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Yersinia enterocolitica in a Brazilian pork production chain: Tracking of contamination routes, virulence and antimicrobial resistance

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ABSTRACT

This study aimed to track *Yersinia enterocolitica* contamination in a pork production chain in Minas Gerais, Brazil, and to characterize the virulence and antibiotic resistance of isolates. Samples were collected from four different steps of the pork production chain (pig farm, carcass, processing environment and end product; $n = 870$), and tested for the presence of *Y. enterocolitica*. The pathogen was detected in 8 samples (palatine tonsils = 5; mesenteric lymph nodes = 2; carcass after bleeding = 1), from which 16 isolates were obtained and identified as *Y. enterocolitica* bioserotype 4/O:3. *Xba*I macrorestriction allowed the clustering of isolates in 5 pulsetypes, and the identification of identical profiles of *Y. enterocolitca* isolated from different samples. All isolates were positive for the virulence related genes *ail*, *virF*, *myfA*, *ystA*, *tccC*, *ymoA*, *hreP* and *sat*, and negative for *ystB*, *ystC*, *fepA*, *fepD* and *fes*. Considering 17 antibiotics from 11 classes, only ciprofloxacin and kanamycin were effective against all isolates, and three multidrug resistance profiles were identified among them, with simultaneous resistance to 9 of 11 classes. All isolates presented positive results for *emrD*, *yfhD* and *marC*, related to multidrug resistance. The results of this study demonstrated the contamination routes of *Y. enterocolitica* within the assessed pork production chain, and highlighted the pathogenic potential and antibiotic resistance of this foodborne pathogen.

1. Introduction

Meat production is considered an important economic activity in Brazil, ranking the country among the main worldwide producers and exporters of beef, poultry and pork (Amaral et al., 2006). Specifically related to pork, processing companies must fully comply with quality and safety standards, which demand constant monitoring during the different stages of production. Pork is the most consumed animal protein in the world, and is often associated with cases of foodborne outbreaks due to contamination by different pathogens throughout the production chain (Borch et al., 1996). *Yersinia enterocolitica* is a pathogen often associated with pork products, mainly in European countries. It is responsible for yersiniosis in humans, an enterocolitis also known as pseudo-appendicitis, which in severe cases may result in septicemia (Fàbrega and Vila, 2012). Most *Y. enterocolitica* infections are caused by the bio-serotype 4/O:3 (Drummond et al., 2012); however, the virulence of *Y. enterocolitica* is determined by different genes that are not restricted to this bio-serotype (Bhagat and Virdi, 2007; Bottone, 2015).

Pigs are considered to be natural reservoirs of *Y. enterocolitica*, which explains its presence in slaughterhouses, and the association between pork consumption and yersiniosis (Bonardi et al., 2013; Vilar et al., 2015). As this pathogen persists in the pork chain from the initial steps of production, contamination of carcasses and pork products can occur, particularly during handling of the head, tongue and palatine tonsils (Van Damme et al., 2015). In addition, *Y. enterocolitica* may be present in the intestinal contents and mesenteric lymph nodes of pigs, which are also considered important sources of contamination during slaughtering (Borch et al., 1996).

Considering the natural presence of *Y. enterocolitica* in pigs, this pathogen is subjected to all preventive and therapeutic procedures adopted during pig production at the farm stage, including the use of

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Table 1
Specifications, numbers and adopted procedures for sampling of different pork production steps for *Y. enterocolitica* isolation.

Production step	Sample	Detail	n	Procedure[a]
Farm production	Barn	–	20	Footprint
Slaughtering	Carcass	A: after bleeding	100	Swab (400 cm^2)
		B: after buckling	100	Swab (400 cm^2)
		C: after evisceration	100	Swab (400 cm^2)
		D: after final washing	100	Swab (400 cm^2)
		Palatine tonsils	100	Portions (12.5 g)
		Mesenteric lymph nodes	90	Portions (12.5 g)
Processing	Knife	–	60	Swab (400 cm^2)
	Hands of employees	–	60	Swab (400 cm^2)
	Boning table	–	20	Swab (400 cm^2)
	End product	Ribs	10	Swab (400 cm^2)
		Shoulder	20	Swab (400 cm^2)
		Ham	100	Swab (400 cm^2)
		Sausage	100	Portions (25 g)

[a] Footprint samples were obtained according Botteldoorn et al. (2003); swab samples were obtained with sterile sponges and diluted in 200 mL of buffered peptone water (BPW, Oxoid, Basingstoke, England); portions were 1:10 diluted in BPW.

antibiotics (Baumgartner et al., 2007). As a consequence, studies have demonstrated the development of resistance of *Y. enterocolitica* to drugs usually adopted in pig production (Jamali et al., 2015; Simonova et al., 2008).

Considering the limited data in Brazil regarding the presence of *Y. enterocolitica* in food products, this study aimed to characterize the distribution, virulence and antibiotic resistance of this pathogen during different stages of the pork production chain.

