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FOOD MICROBIOLOGY – RESEARCH PAPER

Pathogenic potential and antibiotic resistance of *Yersinia enterocolitica*, a foodborne pathogen limited to swine tonsils in a pork production chain from Southern Brazil

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Abstract

In this study, we aimed to characterize the distribution of *Yersinia enterocolitica* in a pork production chain in Brazil, as well as the virulence profile and antibiotic resistance of the obtained isolates. Samples from 10 pig lots obtained from finishing farms (water, feed, and barn floors, $n = 30$), slaughterhouse (lairage floors, carcasses at four processing steps, tonsils, and mesenteric lymph nodes, $n = 610$), and processing (end cuts, processing environment, $n = 160$) were obtained in Paraná state, Brazil, and subjected to *Y. enterocolitica* detection by ISO 10,273. The obtained isolates were identified based on biochemical and molecular features (16 s rRNA, *inv*, bioserotyping) and subjected to PCR assays to detect virulence (*ail*, *ystA*, *ystB*, *virF*, *myfA*, *fepA*, *fepD*, *fes*, *tccC*, *ymoA*, *hreP*, and *sat*) and multidrug resistance–related genes (*emrD*, *yfhD*, and *marC*). Also, isolates were subjected to disk diffusion test to characterize their resistance against 17 antibiotics from 11 classes and to pulsed field gel electrophoresis (PFGE) after XbaI macro-restriction. *Y. enterocolitica* was detected in a single sample (tonsil), and the obtained three isolates were characterized as serotype O:3, harboring *ail*, *ystA*, *virF*, *myfA*, *tccC*, *ymoA*, *hreP*, *emrD*, *yfhD*, and *marC*, and resistant to all tested antibiotics. The three isolates presented identical macro-restriction profiles by PFGE, also identical to isolates obtained from Minas Gerais, other Brazilian state; one selected isolate was identified as biotype 4. Despite the low occurrence of *Y. enterocolitica* in the studied pork production, the virulence potential and the antibiotic resistance profiles of the isolates demonstrated their pathogenic potential, and the macro-restriction profiles indicate strains descending from a common subtype in the pork production chain of two Brazilian States.

Keywords Antibiotic resistance · PFGE · Pork · Virulence · *Yersinia enterocolitica*

Introduction

Pork is the most produced and consumed animal protein in the world [1]. In Brazil, swine production has been showing relevant growth rates since the 1980s, mainly due to the intensive breeding of animals, associated with genetic, nutritional, and health improvement [2]. However, an intensive farming system can enhance the spread of important pathogens in the swine production, such as the use of collective drinking and feeders, high stool density, direct contact between animals, and number of animals per barn [3, 4].

Different foodborne pathogens can be associated to the swine chain. *Yersinia enterocolitica* is considered an emerging pathogen and pigs are described as reservoirs, once they can carry this pathogen asymptomatically into their lymph nodes, tonsils, and intestines [5, 6]. As consequence, poor hygienic conditions and inadequate procedures during pigs

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slaughtering and processing can facilitate the contamination and spread of *Y. enterocolitica* to carcasses, environment, utensils, and end products [7–9].

The pathogenic potential of *Y. enterocolitica* to humans is determined by virulence features encoded by genes located at the bacterial chromosome or in a plasmid: pYV [10, 11]. Virulence activities of chromosomal genes are mainly related to adhesion and invasion (*ail*, *inv*, *myf*, *hreP*), toxin production (*yst*), enterotoxic activity (*tccC*), iron production (*fes*, *fep*), and virulence modulation (*ymoA*) [10, 12–15]. Among the pYV genes, *virF* is described as a transcriptional regulator of genes associated with important proteins, such as YadA (adhesin A) and Yops (other *Yersinia* proteins), related to adherence and activity against host immune cells [16, 17].

