

Full Length Research Paper

Characterization of *Yersinia* spp. strains isolated from pigs in Abidjan, Côte d'Ivoire, West Africa

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The aim of this study was to evaluate the prevalence and determine the molecular virulence of the pathogenic *Yersinia* spp. strains recovered from raw pig samples from a slaughterhouse in Abidjan, Côte d'Ivoire. A total of 460 raw pig samples including 200 tongues, 150 tonsils, 110 feces, were characterized phenotypically and genotypically for virulence genes. The strains were also tested for the presence of virulence gene using polymerase chain reaction (PCR). Nine (2%) *Yersinia* strains were isolated from the 460 samples. The isolates belonged to various bio-serogroup such as *Yersinia enterocolitica* 4/O:3/VIII (33.3%), *Yersinia intermedia* 4/autoagglutinable (44.4%) and *Y. intermedia* 5/O:7,8-8-8,19 (22.2%). Tonsils were the most contaminated organ from pig with 66.7% of isolated *Yersinia* strains. The *Yersinia* strains exhibited phenotypic virulence characteristics and virulence genes such as *ail* (3/9), *ystA* (8/9) and *yadA* (2/9). Two *Y. enterocolitica* 4/O:3 strains carried simultaneously all three virulence genes. Among these bacteria, virulent strains of *Y. enterocolitica* were found, with biotype and serogroup related to human diseases, representing a risk for the consumers of pork. This study is the first that documented the occurrence of pathogenic *Yersinia* and related species in pigs in Côte d'Ivoire. The occurrence of virulence strains of *Y. enterocolitica* shows that pork is a potential source of human infection by this species and the circulation of *Yersinia* spp. in pigs in Côte d'Ivoire.

Key words: *Yersinia*, pigs, virulence, polymerase chain reaction (PCR).

INTRODUCTION

Yersinia enterocolitica is one of the three species within the *Yersinia* genus pathogenic to humans, and is the

species most commonly associated with human disease episodes. It is a zoonotic pathogen causing self-limiting

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gastro-intestinal infection, with disease heavily associated with the consumption of undercooked or contaminated food products of porcine origin (Fredriksson-Ahomaa and Korkeala, 2003a; Milnes et al., 2008). Genotype of *Y. enterocolitica* strains found in pigs and pork are indistinguishable from strains found in humans, further supporting the association between yersiniosis and consumption of pork (Fredriksson-Ahomaa et al., 2001).

This bacterium has been detected in pigs and pork meat (Bhaduri et al., 2009). Pigs thus constitute a potential reservoir of *Y. enterocolitica* strains pathogenic to humans. Pigs develop no clinical signs, but they carry *Y. enterocolitica* in the oral cavity, particularly on the tongue and tonsils and in the lymph nodes, and they excrete the bacterium in their faeces (Nesbakken et al., 2003). Bioserotype 4/O:3 is the most frequently isolated in pig in France (Fondrevez et al., 2010) and in others European countries.

The majority of human pathogenic *Y. enterocolitica* recovered in Europe belong to bioserotype 4/O:3 or less commonly to biotype 2 (serotype O:9, O:5,27) (EFSA, 2011). Pathogenic *Y. enterocolitica* carry a variety of pathogenic genes that are either carried by their plasmids or as a part of their chromosomal DNA (Burnen et al., 1996) which in turn encode certain outer membrane proteins that are involved in their pathogenesis. Many studied human pathogenic serotypes harbor a virulent-associated plasmid pYV of 70 to 75 Kbp (Bhaduri and Cottrell, 1996). Among the chromosomally encoded virulence genes *inv*, *ail*, and *yst* are the most important (Lobato et al., 1998).

All pathogenic *Y. enterocolitica* strains harbor *ail*, which is different from the *inv* sequence (which encodes a protein of similar function), and renders *Y. enterocolitica* capable of invading the intestinal epithelium. In addition, the Ail protein confers a serum resistance phenotype on *Y. enterocolitica* (Pierson and Falkow, 1993). In contrast to *inv*, which exists in non-pathogenic as well as pathogenic strains of *Y. enterocolitica*, *ail* only exists in *Y. enterocolitica* strains epidemiologically related to human disease (Miller et al., 1989) and is therefore an important virulence marker. Environmental isolates not associated with disease have a nonfunctional *inv* and no *ail* genes (Pierson and Falkow, 1990).

