SCIENTIFIC ARTICLE

Campylobacter spp. isolates of swine feces submitted to transport stress: species and antimicrobial resistance

Campylobacter spp. isolados de fezes de suínos submetidos a estresse de transporte: espécies e resistência antimicrobiana

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Roberta Torres de Melo[1], Mônica Ribeiro Gabriel[2], Eliane Pereira Mendonça[3], Jéssica Laura Miranda Peixoto[4], Guilherme Paz Monteiro[5], Phelipe Augusto Borba Martins Peres[6], Edson Campos Valadares Junior[7] & Daise Aparecida Rossi[8]

[1]Veterinary, Dr. Núcleo de Saúde Pública, Faculdade de Medicina Veterinária, Universidade Federal de Uberlândia – UFU, Uberlândia, MG, Brasil
[2]Veterinary, MSc. Laboratório de Epidemiologia Molecular, Faculdade de Medicina Veterinária, Universidade Federal de Uberlândia – UFU, Uberlândia, MG, Brasil
[3]Veterinary, Dr. Laboratório de Biologia Molecular, Faculdade de Medicina Veterinária, Universidade de Uberaba – UNIUBE, Uberaba, MG, Brasil
[4]Veterinary. Laboratório de Epidemiologia Molecular, Faculdade de Medicina Veterinária, Universidade Federal de Uberlândia – UFU, Uberlândia, MG, Brasil
[5]Biologist, MSc. Laboratório de Epidemiologia Molecular, Programa de Pós-graduação em Ciências Veterinárias, Universidade Federal de Uberlândia – UFU, Uberlândia, MG, Brasil
[6]Biologist. Laboratório de Epidemiologia Molecular, Programa de Pós-graduação em Ciências Veterinárias, Universidade Federal de Uberlândia – UFU, Uberlândia, MG, Brasil
[7]Veterinary. Laboratório de Epidemiologia Molecular, Programa de Pós-graduação em Ciências Veterinárias, Universidade Federal de Uberlândia – UFU, Uberlândia, MG, Brasil
[8]Veterinary, Dr. Laboratório de Epidemiologia Molecular, Programa de Pós-graduação em Ciências Veterinárias, Universidade Federal de Uberlândia – UFU, Uberlândia, MG, Brasil

Abstract

The influence of transport stress in the presence of *Campylobacter* spp. and the antimicrobial resistance profile were evaluated in feces of 60 pigs. The samples were collected at the finishing farm and after transport to the slaughterhouse, totaling 120 samples. Isolation was performed by plaque culture and identification of the species was obtained by biochemical tests confirmed with the PCR technique. *Campylobacter* spp. was isolated in 63.3% of the collected samples at the farm and 91.6% at the slaughterhouse, evidencing the influence of transport stress on the increase of the isolates (P<0.05). The species *C. coli* biotype I, *C. jejuni* biotype I and *C. jejuni* subsp. *doylei* were identified, with *C. coli* being more prevalent on the farm and *C. jejuni* in the slaughterhouse. Bacterial resistance was observed for all six classes of antibiotics tested. Among them, the isolates presented greater resistance to lincomycin, tetracycline and nalidixic acid (98.9%), and greater sensitivity to amoxicillin (22.5%). The strains of *C. coli* showed higher antimicrobial resistance than those of *C. jejuni* (P<0.05). The results of this study point to the high isolation rates of *C. coli* and *C. jejuni* in pig feces destinated for slaughter and possible risks related to meat consumption. The high standards of resistance address the risk to public health.

Keywords: antibiotics, campylobacteriosis, feces, swine breeding.