Besides the pathogenic potential of foodborne pathogens, there is a current worldwide concern related to antibiotic resistance [18]. *Y. enterocolitica* is usually susceptible to different antibiotics, such as aminoglycosides, tetracycline, chloramphenicol, extended spectrum cephalosporins, and trimethoprim-sulfamethoxazole [19]. However, as *Y. enterocolitica* can be hidden in lymphatic tissues since early ages of pigs, it is consequently subjected to all antibiotic treatments during the different stages of swine production and it leads to a natural development of resistance against these substances [20, 21]. In addition, the contact with other bacteria allows transference of genetic material, leading to modifications and acquisition of resistance-related genes and plasmids, resulting in increase of the antibiotic resistance characteristics by *Y. enterocolitica* strains [22, 23].

There are no official data or report of human yersiniosis associated to pork consumption in Brazil, but some studies have demonstrated the relevance of the pork production chain in harboring *Y. enterocolitica* [24–27]. Thus, studies that characterize the distribution and the virulence of *Y. enterocolitica* in Brazil are necessary to support the epidemiological characterization of this foodborne pathogen in the Brazilian pork production chain. Here, we characterized the distribution, virulence, and antibiotic resistance of *Y. enterocolitica* in Western Paraná, a Southern Brazilian region known by its relevance in the pork production.

Material and methods

Sampling

A pork production chain from Western Paraná, a Southern Brazilian state, was selected for this study. Ten pig lots from different farms were selected and samples of water ($n = 10$, 25 mL), feed ($n = 10$, 25 g), and barn floors ($n = 10$, footprint, as described by Botteldoorn et al. [28]) were obtained. During the slaughtering of the selected pig lots, carcasses were surface sampled at different stages: after bleeding ($n = 100$), after singeing ($n = 100$), after evisceration ($n = 100$), and after final rinse ($n = 100$): four sterile molds of 100 cm^2 were placed in different carcass sites and swabbed with pre-moistened sponges (3 M Microbiology, St. Paul, MN, USA), as described by ISO 17,604 [29]. Prior to slaughtering, the lairage floors of pig lots were sampled by footprint ($n = 10$). Mesenteric lymph nodes ($n = 100$, 12.5 g) and palatine tonsils ($n = 100$, 12.5 g) were also samples from the selected carcasses. During the processing of the selected lots, surface samples of end cuts ($n = 40$), knives ($n = 40$), steel gloves ($n = 40$), cutting boards ($n = 20$), and conveyor belts ($n = 20$) were also obtained, as described above. All samples were transferred to sterile bags and kept refrigerated until laboratory analysis.

Detection of *Yersinia enterocolitica*

The samples were subjected to *Y. enterocolitica* detection based on ISO 10,273 [30], with modifications. Aliquots of 25 mL of water and 25 g of feed were diluted in 225 mL peptone-sorbitol-bile (PSB, Sigma-Aldrich, St. Louis, MO, USA), homogenized in Stomacher Seward 400® for 1 min (230 rpm). Samples from surface, lymph nodes, and tonsil were diluted in 160 mL and 112.5 mL of 0.1% peptone saline, respectively, and homogenized. Forty milliliters of aliquots of these samples were transferred to falcon tubes and centrifuged at $2{,}000 \times g$ for 15 min. The obtained pellet was suspended in 10 mL of PSB broth and incubated at 25° C for 72 h. After incubation, 0.5 mL aliquots of the PSB cultures were transferred to 4.5 mL 0.5% potassium hydroxide (KOH) solution for 20 s and then streaked on cefsulodin-irgasan-novobiocin agar (CIN, Oxoid, Basingstoke, England). The plates were incubated at 30 °C for 18 to 48 h, when typical colonies of *Y. enterocolitica* were observed (small colonies with "red bull's eyes red" center). Up to three suspected colonies from each plate were selected, purified, and subjected to biochemical tests of urease, indole, citrate, glucose fermentation, glucose gas production, lactose fermentation, H_2S production, mobility, and liquid disposal of lysine to confirm the results [30]. *Y. enterocolitica* subsp. *enterocolitica* ATCC 9610 was used as positive control.