2. Material and methods

2.1. Sampling

Samples from 10 pig production lots were obtained from two pig farms and a slaughterhouse at 15-day intervals. In the pig farms, samples were obtained from the barn floors. In the slaughterhouse, samples were obtained from pig carcasses, slaughtering and processing utensils, and the end products. Further information regarding numbers of samples and sampling procedures are detailed in Table 1. The obtained samples were homogenized for 2 min using a Stomacher (Seward Limited, Worthing, England).

2.2. Yersinia enterocolitica

The presence of *Y. enterocolitica* in samples was determined according to the method described by Van Damme et al. (2013). Aliquots of 40 mL of the diluted samples were centrifuged at 1000 × *g* for 15 min at 4 °C. Then, the supernatant was discarded and the obtained pellet was suspended with 10 mL of peptone-sorbitol-bile (PSB; Sigma-Aldrich, St. Louis, MO, USA) and incubated at 25 °C for 72 h. Then, 0.5 mL of the culture was added to 4.5 mL of KOH solution (0.5% w/v; Sigma-Aldrich) for 20 s at room temperature. After this alkaline treatment, cultures were streaked onto plates containing *Yersinia*-selective agar (Becton, Dickinson and Company - BD, Franklin Lakes, NJ, USA) and incubated at 30 °C for 48 h. Typical *Yersinia* spp. colonies were selected and subjected to further identification analysis.

Identification and serotyping was conducted by PCR according to Garzetti et al. (2014). The *16s rRNA* and *inv* genes were used for identification, and *per*, *wbbU*, *wbcA* and *wzt* genes were considered to identify the serotypes O:9, O:3, O:8 and O:5,27, respectively. DNA was extracted with a Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). PCR reactions were performed using GoTaq Green master mix (Promega Corporation), 200 nMol of each primer, 40 ng DNA and Nuclease free water (Promega Corporation) until the final volume of 25 µL. The PCR products were visualized after electrophoresis of 1.5% agarose gels in Tris-borate-EDTA (TBE) buffer, stained with GelRed™ (Biotium, Inc., Fremont, CA, USA). The primer sequences, PCR conditions and the expected sizes of PCR products are presented in the Supplementary Table 1.

Isolates identified as *Y. enterocolitica* were sent to the Yersinia Research Reference Laboratory of the College of Pharmaceutical Sciences at the University of São Paulo (USP; Ribeirão Preto, SP, Brazil) for further analysis to confirm the identification and to characterize the bioserotype of the isolates.

Isolates identified as *Y. enterocolitica* were subjected to DNA macrorestriction as indicated by PulseNet (Centers for Disease Control and Prevention, Atlanta, GA, USA), and as described by Ribot et al. (2006), with some modifications. Briefly, isolate cultures were diluted up to 0.7 to 0.9 optical densities (λ = 610 nm), treated with 20 µL of proteinase K (20 mg/mL; Sigma-Aldrich) and mixed with 400 µL of low-melting-point agarose (1% w/v; Bio-Rad, Hercules, CA, USA). Plugs were digested with *Xba*I (50 U, Promega Corporation) for 2 h at 37 °C. CHEF-DR III (Bio-Rad) was used to run the gels with the following parameters: initial switch time of 2.2 s, final switch time of 63.8 s, angle of 120°, 6 V/cm, and running time of 16 h. Gels were stained with GelRed (Biotium). Pulsed-field gel electrophoresis (PFGE) profiles of isolates were compared to PFGE profile of *Salmonella* Braenderup ATCC BAA664 (digested with *Xba*I), using Bionumerics 6.6 software (Applied Maths, Ghent, Belgium), considering an optimization of 1% and Dice coefficient of 5%.

2.3. Virulence potential

Isolates identified as *Y. enterocolitica* were subjected to PCR to detect the presence of virulence related genes. Primers for *virF*, *myfA*, *ystA*, *ystC*, *fepA*, *fepD*, *fes*, *tccC*, *ymoA, hreP* and *sat* were described by Bhagat and Virdi (2007), and primers for *ail* and *ystB* were designed in the present study (Primer Blast, National Center for Biotechnology Information - NCBI, Bethesda, MD, USA). PCR reactions were conducted as described above (Section 2.2). Primers sequences, annealing temperatures and PCR conditions are described in the Supplementary Table 1. The PCR products for *ail* were sequenced at the Human Genome and Stem Cell Research Center (HGSCRC) at the University of São Paulo (São Paulo, SP, Brazil).

2.4. Antibiotic resistance

The *Y. enterocolitica* isolates were tested for resistance to 17 antibiotics (11 classes), according protocol described by Bae et al. (2005). Isolates were transferred to Brain Heart Infusion (Becton, Dickinson and Company - BD, Franklin Lakes, NJ, USA) in 96-well microtiter plates, incubated at 30 °C for 24 h, and aliquots of approximately 2 µL were