Resumo

Avaliou-se a influência do estresse de transporte na presença de *Campylobacter* spp. em fezes suínas de 60 animais sua resistência aos antimicrobianos. As coletas foram realizadas na granja de terminação e após o transporte ao abatedouro, totalizando 120 amostras. O isolamento foi realizado pelo cultivo em placa e a identificação das espécies obtida por testes bioquímicos e PCR. *Campylobacter* spp. foi isolado de 63,3% das amostras coletadas na granja e 91,6% no abatedouro, evidenciando a influência do estresse de transporte no aumento da isolamentos (P<0,05). Foram identificadas as espécies *C. coli* biótipo I, *C. jejuni* biótipo I e *C. jejuni* subsp. *doylei*, sendo que *C. coli* foi mais prevalente na granja e *C. jejuni* no abatedouro. Foi observada resistência bacteriana para todas as seis classes de antibióticos testados. Dentre estes, os isolados apresentaram maior resistência à lincomicina, tetraciclina e ácido nalidíxico (98,9%), e maior sensibilidade à amoxacilina (22,5%). As cepas de *C. coli* apresentaram maior resistência antimicrobiana que as de *C. jejuni* (P<0,05). Os resultados deste estudo alertam para os altos índices de isolamento de

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***Correspondence**
Roberta Torres de Melo
Faculdade de Medicina Veterinária,
Universidade Federal de Uberlândia – UFU
Rua Ceará s/n, Bloco 2D sala 43, Campus Umuarama
CEP 38408-018 - Uberlândia (MG), Brasil
E-mail: roberta-melo@hotmail.com

C. coli e *C. jejuni* em fezes de suínos destinados ao abate e possíveis riscos relacionados ao consumo da carne. Os altos padrões de resistência atentam para o risco à saúde pública.

Palavras-Chave: antibióticos, campilobacteriose, fezes, suinocultura.

Introduction

The Brazilian swine breeding occupy a prominent position on the world stage, as the fourth largest producer and the fourth largest exporter of pork. The production of pork in the country in 2016 showed a percentage increase of 2.42% compared to the previous year, producing 3.73 million tons of pork (Associação Brasileira de Proteína Animal, 2017).

Given the importance of the country in the sector, there is a need for special care in relation to food safety to ensure the sanitary quality of food. Providing safe food to consumers is a major challenge, and its neglect can lead to the growth of micro-organisms that cause serious infections and poisoning, such as campylobacteriosis, that can lead from simple intestinal discomfort to neurological disorders and death (Miranda & Barreto, 2012).

Campylobacter is highlighted as one of the main causers of food-borne human gastroenteritis in the world (World Health Organization, 2013). Pigs may be reservoirs for *Campylobacter* spp. acting as a potential biological contaminant in foods of animal origin (Haruna et al., 2013). Infection in humans occurs through direct contact with carrier animals, through ingestion of raw or poorly processed meat from poultry, swine and cattle or untreated or contaminated feces containing the pathogen (Food and Drug Administration, 2008).

In a study on the prevalence of *Campylobacter* spp. in pig feces, Burrough et al. (2013) pointed out that 82.6% of the collected samples were positive for this pathogen. A study conducted in Brazil by Silva et al. (2012) in samples of feces and pig carcasses showed that *Campylobacter coli* was the most prevalent species in swine.

Animal exposure at all stages of production from farm to slaughter may be an important factor in *Campylobacter* infection of pigs (Castillo Neyra et al., 2012). The knowledge of the predisposing factors for contamination and their impact along the production chain allows the introduction of control measures in the production process, which is an alternative to guarantee the safety of the food produced. The aim of this study was to determine the isolation of *Campylobacter* spp. in pig feces before and after transport stress (from finishing farm to slaughterhouse), the diversity of isolated species and their antimicrobial susceptibility profile.

Material and Methods

The study was performed in a pig finishing farm and in a slaughterhouse under federal inspection located in the region of Triângulo Mineiro, Minas Gerais, Brazil. A total of 120 fecal samples were collected through rectal swab.

The evaluated animals came from a farm located approximately 200km from the slaughterhouse, being transported during the night. The period of animals transportation was approximately three hours and the time between arrival at the slaughterhouse and the collection was approximately one hour.

The collections were done in 60 animals in the finishing farm and in these same animals after transportation to the slaughterhouse and already settled in the waiting pen. The collections were divided into three different batches of 20 animals in the finishing phase (age of 138 - 140 days) and with an average weight of 90 kg.

