



Isolation and characterization of *Yersinia intermedia* strains from pig tonsils in Abidjan, Côte d'Ivoire

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ABSTRACT

A total of 150 samples of pig tonsils were collected from slaughterhouse in Abidjan; the pigs were from pig farms located in different areas of Côte d'Ivoire. Samples were examined for the presence of *Yersinia intermedia*. Optimal recovery of *Y. intermedia* was achieved using two step enrichment procedures based on pre-enrichment in trypticase soya broth at 28 °C for 24 h, followed by cold enrichment method at 4 °C for 21 days in phosphate buffered saline broth. Then, Aulisio's alkali treatment method was performed before streaking onto MacConkey agar. Six strains of *Yersinia intermedia* were isolated and tested for the following characteristics associated to the virulence in *Y. enterocolitica* such as pyrazinamidase activity and autoagglutinability. All the six strains were all positive for the pyrazinamidase test and four of them were autoagglutinable. Four strains were biotype 4/autoagglutinable and two were biotype 5/O:7,8-8-8,19. All the six strains of *Yersinia intermedia* were rhamnose negative and not motile at 25 °C. The results of antimicrobial resistance showed that all the strains presented multiple antibiotic resistance. The results indicate that 4% of pig tonsils from different farms collected at the slaughterhouse were contaminated with *Yersinia intermedia*. This study is the first which shows the presence of *Yersinia intermedia* in pigs in Côte d'Ivoire.

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Keywords: *Yersinia intermedia*, pig tonsils, prevalence, biotype, antibiotic resistance, Côte d'Ivoire.

INTRODUCTION

The name *Yersinia intermedia* was suggested because this species has its biochemical and genetic properties intermediate to those of *Yersinia enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*. The species *Yersinia intermedia* is a member of the genus *Yersinia* which belongs to the *Enterobacteriaceae* family. *Y. intermedia* was separated from *Yersinia*

enterocolitica and was defined biochemically and genetically as a new species in 1980. Acid production from alpha-Methyl-D-glucoside, D-melibiose, D-raffinose and L-rhamnose and utilization of Simmons citrate help distinguish it from *Y. enterocolitica* and other *Yersinia* species (Brenner et al., 1980).

Bacteria belonging to this species have been isolated from the environment (freshwater, sewage), various animals (fish,

oysters, shrimp, snails, wild and domestic animals), food (milk, cream, meat), and sometimes, healthy and sick humans, mainly from their stools (Martin et al., 2009).

Y. intermedia apparently are not intestinal pathogens, but have been associated with wound and skin infections (Bottone, 1977; Hawkins and Brenner, 1978; Brenner, 1979). *Yersinia intermedia* is usually considered non pathogenic for humans. Therefore, the pathogenic potential of *Y. intermedia* for humans has not been defined (Punsalung et al., 1987).

Besides *Y. enterocolitica*, two other *Yersinia* species, *Y. pseudotuberculosis* and *Y. pestis*, have long been known to cause human disease (Carniel and Mollaret, 1990; Bottone, 1999). The remaining seven species (*Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. aldovae*, *Y. rohdei*, *Y. bercovieri* and *Y. mollaretii*) have generally been termed as "*Y. enterocolitica*-like". They have not been studied extensively, and because of the absence of classical *Yersinia* virulence markers, they have been generally considered to be environmental and nonpathogenic species. However, an increasing number of these non pathogenic species have been isolated from sick humans, raising the question of their possible pathogenicity (Sulakvelidze, 2000).

The objectives of this work were to study *Y. intermedia* strains isolated from pig tonsils in Côte d'Ivoire and to determine their phenotype, biotype, serotype and evaluate their antibiotic resistance profile.

MATERIALS AND METHODS

Sampling

From January 2009 to September 2010, a total of 150 samples of pig tonsils were collected from apparently healthy pigs at the pig slaughterhouse from Abidjan in Côte d'Ivoire. The tonsil samples were cut out immediately after evisceration and collected in sterile plastic bags. The samples were stored in an ice box at 4 °C within 2 hours during transportation from the site of

collection and taken to the laboratory for immediate processing.

