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Short communication

Virulence-associated gene pattern of porcine and human *Yersinia enterocolitica* biotype 4 isolates

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Abstract

Yersinia enterocolitica 4/O:3 is the most important human pathogenic bioserotype in Europe and the predominant pathogenic bioserotype in slaughter pigs. Although many studies on the virulence of *Y. enterocolitica* strains have showed a broad spectrum of detectable factors in pigs and humans, an analysis based on a strict comparative approach and serving to verify the virulence capability of porcine *Y. enterocolitica* as a source for human yersiniosis is lacking. Therefore, in the present study, strains of biotype (BT) 4 isolated from Swiss slaughter pig tonsils and feces and isolates from human clinical cases were compared in terms of their spectrum of virulence-associated genes (*yadA*, *virF*, *ail*, *inv*, *rovA*, *ymoA*, *ystA*, *ystB* and *myfA*). An analysis of the associated antimicrobial susceptibility pattern completed the characterization. All analyzed BT 4 strains showed a nearly similar pattern, comprising the known fundamental virulence-associated genes *yadA*, *virF*, *ail*, *inv*, *rovA*, *ymoA*, *ystA and myfA*. Only *ystB* was not detectable among all analyzed isolates. Importantly, neither the source of the isolates (porcine tonsils and feces, humans) nor the serotype (ST) had any influence on the gene pattern. From these findings, it can be concluded that the presence of the full complement of virulence genes necessary for human infection is common among porcine BT 4 strains. Swiss porcine BT 4 strains not only showed antimicrobial susceptibility to chloramphenicol, cefotaxime, ceftazidime, ciprofloxacin, colistin, florfenicol, gentamicin, kanamycin, nalidixic acid, sulfamethoxazole, streptomycin, tetracycline and trimethoprim but also showed 100% antibiotic resistance to ampicillin. The human BT 4 strains revealed comparable results. However, in addition to 100% antibiotic resistance to ampicillin, 2 strains were resistant to chloramphenicol and nalidixic acid. Additionally, 1 of these strains was resistant to sulfamethoxazole.

The results demonstrated that *Y. enterocolitica* BT 4 isolates from porcine tonsils, as well as from feces, show the same virulence-associated gene pattern and antibiotic resistance properties as human isolates from clinical cases, consistent with the etiological role of porcine BT 4 in human yersiniosis. Thus, cross-contamination of carcasses and organs at slaughter with porcine *Y. enterocolitica* BT 4 strains, either from tonsils or feces, must be prevented to reduce human yersiniosis.

Keywords: Swine; Pig; Switzerland; Yersiniosis; Antibiotic resistance

1 Introduction

Based on biochemical characteristics, ~~Y.~~ *Yersinia enterocolitica* is divided into several distinct biotypes (BTs) (BT 1A, 1B, 2, 3, 4 and 5), of which the pathogenic BTs (1B, 2, 3, 4 and 5) are responsible for the fourth most common foodborne zoonosis in Europe (EFSA, 2013). The most common human pathogenic *Y. enterocolitica* in the European Union and Switzerland are of bioserotype 4/O:3 (Fredriksson-Ahomaa et al., 2012; EFSA, 2013). Pigs have been characterized as the main reservoir for pathogenic *Y. enterocolitica* BT 4 and are supposed to be the most important infection hazard for humans (EFSA, 2013), but other mammals, e.g., sheep, cattle, cats and dogs, are also discussed as sources of human BT 4 infection (Fearnley et al., 2005; Stamm et al., 2013). BT 4 strains are detected in carcasses and in pig feces at slaughter, as well as in tonsils, where they are present at an up to 6-fold higher detection rate than in feces (Fredriksson-Ahomaa et al., 2001; Nesbakken et al., 2003). However, the route of infection and the impact of different sources (tonsils versus feces) have not been clarified.