For transport, the swabs were kept in transport medium (5mL of sterile buffered peptone water) and sent immediately to the laboratory. For isolation of *Campylobacter* spp. and biochemical identification, the protocol of analysis used was the traditional cultivation in plates with pre-enrichment, according to recommendations of Fernández (2011) with modifications according to ISO 10272-1 (International Organization for Standardization, 2006).

Pre-enrichment was based on the addition of a 2.5mL aliquot of sterile buffered peptone water with 2.5mL of Bolton broth (Oxoid®) supplemented with antibiotic mixture (10mg of cefoperazone, 10mg of vancomycin, 10mg of trimethoprim and 25mg of cycloheximide) (Selective Supplement Oxoid®), both in double concentration and added with 5% of hemolyzed equine blood, incubated

under a microaerophilic atmosphere (Probac do Brasil®) at 37ºC for 4 to 6 hours and 41.5ºC for 44 hours ± 4 hours .

The pre-enriched broth was seeded on *Campylobacter* Blood-Free Selective Medium (modified CCDA) agar (Oxoid®) with antibiotic supplement (16mg of cefoperazone and 32mg of amphotericin B) (Oxoid®) and incubated at 41.5ºC for 44 hours ± 4 hours in microaerophilia (Probac do Brasil®). Colonies suspected of belonging to the genus *Campylobacter* were confirmed by modified Gram staining (use of carboxifuccin replacing safranin) and species were identified by catalase, oxidase, sulfide production (H2S), nitrate reduction (NO3), hippura hydrolysis and DNAse test (for biotyping) (Fernández, 2011).

Campylobacter jejuni (ATCC 33291), *Campylobacter coli* (ATCC 43478) and a *Campylobacter jejuni* strain (IAL 2383) isolated from humans were used as controls.

Identification by molecular polymerase chain reaction (PCR) was performed in parallel with the traditional identification methodology for confirmation of *C. jejuni* positive strains in biochemical tests. The primers and the protocol used in the reaction and amplification were followed according to Hänel et al. (2004). The primers used for identification of *C. jejuni* comprise the *flaA* gene: *flaA*-F (5'ATGGGATTTCGTATTAACAC3') and *flaA*-R (5' CTGTAGTAATCTTAAAACATTTTG3').

The final volume for the amplification reaction was 50µL, composed of 20ng of the bacterial DNA solution (extracted by the Wizard® Genomic DNA Purification kit - Promega®) and by the following reagents: 10mM of Tris-HCL; 50mM of KCl; 200µM of each triphosphate deoxynucleotide (DNTP); 5.5 mM of $MgCl_2$; 20picomoles of flaA-F and flaA-R and 1.25U of Taq DNA polymerase.

The thermocycler amplification (Eppendorf®) obeyed the following cycles: an initial denaturation cycle at 94 ° C for 4 minutes; 25 amplification cycles consisting of 3 stages: denaturation at 94 ° C for one minute, annealing at 47 ° C for one minute and extension at 72 ° C for one minute; completing with another final extension cycle at 72 ° C for 7 minutes. The volume of 5µL of the amplification products were subjected to 1.5% agarose gel electrophoresis stained with Syber Safe (Invitrogen®), using as a molecular weight standard the 100bp marker (Invitrogen®).

For the evaluation of antimicrobial resistance, the disc diffusion test was used with addition of 5% of defibrinated goat blood (Laborclin®) to Mueller-Hinton agar (Difco™) and incubation in a microaerophilic atmosphere at 37 ° C per 48 hours (Clinical and Laboratory Standards Institute, 2010). The antimicrobials tested were: nalidixic acid (30µg), amoxicillin (10µg), erythromycin (15µg), gentamicin (10µg), lincomycin (9/100µg), neomycin (30µg), norfloxacin (10µg) and tetracycline (30µg).

The results were submitted to descriptive statistics, with the calculation of the percentages of isolation and antimicrobial resistance. In order to compare the different proportions of positivity of the batches, the McNemar Test with a significance of 5% was used (Ayres et al., 2000). The confidence limits for the analysis of differences in the resistance ratios of *C. coli* and *C. jejuni* were established through the Binomial Proportions test with 95% of confidence. Calculations were performed using the GraphPadPrism program.