Polymerase chain reaction (PCR) is the accepted method for detecting nucleic acids in a variety of samples in the field of molecular diagnostics. PCR applied to pathogenic *Y. enterocolitica* detection is a rapid method with high specificity and sensitivity (Fredriksson-Ahomaa and Korkeala, 2003b). However, an enrichment step prior to PCR is essential to increase the sensitivity and to decrease the risk of false-positive results due to detection of dead cells. Moreover, positive results obtained by PCR should be confirmed with culture methods because isolation of the strains is essential for confirmation and to enable characterization.

Most *Yersinia* spp. has traditionally been designated as non-pathogenic and the presence of the bacteria in foods is not always associated with disease. Anyway, some authors have showed that they are not as harmless as it is believed; therefore, detection of virulence factors in *Y. enterocolitica* is necessary. Very little information about the presence of enteropathogenic *Yersinia* in food and in human, and about contamination routes is available in developing countries and tropical regions, particularly in Côte d'Ivoire. Moreover, there is no data and information on the yersiniosis in humans in Côte d'Ivoire. Thus, the aim of this study was to collect data on the carriage of pathogenic *Yersinia* isolated from pigs in Abidjan and to characterize phenotypically and genotypically the strains by using molecular methods. In this study, we have investigated the circulation of *Yersinia* spp. in most appreciate meat from pigs in Côte d'Ivoire.

MATERIALS AND METHODS

Sampling

From January 2009 to September 2010, a total of 460 samples (200 tongues, 150 tonsils, 110 fecal) were collected from asymptomatic pigs in a slaughterhouse in Abidjan, Cote d'Ivoire. The pigs are from different farms and slaughtered at slaughterhouse, no data information about practice in the farms was available for this study. The pig samples distribution on the origin and the location of the farms are listed in the Table 1. The tonsil samples were cut out immediately after evisceration and collected in sterile plastic bags. The feces samples and tongues were swabbed with sterile cotton wool. The cotton swab samples were transferred into tubes containing 9 ml of trypticase soya broth (TSB) with Novobiocin (MERCK, Darmstadt, Germany). The samples were stored cold during transportation in an ice box at 4°C within 2 h from collection and taken to the laboratory for immediate processing.

Isolation and identification of *Yersinia* spp.

Yersinia strains was isolated using two stages enrichment procedures including preenrichment in trypticase soya broth with Novobiocin (MERCK, Darmstadt, Germany) overnight at 28°C and selective enrichment using cold method (21 days at 4°C) in PSMB (phosphate buffered saline supplemented with 1% mannitol, 1% sorbitol and 0.15% bile salts). A volume of 0.5 ml of TSB was transferred into 4.5 ml of PSMB, which was incubated at 4°C for 21 days. In order to reduce the background contaminating flora, Aulisio's alkali treatment method (Aulisio et al., 1980) was performed and immediately streaked onto MacConkey agar (Bio-Rad, Marnes-La-Coquette, France) supplemented with 1% sorbitol. The plates were incubated at 25°C for 48 h.

One to five small (diameter < 2 mm) transparent or pale pink colonies with characteristics of *Yersinia* were transferred onto one to five plates of trypticase soy agar (TSA, Oxoid, France) incubated at 25°C for 24 h. All the isolates from pure culture were examined for Gram's staining, oxidase, catalase test, urease activity, tryptophane deaminase, glucose and lactose fermentation, gas formation from glucose, H₂S production, lysine decarboxylase, utilization of Simmons citrate, mannitol fermentation, reduction of nitrate and motility at 25 and 37°C (ISO 10273:2003). The strains were further confirmed using API 20E and API 50CH strips

Table 1. Pigs and samples distribution at the slaughterhouse of SIVAC (Société Ivoirienne d'Abattage et de Charcuterie, Yopougon, Abidjan) according to origin of the pigs.