Although previous studies on molecular typing have demonstrated the genetic relatedness of porcine and human *Y. enterocolitica* (Kuehni-Boghenbor et al., 2006; Laukkanen et al., 2009), the characterization of virulence capability by screening for virulence-associated genes is required to clarify the role of porcine BT 4 in human disease. All pathogenic *Y. enterocolitica* isolates host the 70-kb virulence plasmid (pYV), which is indispensable for full virulence (Cornelis et al.,

1998). Various virulence-associated genes located on the pYV wasere used in previous studies as a target to detect pathogenic *Y. enterocolitica* strains by PCR (Fredriksson-Ahomaa and Korkeala, 2003). The *yadA* gene encodes the *Yersinia* adhesion A protein, relevant for host cell attachment and for complement antibiotic resistance (Galindo et al., 2011). The *virF* gene product is a key factor for transcription of *yop* genes (Cornelis et al., 1998) and is, therefore, fundamental for the type III secretion system. Because pYV can be lost during the culturing process (Thoerner et al., 2003), chromosomal genes should be analyzed as well. The *ail* (attachment invasion locus) gene has been described as a target for virulence screening (Fredriksson-Ahomaa and Korkeala, 2003; Thisted-Lambertz et al., 2008), as it is only found in strains associated with human infection (Miller et al., 1989). Several studies have reported that certain BT 1A isolates may host the *ail* gene as well. For this reason, the potential pathogenicity of BT 1A strains has previously been addressed (Burnens et al., 1996; Kraushaar et al., 2011; Paixão et al., 2013a,b; Sihvonen et al., 2011a). The *inv* gene encodes invasin, a protein functioning in the invasion of and adhesion to the host cell. This gene is present in all *Y. enterocolitica* isolates but non-functional in BT 1A (Pierson and Falkow, 1990). The expression of *inv* is positively regulated by *rovA* (regulator of virulence) and negatively regulated by *ymoA* (*Yersinia* modulating protein) (Ellison et al., 2003; Revell and Miller, 2000). The *ystA* (*Yersinia* stable toxin A) gene is uniquely found in pathogenic BTs, whereas BT 1A frequently bears the *ystB* (*Yersinia* stable toxin B) gene (Bhagat and Virdi, 2007; Stephan et al., 2013). Another virulence-associated gene is the *myfA* (mucoid *Yersinia* factor) gene, which encodes fimbriae (Revell and Miller, 2001).

In previous studies, the characterization of *Y. enterocolitica* bioserotypes by analyzing the virulence gene pattern has produced a strong evidence of swine as the main reservoir for human pathogenic strains. However, several of these studies focused solely on porcine isolates (Bonardi et al., 2013; Paixão et al., 2013a,b) or human strains (Stephan et al., 2013; Fredriksson-Ahomaa et al., 2012). Or only a small number of virulence-associated genes wasere analyzed (Thoerner et al., 2003). To our knowledge, no previous study has used this same approach to analyze BT 4 strains from porcine tonsils, porcine feces and human feces for a complete spectrum of relevant virulence-associated genes.

Y. enterocolitica is known to be highly susceptible to most antibiotics with the exception of penicillin, ampicillin and first-generation cephalosporins. This intrinsic antibiotic resistance is based on 2 well-known chromosomal beta-lactamase genes, *blaA* and *blaB* (Fàbrega and Vila, 2012). The occurrence of multiresistant human pathogenic *Y. enterocolitica* strains and a high proportion of antibiotic resistance to chloramphenicol, streptomycin and sulfonamides in porcine strains in Italy have recently been described (Sihvonen et al., 2011b; Bonardi et al., 2014). These findings raise questions about the current situation in Swiss isolates. Previous studies have used the disc diffusion method to conduct antimicrobial susceptibility testing for Swiss *Y. enterocolitica* isolates (Baumgartner et al., 2007; Fredriksson-Ahomaa et al., 2012). However, this method is known to produce high rates of incorrect results (Meyer et al., 2011). Accordingly, broth microdilution was used in this study.

Thus, the aim of this study was to i) characterize *Y. enterocolitica* BT 4 from human clinical cases in comparison with porcine BT 4 strains for a broad spectrum of relevant virulence-associated genes, ii) to include porcine *Y. enterocolitica* isolates from both tonsils and feces to detect possible source-dependent gene patterns and iii) to characterize *Y. enterocolitica* BT 4 isolates from Switzerland for their antibiotic susceptibility pattern based on the broth microdilution method.