Results

The percentage of *Campylobacter* spp. in feces samples from the farm was 63.3% (38/60) and 91.6% (55/60) after transportation at the slaughterhouse.

There was a significant difference between the groups before and after transport ($P < 0.05$) indicating that the stress period may influence the increase in the excretion rate of the microorganism, resulting in an increase in the number of isolates as presented in Table 1.

Campylobacter coli biotype I was identified in 53 (57.0%) samples (33 from the farm and 20 from the slaughterhouse), *Campylobacter jejuni* biotype I in 20 (21.5%) samples (five from the farm and 15 from the slaughterhouse) and *Campylobacter jejuni* subspecie *doylei* in 20 (21.5%) samples from the slaughterhouse.

In the samples collected after transport to the slaughterhouse there was isolation of *C. jejuni* subspecie *doylei*, which was not previously identified in the animals settled in the termination farm, probably because it was present in an amount insufficient to be detected by the traditional method used.

Table 1. Positivity to Campylobacter spp. in feces samples collected on the farm and at the slaughterhouse in three different batches of finishing pigs.

SAMPLES / COLLECTION POINT	Batch A (n=20)	Batch B (n=20)	Batch C (n=20)	Total (n=60)
	+ (%)	+ (%)	+ (%)	+ (%)
Feces swab/farm	13 (65)	10 (50)	15 (80)	38(63.3) [a]
Feces swab /slaughterhouse	16 (80)	19 (95)	20 (100)	55 (91.6) [b]
TOTAL (n=120)	29 (72.5)	29 (72.5)	35 (87.5)	93 (77.5)

+ positive samples; n - number of collected animals; [a/b] - different letters in the same column indicate that there was significant difference by the McNemar Test (P <0.05 - probability of significance).

Table 2. Distribution and antibiotic resistance profile of strains of *C. coli* and *C. jejuni*, isolated from feces of pigs settled on the farm and at the slaughterhouse pen.

Antibiotics	Farm (n=38)		Slaughterhouse (n=55)		Total (n=93)	
	C. coli (n=33)	*C. jejuni* (n=5)	*C. coli* (n=20)	*C. jejuni* (n=35)	*C. coli* (n=53)	*C. jejuni* (n=40)
	R (%)	R (%)	R (%)	R (%)	R (%)	R (%)
Nalidixic Acid	32 (96.97)	5 (100)	20 (100)	35 (100)	52 (98.11)	40 (100)
Amoxicillin	8 (24.24)	0	6 (30)	7 (20)	14 (26.41)	7 (17.5)
Erythromycin	33 (100)	5 (100)	18 (90)	30 (85.71)	51 (96.23)	35 (87.5)
Gentamicin	26 (78.79)	0	6 (30)	16 (45.71)	32 (60.38)*	16 (40)*
Lincomycin	33 (100)	5 (100)	19 (95)	35 (100)	52 (98.11)	40 (100)
Neomycin	26 (78.79)	1 (20)	16 (80)	17 (48.57)	42 (79.24)*	18 (45)*
Norfloxacin	26 (78.79)	5 (100)	12 (60)	29 (82.86)	38 (71.7)	34 (85)
Tetracycline	33 (100)	5 (100)	19 (95)	35 (100)	52 (98.11)	40 (100)

R - number of resistant strains; % - percentage of resistance; n - number of isolates; * - significant difference (P <0.05).

The results presented by PCR confirmed the presence of *Campylobacter jejuni* in the samples previously identified in the biochemical tests.

Resistance was observed to all eight antibiotics tested, distributed in six classes (Table 2). Most of the strains tested (72/93 - 77.4%) showed multiresistance characteristics, as they showed resistance to three or more antimicrobial classes (Magiorakos et al., 2012). Among the antibiotics tested, the isolates presented higher resistance to lincomycin (lincosamides), tetracycline (tetracycline) and nalidixic acid (fluoroquinolone) (92/93 - 98.9%), followed by erythromycin (macrolide) (83/93 - 92. 4%), norfloxacin (fluoroquinolone) (72/93 - 77.4%), neomycin (aminoglycoside) (60/93 - 64.5%), gentamicin (aminoglycoside) (48/93 - 51.6%) and amoxicillin (beta-lactam) (21/93 - 22.5%).

