and erythromycin, respectively, reported in a recent study with foals in Brazil (SILVA et al., 2013a).

Moreover, the bimodal distribution for erythromycin and tylosin suggests the presence of resistance genes to these compounds. Previous reports have demonstrated that the resistance of *C. perfringens* strains to these antibiotics is commonly associated with the presence of *erm* (erythromycin ribosomal methylase) genes, which encode methylases that inhibit the action of most macrolides (SLAVIĆ et al., 2011). However, to date, there are no studies reporting the detection of antimicrobial resistance genes in *C. perfringens* strains isolated in Brazil.

The antimicrobial, oxytetracycline, had the highest number of resistant strains (12.1 %) in this study, but this percentage is low compared with previously reports in several species (MARKS; KATHER, 2003; SALVARANI et al., 2012; SLAVIĆ et al., 2011; SILVA et al., 2009). Resistance to this class of antibiotics has been attributed to the presence of tetracycline resistance genes, such as *tetA* and *tetB*, which are commonly found in *Clostridium* strains (CHOPRA, ROBERTS, 2001; JOHANSSON et al., 2004).

The five oxytetracycline-resistant strains in this study were isolated from two dogs, two goats (*C. perfringens* type D) and an ocelot. This study provides the first evaluation of the antimicrobial susceptibility of a strain of *C. perfringens* isolated from ocelot and is thus the first report of oxytetracycline resistance in strains isolated from this species. Recently, SILVA et al. (2013b) reported *Clostridium difficile*-associated diarrhea in ocelot after antibiotic therapy, suggesting the importance of the genus *Clostridium* as an enteropathogen in this species, particularly in captive animals.

Previous studies have reported the antimicrobial susceptibility of *C. perfringens* type D (SLAVIĆ et al., 2011) or type A (BRAZIER et al., 1985) strains isolated from goats, but this is the first study to evaluate the antimicrobial susceptibility of *C. perfringens* type D strains from goats with confirmed enterotoxemia. Surprisingly, two out of three tested strains were resistant to oxytetracycline. Notably, these three strains were obtained from animals belonging to three different farms from distant states in Brazil (Bahia, Minas Gerais and Rio Grande do Sul). Unfortunately, there are no data concerning the administration of antimicrobials in these animals. Nevertheless, this result encourages further studies, using a larger number of samples and focusing on the antimicrobial susceptibility of strains of *C. perfringens* type D from goats.

No multidrug resistant strains were identified in the present study, suggesting that no isolate showed simultaneously resistance to three or more drugs (CLSI, 2011). However, in this study, we isolated canine *C. perfringens* strain showing resistance to both erythromycin and tylosin and moderate sensitivity to oxytetracycline, indicating a profile similar to multidrug resistance.

The evaluation of antimicrobial susceptibility might be useful to guide clinicians during the treatment of diseases caused by this pathogen. This study provides the first characterization of the antimicrobial susceptibility of *C. perfringens*

strains from wild carnivores and is the first study to consider isolates from canines, cattle, buffaloes and goats in Brazil. Future studies should evaluate the presence of resistance genes in the tested strains.

## Acknowledgements

This study was supported by funds from Capes, Fapemig and CNPq.

## References

- BRAZIER, J. S.; LEVETT, P. N.; STANNARD, A. J.; PHILLIPS, K. D.; WILLIS, A. T. Antibiotic susceptibility of clinical isolates of clostridia. *Journal of Antimicrobial Chemotherapy*, London, v. 15, n. 2, p. 181-185, 1985.
- CHOPRA, I.; ROBERTS, M. Tetracycline antibiotics: mode of action, application, molecular biology and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews*, Washington, v. 65, n. 2, p. 232-260, 2001.
- CLINICAL AND LABORATORY STANDARDS INSTITUTE - CLSI. Performance standards for Antimicrobial Susceptibility Test. Pennsylvania: Twenty-first Information Supplement, CLSI M100-S21, 2011.
- FACURY-FILHO, E. J.; CARVALHO, A. U.; ASSIS, R. A.; LOBATO, F. C. F.; RACHID, M. A.; CARVALHO, A. A.; FERREIRA, P. M.; NASCIMENTO, R. A.; FERNANDES, A. A.; VIDAL, J. E.; UZAL, F. A. Clinicopathologic features of experimental *Clostridium perfringens* type D enterotoxemia in cattle. *Veterinary Pathology*, London, v. 46, n. 6, p. 1213-1220, 2009.
- GHARAIBEH, S.; AL-RIFAI, R.; AL-MAJALI A. Molecular typing and antimicrobial susceptibility of *Clostridium perfringens* from broiler chickens. *Anaerobe*, London, v. 16, n. 6, p. 586-589, 2010.
- GHOLAMIANDEHKORDI, A.; EECKHAUT, V.; LANCKRIET, A.; TIMBERMONT, L.; BJERRUM, L.; DUCATELLE, R.; HAESBROUCK, F.; VAN IMMERSEEL, F. Antimicrobial resistance in *Clostridium perfringens* isolates from broilers in Belgium. *Veterinary Research Communications*, Edinburgh, v. 33, n. 8, p. 1031-1037, 2009.
- GONÇALVES, L. A.; FREITAS, T. D.; ASSIS, R. A.; FACURY FILHO, E. J.; LOBATO, F. C. F. Primeiro relato no Brasil de mastite necrótica bovina por *Clostridium perfringens* tipo A. *Ciência Rural*, Santa Maria, v. 36, n. 4, p. 1331-1333, 2006.