2 Materials and Mmethods

2.1 *Y. enterocolitica* strains

Eighty-seven BT 4 *Y. enterocolitica* were analyzed. Thirty-eight strains were isolated from Swiss slaughter pigs from March 2012 to February 2013 and another thirteen strains were isolated with fecal swabs from Swiss slaughter pigs between July and December 2013 (Büttner et al., 2013). Isolation and biotyping was based on the International Standards Organization (ISO 10273:2003) isolation method for *Y. enterocolitica* in food (unpublished data). The strains were serotyped for O:3, O:5, O:8, O:9 and O:27 by slide agglutination with commercially available antisera (SIFIN, Berlin, Germany).

Thirty-six human fecesal BT 4 isolates had been collected between 2001 until 2003 at the Federal Office of Public Health (FOPH) (Kuehni-Boghenbor et al., 2006).

All strains were preserved in Trypticase-–Soy Bouillon (TSA) (Becton Dickinson, Franklin Lakes, New Jersey, USA) with 30% glycerol and stored at-– 80 °C until further analysis.

2.2 Detection of virulence-associated genes by PCR

Virulence-associated genes of *Y. enterocolitica* isolates were detected by conventional PCR. DNA was prepared from overnight-growing colonies on TSA at 30 ± 1 °C. A few colonies were transferred to lysis buffer (0.1 M Tris–HCl, pH 8.5, 0.05% Tween 20, 0.24 mg/ml proteinase K) and incubated for 1 h at 60 °C following 15 min at 97 °C. DNA extracts were stored at-– 20 °C for further analyses. Nine virulence-associated genes were tested-– 7 chromosomal genes (*ail*, *inv*, *rovA*, *ymoA*, *ystA*, *ystB* and *myfA*) and 2 plasmid-borne genes (*yadA*, *virF*). For *virF*, *ail*, *inv*, *ymoA*, *ystA*, *ystB* and *myfA*, the primers and cycling parameters described by Bhagat and Virdi (2007) were used. For *rovA* the protocols defined by Divya and Varadaraj (2011) and for *yad*A the protocols by Thoerner et al. (2003) were used (Table 1). Two microliters of template was added to 28 µL of reaction mixture consisting of 1 X× PCR buffer (Solis BioDyne, Tartu, Estonia), 2.5 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia), 200 µM dNTPS (Roche, Rotkreuz, Switzerland), 0.25 µL FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia) and primers (Microsynth, Balgach, Switzerland) at a final concentration of 0.25 µM.

Table 1 *Y. enterocolitica* biotype 4 serotypes and virulence-–gene patterns from strains isolated out of from different sources.

