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SHORT COMMUNICATIONS

Low occurrence of pathogenic *Yersinia enterocolitica* in pig tonsils at slaughter in Southern Brazil

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Abstract *Yersinia enterocolitica* is a foodborne pathogen and pigs are the main reservoir of it in their tonsils. As Brazil is a large producer and exporter of pork meat and information regarding this pathogen is still quite scarce, this study aimed at evaluating the direct detection of *Y. enterocolitica* followed by pathogenic *Y. enterocolitica* (PYE) determination in tonsils of slaughtered pigs. For this purpose, 400 pig tonsils were collected from 15 farms in four federally certified slaughterhouses in Southern Brazil. Initially, samples were screened using conventional PCR targeting of the 16sRNA gene, followed by multiplex PCR (mPCR) in order to detect three virulence genes (*ail*, *yadA*, and *virF*) and quantitative real-time PCR (qPCR) for the detection of the *ail* gene. One hundred and one (25.2%) of the samples tested positive for the 16sRNA gene. However, a PYE was detected in one out of the 101 *Y. enterocolitica* positive samples. The three virulence genes were determined by mPCR and confirmed by partial DNA sequencing. Thus, a significant occurrence of *Y. enterocolitica* was observed in pig tonsils from federally inspected slaughterhouses in Brazil, although the presence of pathogenic strains was quite low.

Keywords Swine · Yersiniosis · Virulence genes · Foodborne pathogen · PCR

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Introduction

Yersinia enterocolitica is a gram-negative bacteria associated with enteric disease in animals and more particularly in humans. The occurrence of foodborne infections caused by this pathogen is an emerging public health problem. Humans that are infected may present gastrointestinal disturbances, such as abdominal pain, often bloody diarrhea, fever, and vomiting (Robins-Browne 2001). *Y. enterocolitica* is widespread in the environment and among animal populations, thus posing a potential source of infection towards humans (Sabina et al. 2011; Bancerz-Kisiel and Szweda 2015). In animals, *Y. enterocolitica* infections are usually asymptomatic. Pigs provide the main source of pathogenic *Y. enterocolitica* (PYE) strains in lymphatic tissues and can shed it through the fecal route (Fosse et al. 2009; Sabina et al. 2011; Virtanen et al. 2011). At slaughter, the pig carcass can be contaminated due to the infected tissues and, consequently, so will the final meat product (Schaake et al. 2013). Just like psychrophilic bacteria, *Y. enterocolitica* is able to grow at low temperatures and produces a heat stable enterotoxin, representing a significant risk factor for human infection when pork is consumed raw or medium rare (Schaake et al. 2013; Bancerz-Kisiel and Szweda 2015). Companion animals are also considered an increasingly important factor in the epidemiology of *Y. enterocolitica* infections in humans (Sabina et al. 2011; Bancerz-Kisiel and Szweda 2015).

Y. enterocolitica is a highly heterogeneous bacteria consisting of six biotypes: 1A, 1B, 2, 3, 4, and 5. Strains of *Y. enterocolitica* biotype 1B are the most virulent while the 1A biotype is widely spread in the environment and considered apathogenic (Robins-Browne 2001; Sabina et al. 2011). However, some 1A biotype isolates have been considered just as causative of gastrointestinal disease, as some virulence markers have also been found in these strains (Sabina et al.

2011; Bancerz-Kisiel and Szweda 2015). The virulence mechanisms presented by pathogenic strains are embodied through virulence markers encoded by chromosomal and plasmid genes (Robins-Browne 2001; Thoerner et al. 2003). The virulence marker most typical of PYE is the Yersinia virulence plasmid (pYV), which encodes for proteins that contribute to intestinal invasion and allow survival and multiplication of the bacteria in macrophages and lymphoid tissues. The plasmidial *virF* gene is responsible for the regulation of the transcription of several important virulence genes. Besides the markers located on the pYV, PYE possess chromosomal markers that are much more genetically stable than plasmid markers. One of them, the attachment invasion locus (*ail*) is involved with the adhesion of the bacteria in the intestine (Thoerner et al. 2003; Schaake et al. 2013; Tadesse et al. 2013).