Isolates that presented biochemical results coherent with *Y. enterocolitica* were subjected to DNA extraction by boiling [31] and PCR assays targeting *inv* and a specific region of 16 s rRNA, for *Y. enterocolitica* identification [32]. Also, PCR assays were performed targeting *per*, *wbbU*, *wbcA*, and *wzt* for characterization of serotypes O:9, O:3, O:8, and O:5,27, respectively [32]. Amplification reactions were conducted using Gotaq Green Master Mix (Promega Corp., Madison, WI, USA), 200 nMol of each primer, 40 ng of extracted DNA, and nuclease free water with 25 µL final volume. PCR products were visualized after 1.5%

agarose gel electrophoresis in GelRed ™ Tris–borate-EDTA (TBE) buffer (Biotium, Inc., Fremont, CA, USA). Primer sequences, conditions used for PCR amplifications, and expected product sizes are specified in the Supplementary Table. *Y. enterocolitica* subsp. *enterocolitica* ATCC 9610 was considered as the positive control for PCR assays.

Isolates identified as *Y. enterocolitica* were subject to DNA macro-restriction with XbaI (Promega) and pulsed field gel electrophoresis (PFGE), as indicated by PulseNet (Centers for Disease Control and Prevention, Atlanta, GA, USA), following the protocol described by Ribot et al. [33]. The obtained band profiles were compared using the software Bionumerics 6.6 (Applied Maths, Ghent, Belgium), considering 5% optimization and 5% Dice coefficient. Band profiles from *Y. enterocolitica* isolates ($n=8$) were included for a comparative analysis. These isolates were obtained from different steps of the pork production chain (tonsils, mesenteric lymph nodes, pork carcasses) in Minas Gerais state, Brazil, using the same isolation protocol adopted in this study and identified as bioserotype 4/O:3 [25].

Based on the obtained band profiles, one isolate was selected and biotyped in the Yersinia Research Reference Laboratory of the College of Pharmaceutical Sciences at the University of São Paulo (USP; Ribeirão Preto, SP, Brazil) using the protocol described by Petersen et al. [34].

Virulence-related genes

Isolates identified as *Y. enterocolitica* were subjected to PCR assays for detection of virulence-related genes, as described by Martins et al. [25]. *virF*, *myfA*, *ystA*, *ystC*, *fepA*, *fepD*, *fes*, *tccC*, *ymoA*, and *hreP* genes were screened by individual PCR assays using primers described by Bhagat and Virdi [35]. Primer sequences, PCR conditions, and expected product sizes are specified in the Supplementary Table.

Antibiotic resistance

Y. enterocolitica isolates were characterized according their antibiotic resistance based on disk diffusion assay, following the recommendations of the Clinical and Laboratory Standards Institute [36]. Seventeen antibiotics from eleven classes were considered: (1) aminoglycosides: gentamicin (10 µg) and amikacin (30 µg); (2) fluoroquinolones: ciprofloxacin (5 µg) and norfloxacin (10 µg); (3) tetracyclines: doxycycline (30 µg) and tetracycline (30 µg); (4) phenicols: chloramphenicol (30 µg); (5) third-generation cephalosporins: ceftriaxone (30 µg); (6) folate pathway inhibitors: trimethoprim (5 µg) and sulfonamide (300 µg); (7) carbapenem: meropenem (10 µg) and imipenem (10 µg); (8) quinolone: nalidixic acid (30 µg); (9) penicillins: ampicillin (10 µg) and amoxicillin (10 µg); (10) macrolides: azithromycin (15 µg); (11) lipopeptides: polymyxin B (300 IU). All antibiotics were purchased from Sigma-Aldrich. *Escherichia coli* ATCC 25,922 was considered as a pan-susceptible quality control. Results were interpreted according to enterobacterial susceptibility standards [36].

Also, the extracted DNA of the *Y. enterocolitica* isolates was subjected to PCR assays for detection of the antibiotic resistance–related genes *yfhD*, *emrD*, *marC* (multidrug resistance), and *sat* (streptogramins), as described previously [25, 35]. Primer sequences, PCR conditions, and expected product sizes are specified in the Supplementary Table.