Material	ST	No.	*ail*	*inv*	*ystA*	*ystB*	*myfA*	*rovA*	*ymoA*	*yadA*	*virF*

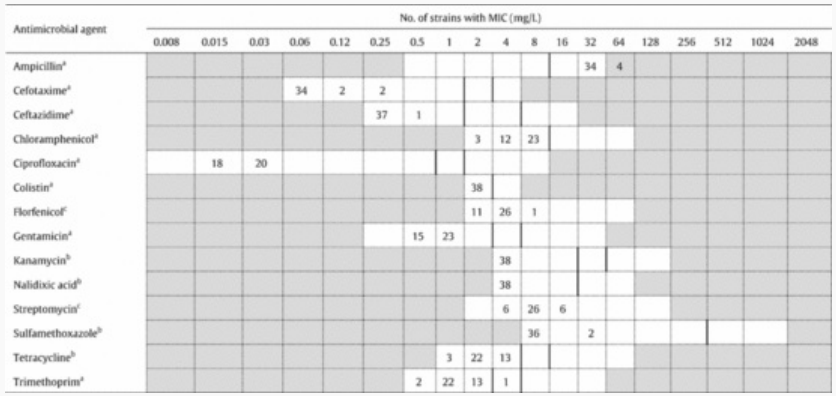
Porcine	Tonsil	O:3	16	+	+	+	–	+	+	+	+	+	
			2	+	–	+	–	+	+	+	–	+	
			1	+	+	+	–	+	+	+	–	+	
		O:3, O:27	15	+	+	+	–	+	+	+	+	+	
		O:8, O:27	3	+	+	+	–	+	+	+	+	+	
		O:3, O:8, O:27	1	+	+	+	–	+	+	+	+	+	
	Feces	O:3	4	+	+	+	–	+	+	+	+	+	
		O:3, O:27	5	+	+	+	–	+	+	+	+	+	
		O:8, O:27	4	+	+	+	–	+	+	+	+	+	
Human	Feces	O:3	17	+	+	+	–	+	+	+	+	+	
		O:3, O:27	16	+	+	+	–	+	+	+	+	+	
		O:27	1	+	+	+	–	+	+	+	+	+	
		O:8	1	+	+	+	–	+	+	+	+	+	
		O:27, O:9	1	+	+	+	–	+	+	+	+	+	

ST serotype.

2.3 Antimicrobial testing

The minimal inhibitory concentration (MIC) of the antibiotics was determined by broth microdilution in cation-adjusted Mueller–Hinton broth using the Sensititre susceptibility plate EUMVS2 (Sensititre, TREK Diagnostic System, Cleveland, Ohio, USA; TREK Diagnostic System, East Grinstead, West Sussex, England). The antibiotics and the concentration ranges are presented in Tables 2 and 3. The plates were incubated for 18–24 h at 30 ± 1 °C. Isolates were classified as susceptible or resistant according to clinical breakpoints for *Enterobacteriaceae* issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines version 4.0 (www.eucast.org). If EUCAST breakpoints were unavailable, human clinical breakpoints for *Enterobacteriaceae* from Clinical and Laboratory Standards Institute (CLSI) documents M100-S22 (Clinical and Laboratory Standards Institute, 2013) were used. For florfenicol and streptomycin, no breakpoints from EUCAST or CLSI were available. Therefore, MIC50 and MIC90 were calculated. *Escherichia coli* ATCC 25922 was used as the control strain with 37 ± 1 °C as the incubation temperature. Measured MICs were in the appropriate range for this strain.

Table 2 Minimal inhibitor concentration (MIC) from of porcine tonsil *Y. enterocolitica* biotype 4 isolates.



Numbers indicate the number of isolates with corresponding MIC value. White areas indicate the range of dilutions tested for each antimicrobial agent; values above or below this range denote MIC values greater than the highest concentration tested and MIC values smaller than or equal to the lowest concentration tested, respectively. Vertical lines indicate clinical breakpoints, when two vertical lines exist, the lower breakpoint indicate susceptibility and the higher breakpoint

resistance with an intermediate range in between. [a]Clinical breakpoint is available according to EUCAST guidelines (clinical breakpoint for version 4.0, 2014 *Enterobacteriaceae*). [b]Clinical breakpoint is available according to CLSI (M100-S22, 2012 human clinical breakpoint for *Enterobacteriaceae*). [c]No EUCAST or CLSI clinical breakpoint available.

Table 3 Minimal inhibitor concentration (MIC) of ~~from~~ humane feces *Y. enterocolitica* biotype 4 isolates.

Antimicrobial agent	No. of strains with MIC (mg/L)																		
	0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Ampicillin[a]													30	6					
Cefotaxime[a]				31	5														
Ceftazidime[a]						36													
Chloramphenicol[b]									3	30	1				2				
Ciprofloxacin[a]		15	19			2													
Colistin[c]									36										
Florfenicol[c]									26	10									
Gentamicin[a]							1	35											
Kanamycin[b]										36									
Nalidixic acid[b]										34					2				
Streptomycin[c]										2	29	1				4			
Sulfamethoxazole[b]											31	2	1		1				1
Tetracycline[b]								5	28	3									
Trimethoprim[a]								23	13										