Isolation and identification of *Yersinia intermedia*

The samples were analyzed at the Laboratory of Biotechnology of the Department of Biosciences, University of Cocody. Tonsil samples were aseptically cut into pieces, 10 g per tonsil sample were inoculated into 90 ml of trypticase soya broth with novobiocin (mTSB-MERCK, Darmstadt, Germany) overnight at 28 °C. Then, 0.5 ml of TSB broth culture was transferred into 4.5 ml of PSMB (Phosphate buffered saline supplemented with 1% mannitol, 1% sorbitol and 0.15% bile salts), which was incubated at 4 °C for 21 days. Aulisio's alkali treatment method (Aulisio et al., 1980) was performed and immediately streaking onto MacConkey agar (BIO-RAD, Marnes-La-Coquette, France) supplemented with 1% sorbitol (SMAC). The plates were incubated at 25 °C for 48 h. After incubation, the plates were examined for characteristics colonies. One to five small (diameter < 2 mm), transparent or pale pink colonies having characteristics of *Yersinia* were transferred onto Trypticase soy agar (TSA, Oxoid, France) plates to create pure culture and incubated at 25 °C for 24 h. All the isolates from pure culture were examined for Gram's staining, oxidase, catalase tests, urease activity, tryptophane deaminase, glucose fermentation, lactose fermentation, H₂S production, lysine decarboxylase, utilization of Simmons citrate, mannitol fermentation, reduction of nitrate and motility at 37 °C and 25 °C. The strains were further identified by the test of API 20E (BioMérieux, Marcy l'Etoile France) incubated for 48 h at 25 °C. Isolates identified as belonging to the genus *Yersinia* were sent for confirmation of the results and the complete characteristics of strains (biotype and serotype) at Pasteur Institut, *Yersinia* Research Unit, *Yersinia* National Reference Laboratory, Paris, France.

Biochemical properties

These biochemical tests were performed at the French *Yersinia* reference laboratory ('Centre National des *Yersinia*, Institut Pasteur', Paris, France). Biochemical tests were performed with API 20E and API 50CH strips (BioMérieux, Marcy l'Etoile-France). In addition, biochemical tests based on acid production from L-rhamnose, D-melibiose, D-raffinose and α -Methyl-D-glucoside and the utilization of Simmons citrate were done according to the biotyping scheme of Brenner (Brenner et al., 1980).

Serotyping

This was performed at Pasteur Institut, *Yersinia* Research Unit, *Yersinia* National Reference Laboratory, Paris, France. All the strains were serotyped with 53 specific antisera. O antigens were detected by slide agglutination with the 53 difference antisera, according to the typing scheme of Wauters et al. (Wauters et al., 1972; Wauters et al., 1991).

Phenotypic virulence markers

Pyrazinamidase

The pyrazinamidase activity was tested as described Kandolo and Wauters (1985). Strains were inoculated over the entire slant of pyrazinamidase agar, inoculated at 28 °C for 48 h, and tested with 1 ml of fresh prepared 1% solution of ferrous ammonium sulfate (aqueous). A positive pyrazinamidase reaction was indicated by a pink to brown color that developed on the slant.

Autoagglutination

The strains were examined for their ability to autoagglutinate at 37 °C in trypticase soya broth (Biokar Diagnostics, Beauvais, France) as suggested by Kandolo and Wauters (1985). Strains were positive if autoagglutination occurred when strains grown at 37 °C.

Antibiotic susceptibilities testing

Antibiotic susceptibilities were determined by the disc diffusion method on Mueller-Hinton agar (Oxoid, France), and the

results were interpreted according to the criteria of the 'Comité de l'Antibiogramme' of the French Society for Microbiology. The plates were incubated (24-48 h at 37 °C) and resistance was scored via visual examination. The antimicrobial drugs tested and their concentrations on the discs (Bio-Rad, Marnes-La-Coquette, France) were as following: Ampicillin (10 µg), ciprofloxacin (5 µg), kanamycin (30 µg), amikacin (30 µg), chloramphenicol (30 µg), penicillin (10 UI), trimethoprim/sulfamethoxazole (1.25/23.75 µg), imipenem (10 µg), amoxicillin (25 µg), ceftriaxone (30 µg), ceftazidime (30 µg), amoxicillin/clavulanic acid (20 µg/10 µg), cephalotin (30 µg), tetracycline (30 UI), cefoxitin (30 µg) and ticarcillin (75 µg).

RESULTS

Sampling and isolation

From 150 pig tonsil samples, 6 (4%) *Yersinia intermedia* strains were isolated.