Results

Considering all samples ($n=800$), 108 (13.5%) presented characteristic colonies on CIN agar, allowing the selection of 307 typical isolates. After biochemical assays, 91 isolates from 48 samples presented results coherent with *Y. enterocolitica*, but only three, from a single sample (tonsil), were confirmed as such after PCR targeting 16 s rRNA and inv (Table 1). Based on these results, the frequency of *Y. enterocolitica* was 0.12% in the pork production chain, and 1.0% among tonsil samples. In addition, the three *Y. enterocolitica* isolates presented positive results only for *wbbu*, what characterize them as belonging to serotype O:3. The macrorestriction profiles of the three isolates are presented in Fig. 1. The isolates presented identical band profiles, also identical to 6 out of the 8 isolates obtained in Minas Gerais state [25]. These obtained isolates were grouped into two clusters with more than 86% genetic similarity between them. Once the obtained isolates presented identical macrorestriction profiles, one was selected and identified as belonging to biotype 4.

All isolates presented PCR amplification production for *inv*, *ail*, *ystA*, *myfA*, *hrep*, *ymoA*, *tccC*, and *virF*. However, *fepA*, *fepD*, and *fes* were not found in these isolates. Based on antibiotic resistance assays, the isolates presented PCR amplification products for *yfhD*, *emrD*, and *marC*. The three isolates were characterized as resistant to amoxicillin, ampicillin, and trimethoprim.

Discussion

The identification of the steps that have major influence on *Y. enterocolitica* contamination, from the farms to the animals' slaughtering, is important for specific control measures and to monitor this pathogen along the production chain, resulting in safety of the end products [37]. Despite the low frequency of isolation, the identification of *Y. enterocolitica* in a palatine tonsil sample is consistent with the literature data, which indicates this site as the main location of this pathogen in pigs [8, 20, 25, 38, 39]. The presence

Table 1 Frequencies of *Y. enterocolitica* positive samples (isolates) obtained from a pork production chain in Western Paraná, Southern Brazil, based on the presence of suspect and confirmed isolates

Site	Sample	*n*	Positive samples (isolates) based on:		
			Typical colonies	Biochemical	16 s rRNA and *inv*
Farm	Barn floor	10	7 (21)	4 (5)	0 (0)
	Feed	10	4 (12)	0 (0)	0 (0)
	Water	10	4 (12)	2 (6)	0 (0)
Slaughterhouse	Lairage floor	10	7 (20)	5 (11)	0 (0)
	Carcass after bleeding	100	0 (0)	0 (0)	0 (0)
	Carcass after singeing	100	2 (3)	0 (0)	0 (0)
	Carcass after evisceration	100	0 (0)	0 (0)	0 (0)
	Carcass after final rinse	100	1 (3)	0 (0)	0 (0)
	Tonsils	100	52 (149)	25 (40)	1 (3)
	Mesenteric lymph nodes	100	19 (55)	12 (29)	0 (0)
Processing	Knives	40	2 (6)	0 (0)	0 (0)
	Steel gloves	40	3 (8)	0 (0)	0 (0)
	Cutting boards	20	2 (5)	0 (0)	0 (0)
	Conveyor belts	20	1 (3)	0 (0)	0 (0)
	End cuts	40	4 (10)	0 (0)	0 (0)
Total		800	108 (307)	48 (91)	1 (3)