The antibiotic resistance was compared between *C. jejuni* and *C. coli* isolates (Table 2). There was a significant difference between the two species, with *C. coli* presenting higher resistance than *C. jejuni* for gentamicin (P = 0.0258) and neomycin (P = 0.0003).

Discussion

The high percentage of *Campylobacter* isolation was expected, considering the literature findings. *Campylobacter* spp. were found by Matthew-Belmar et al. (2015), Gwimi et al. (2015), Wysok et al. (2015), equivalent to 95.6% (172/180), 92.67% (278/300) and 29.8% (52/174), respectively, in pig feces samples.

In Brazil, *C. coli* and *C. jejuni* have been isolated from carcasses and feces of apparently healthy pigs abated in slaughterhouses, as well as from animals with clinical symptoms of enteric disorders manifested as diarrhea (Scarcelli et al., 1991; Gabriel et al., 2010; Silva et al., 2012).

The increase in the number of animals excreting *Campylobacter* proved that there is a possibility of the influence of transport stress on the increase of excretion of the agent and consequent infection of the animals. Harvey et al. (2001) found that transport of pigs may affect the prevalence of *Campylobacter* spp. under conditions of high temperatures and prolonged fasting. According to the same authors besides the increase in the number of infected animals there was also an increase in the amount of *Campylobacter* spp. with a variation of 5 to 7.2 log10 CFU / g, due to increased of cecal pH, which reduces bacterial competition and allows *Campylobacter* spp. to proliferate more quickly, data also analyzed by the authors.

Alter et al. (2005) that found a prevalence of *Campylobacter* spp. in 24-week-old pigs, ranging from zero on the farm to 78% after transporting the animals to the slaughterhouse.

According to Dalla Costa et al. (2010) pigs submitted to transport present at the end of the journey obvious symptoms of stress. The differences in the way that animals deal with stress are reflected in the immunological reactivity that may be reduced in order to facilitate the proliferation of enteric bacteria and their consequent spread in the environment (Tizard Ian, 2014).

Pigs are often asymptomatic carriers of *Campylobacter* spp. and this carrier status may increase the likelihood of contamination of carcasses during the slaughter process (Malakauskas et al., 2005). Studies by Rosenvold & Andersen (2003) have added that stress during transport is considered a favorable factor in the excretion of pathogenic microorganisms in production animals, corroborating the increase of the isolates after transport.

It is known that the greater amount of microorganisms in the sample raises the chances of their isolation in traditional methods of cultivation. Therefore, the increase in the number of isolates at the moment after transport to the slaughterhouse reflects the greater quantity and probability of contamination of the carcasses during the slaughter process, being this information relevant to the pathogen reduction / prevention policies in the industry.

Although there is still no legislation to control the microorganism in pork, the presence of *Campylobacter* began to be quantified in chicken carcasses in southern Brazil at the request of the European Union (União Europeia, 2017). Given that pork can also be a source of human campylobacteriosis, it is possible that in future importing countries will also extend the requirements for pork.

Among the species identified and unlike birds and cattle, *Campylobacter coli* was the most prevalent species on the farm. Silva et al. (2012), Haruna et al. (2013), Gwimi et al. (2015), also reported a higher prevalence of *C. coli* compared to *C. jejuni* in their findings. Alter et al. (2005) reported that *Campylobacter coli* can colonize 75% of piglets in the first week of life, and it is possible that this strain is part of the microbiota of these animals.

The isolation of *C. jejuni* biotype I and subsp. *doylei* of the samples collected after transport to the slaughterhouse probably indicates that they were already part of the faecal microbiota of these animals, in small numbers, and that factors such as stress or fasting were able to favor their multiplication to numbers identifiable by the techniques used.