Biochemical properties

All *Yersinia intermedia* strains gave positive reactions for catalase, nitrate reduction, β -galactosidase, ornithine decarboxylase, D-glucose, D-mannitol, Inositol, D-sorbitol, D-sucrose, D-melibiose, amygdalin and pyrazinamidase activity. The following tests revealed negative reactions for Gram staining, oxidase, tryptophane deaminase, lysine decarboxylase, lysine deaminase, citrate de Simmons utilization, H₂S production, arginine dihydrolase, gelatinase, lactose, L-rhamnose, gas formation from glucose, motility at 25 °C and motility at 37 °C. All the six strains studied here were initially identified as *Yersinia enterocolitica* by an API 20E commercial identification kit and finally identified as *Y. intermedia* at the French *Yersinia* Reference Laboratory ('Centre National des *Yersinia*, Institut Pasteur', Paris, France).

Biotyping and serotyping of *Y. intermedia* strains

The six strains of *Y. intermedia* could be divided into two biotypes (biotype 4 and

biotype 5). Therefore, four strains of *Y. intermedia* biotype 4/autoagglutinable and two biotypes 5/O:7,8-8-8,19 were isolated from pig tonsils (Table 1).

Antibiotic susceptibilities

Antibiograms were determined for the six isolates. All *Y. intermedia* strains were resistant to ampicillin, amoxicillin,

amoxicillin/clavulanic acid, cephalotin, penicillin and ticarcillin. They were susceptible to ciprofloxacin, kanamycin, amikacin, chloramphenicol, trimethoprim/sulfamethoxazole, imipenem, ceftriaxone, ceftazidime and tetracycline. The susceptibilities of the six *Y. intermedia* strains to 16 antibiotics are shown in Table 2.

Table 1: Scheme used to biotype *Yersinia intermedia* isolated from pig tonsils.

Reference	IP number	Acid production from				Utilization of Simmons citrate	Biotype	Serotype
		Mel	Rham	αMG	Raf			
A644	IP33928	+	-	+	+	-	4	Aag
A681	IP33929	+	-	+	+	-	4	Aag
A682	IP33930	+	-	+	+	-	4	Aag
A684	IP33931	+	-	+	+	-	4	Aag
A694	IP33932	+	-	-	+	-	5	O:7,8-8-8,19
A700	IP33933	+	-	-	+	-	5	O:7,8-8-8,19

+, positive reaction; -, negative reaction; IP, Institute Pasteur, Mel, melibiose; Rham, L-rhamnose; αMG, alpha-Methyl-D-glucoside; Raf, raffinose; Aag, autoagglutinable

Table 2: Antibiotic susceptibilities of *Yersinia intermedia* strains isolated from pig tonsils.

Antibiotic	Disc potency	<i>Y. intermedia</i> (6) Number (%) of isolates		
		Resistant	Intermediate	Sensitive
Ampicillin	10 µg	6 (100)	0	0
Ciprofloxacin	5 µg	0	0	6 (100)
Kanamycin	30 µg	0	0	6 (100)
Cefoxitin	30 µg	0	1 (16.67)	5 (83.33)
Chloramphenicol	30 µg	0	0	6 (100)
Penicillin	10 UI	6 (100)	0	0
Trimethoprim/sulfamethoxazole	1.25/ 23.75 µg	0	0	6 (100)
Tetracycline	30 UI	0	0	6 (100)
Amoxicillin/ clavulanic acid	20/10 µg	6 (100)	0	0
Ceftriaxone	30 µg	0	0	6 (100)
Imipenem	10 µg	0	0	6 (100)
Ceftazidime	30 µg	0	0	6 (100)
Ticarcillin	75 µg	6 (100)	0	0
Amoxicillin	25 µg	6 (100)	0	0
Amikacin	30 µg	0	0	6 (100)
Cephalotin	30 µg	6 (100)	0	0

DISCUSSION

A total of 150 pig tonsils were examined for the presence of *Yersinia intermedia*. The strains of *Yersinia intermedia* were isolated in 6 samples (4%) of the tested pig tonsils from apparently healthy pigs. Likewise, Siriken (2004) examined raw ground beef samples and reported that 2 out of 61 raw beef samples (3.3%) were positive for *Y. intermedia*. In another study by Punsalang et al. (1987) isolated 6 *Y. intermedia* from 860 stool cultures (0.7%). However, our studies generated higher isolation (4%) than the results in these studies. Some researchers reported that even higher isolation rate were detected compared to the results of this study. For instance, Nesbakken et al. (1985) in Norway and Franzin et al. (1984) in Italy found that *Y. intermedia* were recovered from 7.9% of retail pork products and 23.3% from raw milk. 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.* as Siriken (2004) revealed. In addition, the present study was performed in Abidjan, where the climate is generally warm. Also, it is known that the isolation ratio of *Y. intermedia* is higher in cold climate. The best results of isolation were obtained by combining pre-enrichment in Trypticase Soya broth with novobiocin overnight at 28 °C, cold enrichment method in PSMB broth for 3 weeks at 4 °C, Aulisio's alkali treatment method and SMAC agar, as previously reported by others (Mehlman et al., 1978; Doyle and Hugdahl, 1983; Martinez, 2010). It appeared that some enrichment procedures were better than others. For example, Jiang et al. (2000) reported that the highest isolation rate was 32% for the 4 °C/3-week enrichment, followed by 28% for the 4 °C/2-week enrichment, 26% for the 25 °C/24-h enrichment, 22% for the 4 °C/1-week enrichment, and 10% for direct plating.