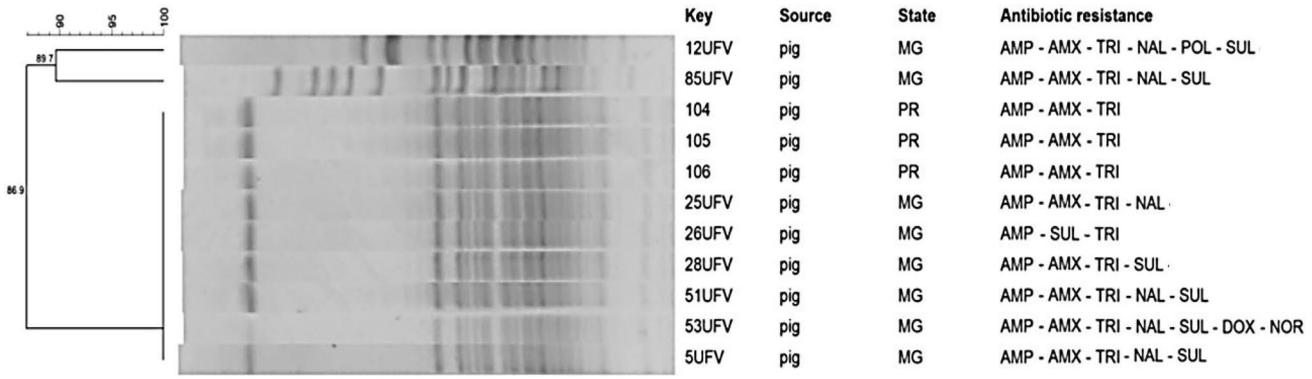

Fig. 1 Schematic representation of macrorestriction profile (XbaI) by PFGE (Dice coefficient with 5% tolerance) and phenotypic profiles of antimicrobial resistance in disk diffusion test (CLSI, 2017) of *Y. enterocolitica* isolates obtained from state of Paraná and Minas Gerais. AMP, ampicillin; AMX, amoxicillin; TRI, trimethoprim; NAL, nalidixic acid; POL, polymyxin B; SUL, sulfonamides; DOX, doxycycline; NOR, norfloxacin

of *Y. enterocolitica* in swine tonsils is considered as one of the main risk factors for carcass contamination in the slaughtering process and consequently in the end products, as these tissues are incised during inspection and slaughtering, increasing the chances of cross contamination [40, 41].

Low frequencies of *Y. enterocolitica* in pig slaughtering, especially in tonsils, have been reported in other studies conducted in Brazil. However, in São Paulo state, the occurrence of *Y. enterocolitica* positive samples was 8.0% in tonsils, tongues, submandibular, and mesenteric lymph nodes and knives [42]. Another study in São Paulo isolated 442 strains of *Y. enterocolitica* from a total of 792 samples collected from slaughtered pigs, slaughterhouse environment, and retail market [27]. From 400 samples of swine tonsils from Western Santa Catarina, 101 (25.25%) were positive for *Y. enterocolitica* [43]. In a similar study in Minas Gerais state, approximately 5% of swine tonsil samples were positive for *Y. enterocolitica* [25]. In other countries, especially in Europe, the presence of *Y. enterocolitica* in pig tonsils can be considered as high when compared to Brazilian studies, ranging from 13 to 90% [12, 38, 39, 44–46], while in the USA this prevalence was reported as 10% [47] and in China as 19.5% [48]. It is important to highlight that these differences can occur due to different isolation and

detection approaches [17]. Conventional PCR and real-time PCR assays directly from samples and pre-enrichment broths have shown higher detection capacity of *Y. enterocolitica* than conventional procedures, and some studies already described that although the pathogen is present in samples, it is not always able to form colonies, which undermines the results obtained exclusively by culture plating [20, 21, 49]. Pork products usually contain a rich and diverse background microbiota, what can jeopardize the proper isolation of *Y. enterocolitica* by culture dependent methods [50]. Also, the low occurrence of *Y. enterocolitica* can be associated with the competing microbiota in the tested samples. It was already demonstrated that the serological prevalence of *Y. enterocolitica* is inversely proportional to the serological prevalence of *Salmonella* spp. in swine herds [51, 52]. In a parallel study with the same samples, Viana et al. [53] observed *Salmonella* occurrence of 45% in palatine tonsils, supporting this hypothesis.