The use of biochemical tests to identify the species of *Campylobacter* is limited by the occurrence of strains with atypical reactions. Small alterations in the amount of the inoculum, excessive subcultures, deletions of the hip gene and absence of its transcription can interfere in the correct identification of the species (Kolackova & Karpiskova, 2005). Although 100% of concordance was observed in the identification of *C. jejuni* by biochemical identification and PCR, the use of a genotypic identification method is useful for the rapid results and can be used in the case of strains with atypical responses in the identification phenotype.

The resistance identified for all antimicrobials indicates the difficulty in treatment in case of clinical disease in humans. Haruna et al. (2013) reported that resistance to these antimicrobial agents (nalidixic acid, enrofloxacin, oxytetracycline and dihydrostreptomycin) has already been observed in *Campylobacter* isolates obtained from pig feces samples. Resistance to antibiotics is relevant information, since campylobacteriosis is a disease transmitted primarily by the consumption of contaminated foods, particularly those of animal origin.

The results of this study are similar to the findings of Fraqueza et al. (2014) who observed high resistance to fluoroquinolone, tetracycline and macrolides and sensitivity to gentamicin and amoxaciclin in *Campylobacter* spp. in pigs. Sasaki et al. (2013) observed resistance in *Campylobacter* spp. collected from porcine liver to nalidixic acid, dihydrostreptomycin and oxytetracycline. Nguyen et al. (2016) found resistance to nalidixic acid, tetracycline and ciprofloxacin in percentages of 88.9%, 77.8% and 66.7%, respectively, in samples from chicken and pork meat.

Thakur & Gebreyes (2005) tested six antibiotics in *C. coli* strains isolated from an antimicrobial-free pig production system. The antibiotics, to which 97% of the microorganisms tested presented higher resistance, were: erythromycin, nalidixic acid and tetracycline. Ekkapobyotin et al. (2008) also found similar values when analyzing strains of *C. coli* isolated from swine. The strains had high levels of resistance to nalidixic acid (84%), tetracycline (81%) and erythromycin (66%).

The fact that *C. coli* has higher antimicrobial resistance when compared to *C. jejuni* has already been described by other authors. Pezzotti et al. (2003) in a study on the occurrence of *C. coli* and *C. jejuni* in cattle, pigs and chickens, compared antimicrobial resistance between the two species and concluded that *C. coli* was more resistant than *C. jejuni*, with tetracycline and streptomycin being the antibiotics with the greatest divergence. Little et al. (2008) also found resistance of *C. coli* to antibiotics: ampicillin, chloramphenicol, tetracycline, furazolidone, gentamicin, kanamycin, neomycin, nalidixic acid, ciprofloxacin and erythromycin.

The resistance patterns observed for antimicrobials of the macrolide (erythromycin) and fluoroquinolones (nalidixic acid and norfloxacin) classes are difficult to treat in case of infection, since they are drugs of choice for the treatment of campylobacteriosis (Ternhag et al., 2007). The increased occurrence of macrolide and fluoroquinolone-resistant infections in man has been reported in several countries (Thakur & Gebreyes, 2005; Ekkapobyotin et al., 2008; Fraqueza et al., 2014).

The routine practice of providing antibiotics to animals on farms for both prevention and therapy is an important factor in the emergence of bacterial resistance to antibiotics and can therefore be transferred to humans through the food chain (Angulo et al., 2000).

Due to the common appearance of pathogens that express multiresistance, there is an increase in cases of mortality in bacterial diseases, complications in treatments, and costs for the health system. The prudent use of antimicrobials, appropriate choice, dosage and appropriate duration helps to reduce the selective pressure of resistant microorganisms (Tenover, 2006).

Conclusion

There was a significant increase in the isolates of *Campylobacter* spp. in feces of finishing pigs evaluated after stress of transport of the animals to the slaughterhouse, which may reflect in the contamination of the carcasses during the slaughter. The identified species: *C. coli* biotype I, *C. jejuni* biotype I and *C. jejuni* subsp. *doylei* are etiological agents of human campylobacteriosis demonstrating that the findings are relevant to public health. Most of the strains studied presented a multiresistance profile to the antimicrobials, with amoxicillin being the drug indicated as the most effective for the studied species.

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