Numbers indicate the number of isolates with corresponding MIC value. White areas indicate range of dilutions tested for each antimicrobial agent; values above or below this range denote MIC values greater than the highest concentration tested and MIC values smaller than or equal to the lowest concentration tested, respectively. Vertical lines indicate clinical breakpoints, when two vertical lines exist, the lower breakpoint indicate susceptibility and the higher breakpoint resistance with an intermediate range in between. [a]Clinical breakpoint is available according to EUCAST guidelines ~~(~~(version 4.0, 2014 *Enterobacteriaceae*). [b]Clinical breakpoint is available according to CLSI (M100-S22, 2012 *Enterobacteriaceae*). [c]No EUCAST or CLSI clinical breakpoint available.

3 Results and ~~D~~discussion

3.1 Detection of virulence-associated genes

Pigs are the focus of interest as the primary reservoir for human pathogenic *Y. enterocolitica*, especially for BT 4, which is predominantly found in porcine tonsils and also represents the principal BT in human isolates (EFSA, 2013). Therefore, the current study focused on BT 4. The virulence genes chosen (*yadA*, *virF*, *ail*, *inv*, *rovA*, *ymoA*, *ystA*, *ystB and myfA*) covered those involved in important steps during host infection (Fàbrega and Vila, 2012). In total, 87 porcine and human isolates were tested for virulence-associated gene patterns. Eighty-four of the strains (< 96%) possessed the *yadA*, *virF*, *ail*, *inv*, *rovA*, *ymoA*, *ystA* and *myfA* genes, whereas none of them possessed the *ystB* gene, demonstrating the high potential of Swiss porcine BT 4 isolates to be pathogenic for humans. Only 1 strain isolated from porcine tonsils (1/38) lacked *yadA*, and another 2 isolates from porcine tonsils (2/38) were *yadA*- and *inv*-negative, whereas isolates from porcine feces showed no difference in their virulence-associated gene patterns in comparison to the human isolates (Table 1). In part, these results contradict the findings of previous studies. Bonardi et al. (2013) detected 12.5% Italian porcine BT 4 *ail* negative strains, and Zheng et al. (2008) also detected *ystA*-negative and *ystB*-positive human BT 4 strains in China, in contrast to our data. The detection rate in the current study, with 92.1% of *yadA*-positive porcine tonsil strains, was still higher than that in recent studies on porcine or human BT 4 strains (54–83%) (Zheng et al., 2008; Stephan et al., 2013; Bonardi et al., 2013). As we tested 2 plasmid-borne genes and the strains still carry the *virF* gene, it is unlikely that the lack of the *yadA* gene is a consequence of a plasmid loss during the cultivation process. Two tested BT 4 strains from porcine tonsils lacked the *inv* gene. It has been reported that *inv* negative strains still have the ability to invade the host cells (Pepe and Miller, 1993). Therefore it is possible that the 2 porcine isolates lacking *inv* are still pathogenic. In addition to the abovementioned virulence-associated genes, the *ymoA* and *rovA* genes were tested. Both of these genes are involved in the regulation of *inv* expression. Moreover, *ymoA* is also relevant for the modulation of the expression of the *virF* and *yst* genes (Revell and Miller, 2000) (Ellison et al., 2003). Our results showed that, independent of the source, all tested isolates possessed both effector and regulator genes. To the authors' knowledge these findings are not demonstrated up to now elsewhere.

3.2 Serotype distribution

The tested isolates comprised a broad spectrum of known STs (O:3; O:8; O:27; O:3,27; O:3,8,27; O:8,27 and O:9,27). The most frequent STs were O:3 and O:3,27, followed by O:8,27, except for human isolates, which did not show ST O:8,27 (Table 1). This finding is in contrast to the results of other studies that determined almost 100% bioserotype 4/O:3 for both porcine and human strains. This discrepancy can be explained by methodological differences of serotyping. In other studies, only antiserum O:3 (Laukkanen et al., 2009) or a different panel of antisera was used (Bonardi et al., 2013). In the present study, the virulence-associated gene pattern was independently detected from STs for both porcine and human isolates. Thus, determination of STs is of limited value for the characterization of pathogenic *Y. enterocolitica* isolates.