Y. enterocolitica is described as an emerging pathogen in Europe due to the high number of reported yersiniosis cases in recent decades [5]. Fosse et al. [54] estimate that 77.3% of worldwide cases of yersiniosis may be associated with pork consumption. In Brazil, only a few studies describe the isolation and characterization of *Y. enterocolitica* from food, and the lack of epidemiological data from foodborne disease cases and outbreaks does not allow a proper characterization of the described yersiniosis, neither their potential link with contaminated pork products [26]. *Y. enterocolitica* isolates belonging to bioserotype 4/O:3 are described as the main pathogenic agents of yersiniosis in humans and animals worldwide, including in Brazil [10, 24, 32, 48, 55]. Martins et al. [25] identified *Y. enterocolitica* isolates from this bioserotype in a pork production chain in Minas Gerais, Brazil, and Paixão et al. [27] described that among 442 *Y. enterocolitica* isolates, all obtained from swine tonsils were characterized as serotype O:3.

The presence of the main virulence plasmid, pYV, is indicated by positive results for *virF*, responsible for coding one of the major pathogenicity factors of *Y. enterocolitica*, the type III secretion system [14, 17]. Despite presenting pYV, other chromosomal virulence genes are required for full pathogenicity capacity of *Y. enterocolitica*, such as *inv*, *ail*, *ystA*, *myfA*, *hrep*, *ymoA*, and *tccC* [14, 56]. These genes encode proteins that act in synergy for adhesion, internalization, and production of molecules necessary for enterotoxic activity to occur and to escape the host immune system [10, 12, 14, 24, 56, 57]. However, the absence of *fepA*, *fepD*, and *fes* indicates that the isolates obtained in this study have limited capacity for iron utilization, an essential factor for the development of most microorganisms [58].

Resistance to amoxicillin and ampicillin was already expected, once *Y. enterocolitica* is intrinsically resistant to these drugs [36]. All antibiotics usually recommended for yersiniosis treatment were effective against the obtained isolates: tetracycline, chloramphenicol, gentamicin, and third-generation cephalosporins [13]. Some studies have demonstrated increasing frequencies of multidrug-resistant *Y. enterocolitica*, as we observed based on phenotypical and molecular assays [25, 28, 31, 59]. Besides presenting amplification for *yfhD*, *emrD*, and *marC*, related to multidrug resistance, the three isolates also presented positive results for *sat*, related to resistance to streptogramins (virginiamycin), an antibiotic used as a feed additive [35, 60]. The use of antibiotics in animal production as growth promoters and prophylaxis is considered one of the main causes for the development of resistance, and the pork production is known by the wide use of drugs with these purposes when compared to other livestock systems [61]. Considering the strong epidemiological link between *Y. enterocolitica* and the pork production chain, monitoring the antibiotic resistance profiles of pork-related bacteria can be considered critical with regard to food safety and the performance of yersiniosis treatments in humans [19].

The results from the Minas Gerais' strains are different probably because of the variances in pork production between these Brazilians states, and distinct drugs are probably being considered during production and resulting in these different profiles. Identification of *Y. enterocolitica* from Paraná and Minas Gerais states from a same serotype (O:3) and sharing identical band patterns suggests the low variability of this pathogen circulating in these two Brazilian states. Rusak et al. [26] reported a high similarity based on PFGE after XbaI macro-restriction among *Y. enterocolitica* from different bioserotypes and samples (swine, food, and clinical patients) in Brazil: all isolates identified as O:3 were grouped in a single cluster, with high similarity index.

Here we reported a low occurrence of *Y. enterocolitica* in a pork production chain, specifically in Western Paraná, a relevant pork production region in Southern Brazil. Despite the low occurrence, the *Y. enterocolitica* 4/O:3 isolates obtained presented high pathogenic potential and resistance to three antibiotics, but identical XbaI macro-restriction patterns with isolates obtained from other Brazilian state, Minas Gerais. These results suggest that strains descending from a common subtype may be circulating in the pork production chain of two states from different regions of Brazil, leading to further studies to elucidate their genomic profiles and potential clonality.

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Declarations

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