- JOHANSSON, A.; GREKO, C.; ENGSTRÖM, B. E.; KARLSSON, M. Antimicrobial susceptibility of Swedish, Norwegian and Danish isolates of *Clostridium perfringens* from poultry, and distribution of tetracycline resistance genes. *Veterinary Microbiology*, Amsterdam, v. 99, n. 3-4, p. 251-257, 2004.
- MARKS, S. L.; KATHER, E. J. Antimicrobial susceptibilities of canine *Clostridium difficile* and *Clostridium perfringens* isolates to commonly utilized antimicrobial drugs. *Veterinary Microbiology*, Amsterdam, v. 94, n. 1, p. 39-45, 2003.
- MOODY, J. A.; FASCHING, C. E.; SINN, L. M.; GERDING, D. N.; PETERSON, L. R. Comparative efficacy of cefoperazone, cefoperazone plus sulbactam, ciprofloxacin, clindamycin, metronidazole, and penicillin G against anaerobic bacteria in an animal model. *Journal of Laboratory and Clinical Medicine*, Milwaukee, v. 115, n. 2, p. 190-195, 1990.
- RAMSEY, I. K.; TENNANT, B. J. *Manual de doenças infecciosas em cães e gatos*. 3. ed. São Paulo: Roca, 2010.
- SALVARANI, F. M.; SILVA, R. O. S.; PIRES, P. S.; CRUZ JÚNIOR, E. C. C.; ALBEFARO, I. S.; GUEDES, R. M. C.; LOBATO, F. C. F. Antimicrobial susceptibility of *Clostridium perfringens* isolated from piglets with or without diarrhea in Brazil. *Brazilian Journal of Microbiology*, São Paulo, v. 43, n. 3, p. 1030-1033, 2012.
- SILVA, R. O. S.; SALVARANI, F. M.; ASSIS, R. A.; MARTINS, N. R. S.; PIRES, P. S.; LOBATO, F. C. F. Antimicrobial susceptibility of *Clostridium perfringens* strains isolated from broiler chickens. *Brazilian Journal of Microbiology*, São Paulo, v. 40, n. 2, p. 262-264, 2009.
- SILVA, R. O. S.; RIBEIRO, M. G.; PALHARES, M. S.; BORGES, A. S.; MARANHÃO, R. P.; SILVA, M. X.; LUCAS, T. M.; OLIVO, G.; LOBATO, F. C. F. Detection of A/B toxin and isolation of *Clostridium difficile* and *Clostridium perfringens* from foals. *Equine Veterinary Journal*, Lexington, v. 45, n. 6, p. 671-675, 2013a.
- SILVA, R. O. S.; D'ELIA, M. L.; SOARES, D. F. M.; CAVALCANTI, A. R.; LEAL, R. C.; CAVALCANTI, G.; PEREIRA, P. L. L.; LOBATO, F. C. F. *Clostridium difficile*-associated diarrhea in an ocelot (*Leopardus pardalis*). *Anaerobe*, London, v. 20, p. 82-84, 2013b.
- SILVA, R. O. S.; LOBATO, F. C. F. *Clostridium perfringens*: a review of enteric diseases in dogs, cats and wild animals. *Anaerobe*, London, v. 33, p. 14-17, 2015.
- SLAVIĆ, Đ.; BOERLIN, P.; FABRI, M.; KLOTINS, K. C.; ZOETHOUT, J.; WEIR, P. E.; BATEMAN, D. Antimicrobial susceptibility of *Clostridium perfringens* isolates of bovine, chicken, porcine, and turkey origin from Ontario. *Canadian Journal of Veterinary Research*, Ottawa, v. 75, n. 2, p. 89-97, 2011.
- TANSUPHASIRI, U.; MATRA, W.; SANGSUK, L. Antimicrobial resistance among *Clostridium perfringens* isolated from various sources in Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, Bangkok, v. 36, n. 4, p. 954-961, 2005.
- VIEIRA, A. A. S.; GUEDES, R. M. C.; SALVARANI, F. M.; SILVA, R. O. S.; ASSIS, R. A.; LOBATO, F. C. F. Genotipagem de *Clostridium perfringens* isolados de leitões diarreicos. *Arquivos do Instituto Biológico*, São Paulo, v. 75, n. 4, p. 513-516, 2008.
- WEESE, J. S.; GREENWOOD, S. J.; STCEMPFLI, H. R. Recurrent diarrhea associated with enterotoxigenic *Clostridium perfringens* in 2 dogs. *Canadian Veterinary Journal*, Ottawa, v. 42, n. 4, p. 292-294, 2001.
- ZEIRA, O.; BRIOLA, C.; KONAR, M.; DUMAS, M. P.; WRZOSEK, M. A.; PAPA, V. Suspected neurotoxicity due to *Clostridium perfringens* type B in a tiger (*Panthera tigris*). *Journal of Zoo and Wildlife Medicine*, Yulee, v. 43, n. 3, p. 666-669, 2012.