The isolation of *Y. enterocolitica* is laborious and time consuming. Additionally, the encountered difficulty in precisely determining whether or not a strain is pathogenic poses problems in the traditional diagnostic approach (Petsios et al. 2016). Therefore, molecular methods, such as the polymerase chain reaction (PCR) method, including multiplex PCR and real-time PCR, are often used due to their rapidity and increased sensitivity compared to cultural methods (Bancerz-Kisiel and Szweda 2015; Petsios et al. 2016). The variety in virulence markers presented in PYE seems to be particularly useful in order to differentiate PYE strains from non-pathogenic strains (Thoerner et al. 2003; Thisted Lambertz and Danielsson-Tham 2005; Falcao et al. 2006).

Yersiniosis caused by PYE is one of the most frequently reported zoonoses in the European Union (Eurosurveillance editorial 2015). According to Fosse et al. (2009), *Y. enterocolitica* feature in the three most frequently reported hazardous clinical cases related to human consumption of pork, as well as in one of the two main hazards such as identified in pork carcasses. Brazil has no official data regarding the incidence of PYE in humans, and only sporadic cases have been reported (Silva et al. 2010; Rusak et al. 2014). In addition, little information is available regarding the occurrence of this bacteria in pig slaughterhouses and of its pathogenic potential. Brazil is currently the fourth largest producer and exporter of pork meat in the world, with the pork production being concentrated in the Southern region (ABPA 2017). The aim of this study was to evaluate the direct detection of *Y. enterocolitica* followed by PYE determination in pig tonsils through the investigation of the virulence genes *ail*, *yadA*, and *virF*, in Southern Brazil.

Material and methods

Four hundred tonsils of 168-day-old pigs were randomly collected at four federally inspected slaughterhouses, located at Concórdia (A, n = 100), Seara (B, n = 104), Chapecó (C, n = 98), and Forquilhinha (D, n = 98), in the state of Santa Catarina, Brazil. These locations represent the main sites of pork production in the State. At slaughterhouse A, B, C, and D, sampled pigs were from five, four, one, and five herds, respectively, amounting to a total of 15 all-in all-out growing-finishing farms.

In order to perform molecular tests, DNA extraction of the tonsils was performed with a Prep/Preamp Kit (NewGene), according to the manufacturer's recommendations. A conventional PCR (cPCR) was used for the detection of the 16sRNA gene of *Y. enterocolitica* (Wannet et al. 2001). A multiplex PCR (mPCR) was standardized for the detection of the virulence genes (*ail*, *yadA*, and *virF*) using previously described primers (Thoerner et al. 2003; Thisted Lambertz and Danielsson-Tham 2005). The 25 μL mPCR was performed with 1.5 mM $MgCl_2$, 1× PCR buffer, 0.2 mM dNTP, 0.4 μM of each primer, and 1.25 U taq DNA polymerase (Invitrogen). The mPCR reaction was carried out at 94 °C during 3 min followed by 30 cycles of 94 °C for 30 s per cycle, 60 °C during 1 min, 72 °C during 1 min, and then a final extension of 72 °C during 5 min. Quantitative real-time PCR (qPCR) using SYBRGreen fluorophore was standardized for *ail* gene detection, also using primers (250 mM each) as described in literature (Thisted Lambertz and Danielsson-Tham 2005) (Table 1). The reaction took place using an ABI Prism 7500 Sequence Detection System (Life Technologies) under the following conditions: 95 °C during 10 min, then 40 cycles at 95 °C for 15 s per cycle, and finally 60 °C during 1 min.

An isolate of *Y. enterocolitica* (8081/NCTC 13174) that tests positive for the *ail*, *yadA*, and *virF* genes was used as a positive control. For its use in qPCR, the amplified product of the *ail* gene was cloned using a TOPO TA Cloning Kit (Invitrogen). The number of copies of the cloned plasmid was calculated according to Yun et al. (2006).