Location	Total of Samples collected				
	Pigs	Sample	Tonsil	Tongue	Feces
Azaguié	59	66	21	28	17
Anyama	40	46	15	20	11
Bouaké	15	18	6	7	5
Bingerville	78	89	28	41	20
Divo	67	75	26	33	16
Grand-Bassam	57	64	22	29	13
Tiassalé	20	23	8	9	23
Yopougon	71	79	24	33	22
Total	407	460	150	200	110

(BioMerieux, Marcy l'Etoile- France) and incubated for 24 to 48 h at 25°C.

Bioserotyping and phagotyping

The isolated strains of *Yersinia* spp. were serotyped with specific antisera. O antigens were determined by slide agglutination with the 53 difference antisera, according to the typing scheme of Wauters et al. (1991). Phage typing was carried out with a set of 12 lysogenic phages and 16 sewage phages (Nicolle et al., 1976). The assays were performed in Institute Pasteur Paris (Centre National des *Yersinia*, Institut Pasteur, Paris, France) for confirmation and phagotyping.

Detection of virulence genes by PCR

DNA extraction

DNA extraction from *Yersinia* spp. was accomplished by using the phenol-chloroform method as described by Sambrook and Russel (2001) and modified. Briefly, fresh colonies of bacterial cells on trypticase soy agar (TSA, Oxoid, France) were added to 500 µl lysis buffer (BioMerieux, Boxtel) and incubated for 2 h at 56°C. DNA was separated with an equal volume of phenol : chloroform : isoamylalcohol (25:24:1) and centrifuged at 13000 xg for 2 min. 500 µl of supernatant were transferred to a new sterile tube. The DNA was precipitated by adding 100% ethanol to the supernatant. The DNA was washed twice in cold 70% ethanol and then the pellet was resuspended in 50 µl TE buffer (BioMerieux, Boxtel) and stored at -20°C.

Gene amplification

The detection of the *Yersinia* heat-stable enterotoxin gene (*ystA*) were performed using the protocol of Ibrahim et al. (1997) to detect all pathogenic strains of *Y. enterocolitica*. The detection of the attachment invasion gen (*ail*) and *Yersinia* Adhesine A gene (*YadA*) were performed using the protocol of Thoerner et al. (2003). PCR amplifications of *Yersinia* spp. DNA were routinely carried out in a final volume of 50 µl reaction mixture containing 1X Go Taq Reaction Buffer (Promega, USA), 1.5 mM MgCl₂ (Promega, USA), 0.2 mM of dNTP, 1 Unit Go Taq polymerase (Promega, USA), 0.4 µM of each forward and reverse primer (Table 2) and 5 µl DNA. The

amplification was run by an initial denaturation step at 94°C for 2 min, which was followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing of primers at 55°C for 1 min and extension at 72°C for 30 s. A final extension was performed at 72°C for 5 min. *Yst A* and *ail* gene were amplified in duplex reaction while *YadA* was performed in simplex reaction. The amplification was performed in a Thermal Cycler (GeneAmp 9700, Applied Biosystems, Singapore). A negative control and positive control with each of the reaction were included in all experiments.

Visualization of amplified products

After PCR amplification, 10 µl of the PCR amplified product were analyzed on 2% agarose gels (Invitrogen, Scotland). The PCR products were analyzed after agarose gel electrophoresis and visualized under ultraviolet-light trans-illumination after staining with ethidium bromide (0.7 µg/ml) (Eurobio, France).

RESULTS

Prevalence of *Yersinia* spp.

A total of 460 pig samples, nine (2%) strains of *Yersinia* were isolated and distributed in three (33.3%) *Y. enterocolitica* and six (66.7 %) *Y. intermedia*. *Yersinia* spp. strains were isolated in 2 (22.2%) tongue, 6 (66.6%) tonsil and 1 (11.1) feces (Table 3). The isolates belonged to various bioserogroup such as *Y. enterocolitica* 4/O:3/VIII (33.3%), *Y. intermedia* 4/autoagglutinable (44.4%) and *Y. intermedia* 5/O:7,8-8-8,19 (22.2%) (Table 4).