3.3 Antimicrobial testing

In general, the majority of all strains of this study were susceptible to almost all the tested antibiotics. In contrast, 100% antibiotic resistance to ampicillin was found (Tables 2 and 3). The high rate of resistance to β-lactam antibiotics has been well described (Fàbrega and Vila, 2012), and these findings also agree with previous studies of the antimicrobial antibiotic resistance of porcine BT 4 strains from Switzerland (Fredriksson-Ahomaa et al., 2007; Baumgartner et al., 2007). Although human BT 4 isolates shared the overall antibiotic susceptibility phenotype with porcine isolates, 2 human BT 4 isolates possessed antibiotic resistance to chloramphenicol and nalidixic acid. One strain was also resistant to sulfamethoxazole. *Y. enterocolitica* antibiotic resistance to chloramphenicol is rarely described in the literature, but Bonardi et al. (2014) recently found BT 4 strains resistant to chloramphenicol. Additionally, Sihvonen et al. (2011b) detected antibiotic resistance to chloramphenicol in human *Y. enterocolitica* strains. For streptomycin, the human and the porcine tonsil strains showed an MIC50 of 8 mg/L and an MIC90 of 16 mg/L. In contrast, the 2 chloramphenicol-resistant strains showed an elevated MIC of > 128 mg/L, which indicated that they are resistant to streptomycin as well. The origin of this antibiotic resistance pattern (chloramphenicol, streptomycin and sulfonamide) is most likely a conjugative plasmid, as Sihvonen et al. (2011b) have indicated. The 2 chloramphenicol-resistant strains also possessed antibiotic resistance to nalidixic acid. Antibiotic resistance of *Y. enterocolitica* to nalidixic acid has been described in Switzerland (Fredriksson-Ahomaa et al., 2012), and a substantial increase in nalidixic acid resistance in human clinical isolates has been detected in Spain between 1995 and 2002 (Capilla et al., 2004). MIC50 and the MIC90 of florfenicol were 4 mg/L each for isolates from porcine tonsils and from porcine feces. Only 1 porcine isolate exhibited an elevated MIC to florfenicol (MIC = 8 mg/L). For human isolates MIC50 of florfenicol was 2 mg/L and the MIC90 = 4 mg/L, respectively.

4 Conclusions

The virulence-associated gene pattern detected in the human strains from clinical cases is supposed to be sufficient for human infection. The same virulence-associated gene pattern was found in the strains isolated from porcine tonsils as well as porcine feces, demonstrating the potential to cause human infection for all of the Swiss porcine *Y. enterocolitica* BT 4 strains tested. Therefore, measures must be taken at slaughter to avoid *Y. enterocolitica* cross-contamination from tonsils as well as fecal contamination of the carcasses.

Recent studies in Europe have reported increasing antibiotic resistance of both human and porcine *Y. enterocolitica* isolates (Sihvonen et al., 2011b; Bonardi et al., 2014). Low level of antibiotic resistance found in Swiss human strains from 2001 to 2003 underline the increasing tendency in human strains, whereas the rate of acquired antibiotic resistance in recently isolated predominant Swiss porcine *Y. enterocolitica* BT 4 strains is currently very low. However, the use of antibiotics in Swiss livestock is still considerable. Therefore, the prudent use of antimicrobials in veterinary medicine is important for the control of antibiotic resistance phenomenon in Swiss *Y. enterocolitica* isolates.

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Highlights

- ~~The same v~~Virulence-associated gene pattern ~~were~~ found in *Y. enterocolitica* BT 4 strains ~~isolated~~ from porcine tonsils and~~as well as from~~ porcine feces are equal
- Porcine *Y. enterocolitica* BT 4 strains are capable to infect humans despite the source of isolation and serotype
- ~~t~~The rate of acquired resistance in predominant Swiss porcine *Y. enterocolitica* BT 4 strains is currently low

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