DNA sequencing of amplified genes in mPCR was performed directly from the original sample through the Sanger method using the primers described in Table 1. The assembled sequences were compared based on similarity to other known sequences using BLAST (Altschul et al. 1997). Phylogenetic analyses for each gene were obtained using the Neighbor-Joining method (MEGA 6.0 software) (Tamura et al. 2013).

Results

A hundred and one (25.2%) out of 400 tonsil samples analyzed through the cPCR technique tested positive for the 16sRNA gene specific for *Y. enterocolitica*. Of these samples, 43 were detected in slaughterhouse A, 34 in slaughterhouse B, 7 in slaughterhouse C, and 17 in slaughterhouse D. Although 101 samples tested positive for *Y. enterocolitica*, only one sample from slaughterhouse A was pathogenic, based on *ail*,

Table 1 Indicators used for cPCR, mPCR, and qPCR reactions for the detection of pathogenic *Y. enterocolitica*

Molecular technique	Target gene and primer direction	Sequence (5'-3')	Fragment size (pb)	Reference
cPCR	16sRNA		330	Wannet et al. 2001
	Forward	AATACCGCATAACGTCTTCG		
	Reverse	CTTCTTCTGCGAGTAACGTC		
mPCR/qPCR	*ail*		454	Thisted Lambertz and Danielsson-Tham 2005
	Forward	GTTTATCAATTGCGTCTGTTAATGTGTACG		
	Reverse	CTATCGAGTTTGGAGTATTCATATGAAGCG		
mPCR	*virF*		700	Thisted Lambertz and Danielsson-Tham 2005
	Forward	AAGGTTGTTGAGCATTCACAAGATGG		
	Reverse	TTTGAGTGAAAATAAGACTGACTCGAGAACC		
mPCR	*yadA*		849[a]	Thoerner et al. 2003
	Forward	CTTCAGATACTGGTGTCGCTGT		
	Reverse	ATGCCTGACTAGAGCGATATCC		

[a] PCR products from the *yadA* gene may show fragments of 759 bp for *Y. enterocolitica* serotype O: 8 and 849 bp for the other serotypes (Thoerner et al. 2003)

yadA, and *virF* gene detection. In addition, the *yadA* gene fragment presented 849 bp, indicating that the strain is not part of the O:8 serotype group. When the 400 samples were submitted for qPCR analysis, only the sample that had already tested positive in cPCR for the *ail* gene was again positive for this gene. This sample had 11,058,398 copies/μL and a quantification cycle of 26.64.

Partial sequences of the three genes were obtained and submitted to the GenBank database under accession numbers KU672532, KU672533, and KU711832 for the *ail* (453pb), *yadA* (208pb), and *virF* (474pb) genes, respectively. When these sequences were compared with other *Y. enterocolitica* sequences available in the GenBank, nucleotide identity ranged from 93 to 100% for the *ail* gene, 99% for the *virF* gene, and 95 to 97% for the *yadA* gene. Multiple alignments for the *ail*, *virF*, and *yadA* genes showed that the sample sequenced here is similar to other samples isolated from humans and animals in several countries.

Discussion

This study describes a direct genetic detection of *Y. enterocolitica* complemented by a low occurrence of PYE in pig tonsils. *Y. enterocolitica* is not frequently isolated and studied in Brazil, which makes it difficult to establish this agent as a cause of disease, as well as to estimate the impact of the bacteria's presence in pigs. In Brazil, Teodoro et al. (2006) determined an occurrence of 10% of PYE in pig tonsils from slaughterhouses without federal inspection. In another study, the bacteria was not detected in pork sausages (Albuquerque and Cardoso 1999). Other few studies in Brazil have characterized phenotypic and genotyped isolates from different sources, such as food, water, sludge, and human feces (Falcao et al. 2004; Falcao et al. 2006). The incidence of *Y. enterocolitica* in pig tonsils has been reported as well as its risk factor for the contamination of the carcass and, consequently, the final food product (Bhaduri et al. 2005; Teodoro et al. 2006; Ortiz Martinez et al. 2010; Virtanen et al. 2011; Vanantwerpen et al. 2014).