Bioserotypes of *Yersinia* strains

The distribution of the isolated *Yersinia* strains in species, bioserotype, sources of isolation and origin of slaughtered pigs are presented in Table 4. From 9 isolates of *Yersinia* strains, the bioserotyping assay has identified the bioserotype 4/O:3/VIII in 3 *Y. enterocolitica* strains. In contrast, by *Y. intermedia* the both bioserotypes 4/Aag and 5/O:7,8-8-8,19 have been detected in 6 strains. The tonsils were the source of bioserotypes 4/Aag and 5/O:7,8-8-8,19 and have the higher contamination with 66.6% of the isolated strains. Tongues from Bingerville and fecal samples from Yopougon were contaminated with the bioserotype 4/O:3/VIII (Table 4).

Virulence genes detected

Using duplex detection for *ail* and *yst A* genes, 88.8 % (8/9) of isolated strains were positive for *yst A* gene and only 33.3% (3/9) of strains were positive for *ail* gene with amplified bands of 351 and 145 bp, respectively (Figure 1 and Table 5). Two *Y. enterocolitica* strains (L274, L275) possessed simultaneously all 3 virulence genes, while

Table 2. Primers for the detection of virulence genes in *Yersinia* strains.

Gene	Primer	Sequence (5'-3')	Amplicon length (bp)	Reference
<i>ail</i>	ail-F	TAATGTGTACGCTGCGAG	351	Thoerner et al., 2003
	ail-R	GACGTCTTACTTGCACTG		
<i>yst</i>	Pr2a	AATGCTGTCTTCATTTGGAGC	145	Ibrahim et al., 1997
	Pr2c	ATCCCAATCACTACTGACTTC		
<i>yadA</i>	yadA-F	CTTCAGATACTGGTGTCTGCTGT	849	Thoerner et al., 2003
	yadA-R	ATGCCTGACTAGAGCGATATCC		

Table 3. Prevalence of *Yersinia* spp. strains in pigs samples.

Source	No. of samples	No. of <i>Y. enterocolitica</i>	No. of <i>Y. intermedia</i>	Total (%)
Tongue	200	2	0	2 (22.2)
Tonsil	150	0	6	6 (66.7)
Feces	110	1	0	1 (11.1)
Total (%)	460 (100)	3 (33,3)	6 (66.7)	9 (100)

Table 4. Bioserotypes of *Yersinia* strains isolated from pigs.

Strain	Source	<i>Yersinia</i> species	Bioserotype	Location of pigs
L 274	Tongue	<i>Y. enterocolitica</i>	4/O:3/VIII	Bingerville
L 275	Tongue	<i>Y. enterocolitica</i>	4/O:3/VIII	Bingerville
F 276	Faecal	<i>Y. enterocolitica</i>	4/O:3/VIII	Yopougon
A 644	Tonsil	<i>Y. intermedia</i>	4/Aag [*]	Azaguié
A 681	Tonsil	<i>Y. intermedia</i>	4/Aag [*]	Divo
A 682	Tonsil	<i>Y. intermedia</i>	4/Aag [*]	Anyama
A 684	Tonsil	<i>Y. intermedia</i>	4/Aag [*]	Divo
A 694	Tonsil	<i>Y. intermedia</i>	5/O:7,8-8-8,19	Grand-Bassam
A 700	Tonsil	<i>Y. intermedia</i>	5/O:7,8-8-8,19	Grand-Bassam

*Aag, Autoagglutinable

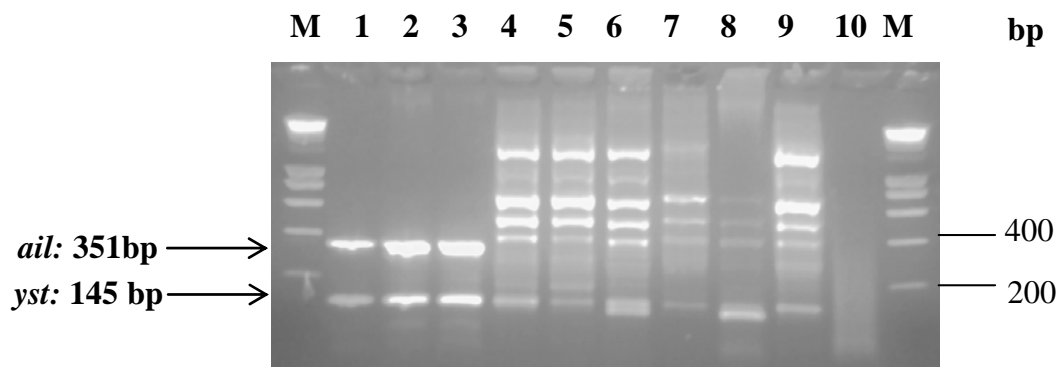
**Figure 1.** Profile detection of virulence genes (*ail* and *yst*) in *Yersinia* strains isolated from pigs. M: DNA Molecular weight markers (Eurogentec, France); Lane 1: L274; Lane 2: L275; Lane 3: S276; Lane 4: A644; Lane 5: A681; Lane 6: A682; Lane 7: A684; Lane 8: A694; Lane 9: A700; Lane 10: negative control.

Table 5. Distribution of virulence genes in *Yersinia* isolates.

Strain	Virulence gene		
	<i>ail</i>	<i>yst A</i>	<i>yadA</i>
L274	+	+	+
L275	+	+	+
F276	+	+	-
A644	-	+	-
A681	-	+	-
A682	-	+	-
A684	-	+	-
A694	-	-	-
A700	-	+	-
Total (%)	3/9 (33.3)	8/9 (88.8)	2/9 (22.2)

strain F276 possessed two virulence genes (Table 4). No specific bands were amplified in some strains and these cannot be classified as positive bands (Figure 1).

DISCUSSION

Y. enterocolitica is a common cause of acute enteritis in temperate and cold countries worldwide. The main symptoms of human yersiniosis are diarrhea, fever and abdominal pain (Savin et al., 2012). Pigs are considered the principal reservoir for the types of *Y. enterocolitica* pathogenic to humans but do not develop clinical signs (Fondrevez et al., 2010).

In this study, *Yersinia* spp. isolates were isolated from 9 (2%) of the 460 pig samples examined from a single slaughterhouse. All *Y. enterocolitica* isolated strains were identified as serotype O:3. It is worth to mention that *Y. enterocolitica* serotype O:3 is isolated more than 90% during human yersiniosis outbreaks. The reason for such presence of pathogenic *Y. enterocolitica* can be cross-contamination possibility during the slaughter process. Slaughter techniques and slaughter hygiene may influence the contamination rate (Andersen, 1988). Moreover, bioserotype 4/O:3 is generally the predominant bioserotype in pig production systems (Van Damme et al., 2010), but a recent study identified bioserotypes 2/O:5 and 2/O:9 as the most prevalent on English pig farms (Martínez et al., 2010). In Nigeria also, the bioserotype 4/O:3 were found in the fecal samples of one pig and one sheep (Okwori et al., 2009). Likewise, in South Africa, *Y. enterocolitica* was isolated in 1% of 1634 fecal examinations (Jennings et al., 1987). Also, in Norwegian study investigation, Johannessen et al. (2000) monitoring the occurrence of *Y. enterocolitica* pathogenic strains in 249 pig samples from five different slaughterhouses, found in 15.2% of cases the pathogenic strains of *Y. enterocolitica* O:3. In the study of Fredriksson-Ahomaa et al. (2009), prevalence of enteropathogenic *Y.*

enterocolitica in individual pigs was significantly lower by culturing (9%) as compared to PCR (35%). The authors explained the results by the low sensitivity of culture method as compared to PCR. However, this difference could be explained by the capacity of the PCR method to detect also death cells.

Moreover, while PCR can be useful to quickly detect suspected positive samples, only culture method enable recovery of isolates. The low rate of our isolation of pathogenic *Y. enterocolitica* in samples may be due to the limited sensitivity culture methods. The apparently low prevalence of pathogenic *Y. enterocolitica* in food may be due to lack of suitable selective methods, as reported by Magras et al. (2008). For Fredricksson-Ahomaa and Korkeala (2003a), there are difficulties associated with the isolation of pathogenic *Y. enterocolitica* strains from the small number of pathogenic strains in the samples and the large number of organisms in the background flora, especially in food and environmental samples, but one of the major factors was the difficulty to recover *Yersinia* strains from poly-contaminated biological samples (Savin et al., 2010), because of their peculiar growth characteristics such as slow growth rate and optimal temperature of 28°C. The differences between the findings of various authors and those of this study might be due to several factor such as isolation methods, number of analyzed samples, season, and geographical location. These factors may cause an increase or decrease in the prevalence of the *Yersinia* spp. (Siriken, 2004). In addition, the present study was performed in Abidjan, where the climate is generally warm by median temperature over 25°C.