In addition, a rapid detection PYE in pork-based products is of great importance in order to avoid the occurrence of food infections in humans. The mPCR technique described in this study can be a useful tool to determine the crucial production process intervals at which contamination occurs and develop control measures to reduce or even eliminate this pathogen in the final product. Different methodologies are available to detect *Y. enterocolitica,* from culture methods, including some standardized reference methods for bacterial isolation, to molecular techniques such as PCR and sequencing. The sensitivity and specificity of these methodologies are very different, and the selection of the most appropriate diagnostic approach

depends on the purpose of the study (Bancerz-Kisiel and Szweda 2015; Petsios et al. 2016).

The PCR technique is fast, inexpensive, sensitive, and specific, and it can be used to differentiate pathogenic from non-pathogenic *Y. enterocolitica* strains by screening for virulence genes (Wannet et al. 2001; Thoerner et al. 2003; Thisted Lambertz and Danielsson-Tham 2005; Petsios et al. 2016). As not all strains present all virulence genes, a technique that could detect one or more genes associated with virulence at the same time, such mPCR, is a great of interest. Real-time PCR is more rapid and sensitive than conventional PCR due to reduced cycle times, and the removal of separate post-PCR detection procedures and the use of sensitive fluorescence detection equipment allow for better target quantification. Although PCR techniques have several advantages compared to traditional culture methods, still several drawbacks exist. A gene-based assay can only determine a pathogen as potentially pathogenic because some genes may be unexpressed (Thisted Lambertz and Danielsson-Tham 2005). PCR methods are also unable to differentiate between viable and non-viable cells and do not yield bacterial isolates, which are essential for supplementary epidemiological studies (Bancerz-Kisiel and Szweda 2015; Petsios et al. 2016). The use of additional sample preparation step prior to the PCR could avoid problems with PCR inhibitors or when the pathogen is present at a low concentration in a food sample (Petsios et al. 2016). However, culture-based methods could result in a possible plasmid loss during subculture and storage (Nesbakken et al. 2006).

Notwithstanding, a low occurrence of PYE was observed in the pig tonsil samples evaluated in this study, and the three genes associated with pathogenicity were detected in the same strain and confirmed by sequencing. Falcao et al. (2006) investigated the virulence genes *ail* and *virF* in 106 strains of *Y. enterocolitica* that were isolated from human ($n = 37$), animal ($n = 34$), and food ($n = 35$) samples between 1969 and 2000 from different regions of Brazil. The *ail* gene was detected in all strains that were isolated from humans and animals, however only in two strains from food. The *virF* gene showed a variable incidence, with 94.6, 61.8, and 2.9% of isolates from humans, animals, and food, respectively. Tadesse et al. (2013) also reported the presence of virulence genes (*ail* and *yadA*) in strains of *Y. enterocolitica* isolated from feces and slaughtered pig carcasses between 2002 and 2005 in the USA. Twenty out of 172 (11.6%) strains had both genes, while 40% only had the *ail* gene. Nevertheless, *ail* gene, a chromosomal marker, is usually the most studied gene due to its genetic stability (Tadesse et al. 2013). Also, the presence of a virulence plasmid, like virF, is a prerequisite for full virulence of *Yersinia* spp. (Tadesse et al. 2013).

The partial sequencing of the three virulence genes from the unique PYE detected here led us to assume its pathogenic character. However, due to the small gene fragment sequenced as well as the low number of *Y. enterocolitica* sequences available in the GenBank, it is difficult to make any comparison or statement that could potentially impact both the swine and human population.

In summary, this study demonstrated an occurrence of *Y. enterocolitica* DNA-based of 25.2% in tonsils of slaughter pigs. Preliminarily, only one sample was characterized as PYE and showed the chromosomal virulence gene (*ail*) and the plasmid-borne virulence genes (*yadA* and *virF*).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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