It is important to emphasize that 33.33% (3 strains) of the *Yersinia* spp. isolates in this study belong to biotypes and serogroups with variable degrees of clinical and epidemiological significant. *Y. intermedia* isolates were initially identified as *Yersinia enterocolitica* by an API 20E commercial identification kit and finally identified as *Y. intermedia* by *Yersinia* National Reference Laboratory at Pasteur Institute, with various bioserogroups (4/autoagglutinable; 5/O:7,8-8-8,19). According to Robins-Browne (2001), *Y. intermedia* species are not related to human disease but are commonly obtained from terrestrial and freshwater. However, there is growing epidemiological and experimental evidence to suggest that some *Y. intermedia* isolated from human can cause disease (Agbonlahor, 1986). These authors suggest that there may be two subgroups of *Y. intermedia*: pathogenic strains of clinical origin and nonpathogenic strains that occur in the environment.

From nine *Yersinia* isolates, three strains of *Y. enterocolitica* showed pathogenic phenotypic profile when tested for autoagglutination and pyrazinamidase production. Moreover, these strains were also positive for chromosomal virulence gene *ail* related to invasion, and for *yadA*, located on the virulence plasmid, pYV (Fredriksson-Ahomaa, 2001). The gene *ail* shows an

isolate to be virulent, as it occur only in pathogenic strains (Carniel, 1995). Additionally, most of the isolates were positive for the presence of the *yst* A gene related to the production of the heat stable enterotoxin (Yst), which is largely restricted to the classical pathogenic biotypes of *Y. enterocolitica* (Delor et al., 1990). According to Ibrahim et al. (1997), the chromosomal gene *yst*, which encodes a heat-stable enterotoxin of *Y. enterocolitica*, is a useful diagnostic marker because it occurs only in invasive strains of this species.

In summary, the results presented here indicate that PCR technique can be successful for the detection of pathogenic *Y. enterocolitica* organism in pig samples and useful tool for examining the prevalence.

The presence of strains carrying important virulence marker is totally undesirable in pig samples used by a large number of people, including poor, malnourished slum-dwellers and people with suppressed immunity (Falcao et al., 2004). In addition, the presence of these strains in pigs exposes the precariousness of pork which may lead, for instance, to future contamination of others animals, water or food used for human consumption. It is possible that the pathogenic strains from pig contaminate the ready-to-eat food. This should be a warning that sanitary control measures have to be taken.

Therefore, there is no data on yersiniosis in human in Côte d'Ivoire. Thus, *Yersinia* strain has been detected in human after infections. In our country, yersiniosis is clinical underestimated and the detection of pathogenic strains in animals, particularly in pigs, represents a real risk to human health. Indeed, Ostroff et al. (1994) in Norway reported the association between yersiniosis in man and the consumption of pork and identified raw or undercooked pork as main source of infection. The presence of pathogenic *Y. enterocolitica* 4/O:3/VIII in pork, second most consumed meat in Côte d'Ivoire after beef must be represented a risk for the consumers.

Conclusion

Our results showed the circulation of pathogenic *Yersinia* strains in animals, particularly in pigs, which is the main host of yersiniosis. Therefore, 2% of samples from pigs slaughtered at slaughterhouse from different pig farms were contaminated with *Yersinia* spp. This study revealed that pathogenic *Y. enterocolitica* 4/O:3 which is the most frequent cause of sporadic yersiniosis in Europe is present in Côte d'Ivoire and this bioserotype species is from pigs, the main reservoir. The occurrence of virulent strains of *Y. enterocolitica* shows that the pigs can be potential sources of human infection by these bacteria in Côte d'Ivoire. Virulent strains of *Y. enterocolitica* 4/O:3/VIII were found, even though in low percentage and thereby represented a risk for the consumers with regards to yersiniosis. Therefore, it is necessary to educate the public about the consumption of raw or under-

cooked pork. The results obtained in the present study could serve for future investigations on pathogenic *Yersinia*, mainly focusing on the possible contamination routes in the pork production and possibility prevention at farm level.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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