Therefore, the isolation ratio may depend on the procedure. Furthermore, the identification of *Y. enterocolitica sensu stricto* as *Y. intermedia* by traditional agar plate techniques (ISO standard 10273:2003) is complicated by the fact that on the commonly used selective agar plates, especially the cefsulodin-irgasan-novobiocin (CIN) agar, several unrelated bacteria also grow (Arnold et al., 2004; Neubauer et al., 2001). In addition, some *Yersinia* strains are inhibited by CIN agar (Fukushima and Gomyoda, 1986).

In our study, we found that phenotypic identification of *Yersinia intermedia* was problematic. All the six strains studied here 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. Likewise, the six strains of Punsalang et al. (1987) were all misidentified as *Yersinia enterocolitica* by the API 20E system and finally identified as *Y. intermedia*. In fact, *Y. intermedia* was proposed by Brenner and his colleagues (Brenner et al., 1980) for *Yersinia enterocolitica*-like bacteria which are rhamnose, melibiose, raffinose and alpha-Methyl-D-glucoside positive strains. After biochemical and DNA hybridization studies, these workers subdivided *Y. intermedia* into eight biogroups. Therefore, according to this scheme, which is based on the acidification of L-rhamnose, D-melibiose, D-raffinose and α -Methyl-D-glucoside and Simmons citrate utilization, the six *Y. intermedia* strains which were obtained from pig tonsils in this study belong to two biotypes (biotype 4 and 5). Biotype 4 was found to be predominant among the isolates. All the *Y. intermedia* strains were rhamnose negative and these constitute a rare biotype. Also, all the isolates were non motile at 25 °C and 37 °C. Unlike, most strains isolated in Europe and America were motile at 25 °C (Brenner et al., 1980). The 6 isolates fit into two biotypes and two serotypes, four were biotype 4/autoagglutinable and two strains of biotype

5/O:7,8-8-8,19. In a previous study, Singh and Viridi (1999) reported the isolation of *Y. intermedia* biotype 5/O:7,8-8-8,19 in pork from India. None of *Y. intermedia* strains examined by Kay et al. (1983), Prpic et al. (1985) and Punsalang et al. (1987) were autoagglutinable. In contrast to these findings, our study reported that four of our six strains were autoagglutinable. Likewise, Agbonlahor (1986) reported that four of five *Y. intermedia* isolated from patient with diarrhea in Nigeria were autoagglutinable.

Kandolo and Wauters (1985) reported that *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, the nonpathogenic serotypes of *Y. enterocolitica* elaborate the enzyme of pyrazinamidase, whereas strains belonging to pathogenic biotype do not. This observation suggests that examining *Yersinia* for pyrazinamidase activity could form the basis of a useful screening test for virulence. So, the pyrazinamidase test was a valuable tool to distinguish potentially pathogenic from nonpathogenic strains of *Yersinia sp.* The six strains described in our study were all positive in the pyrazinamidase test. These results indicate that *Y. intermedia* biotype 4/autoagglutinable and biotype 5/O:7,8-8-8,19 didn't possess the virulence genes usually found in pathogenic *Yersinia* species (lack of pyrazinamidase activity). For the present, our *Y. intermedia* isolates should be considered as nonpathogenic strains or environment species. Although, the strains were usually considered to be non pathogenic for humans, it has been suggested that they may become host-adapted for humans as opportunistic pathogens and be clinically significant in the future (Brenner et al., 1980; Kay et al. 1983). Otherwise, testing for markers of pathogenicity like pyrazinamidase activity and autoagglutinability provide additional information. Markers are not perfectly correlated with pathogenicity but provide useful information under conditions where animal testing is undesirable or impractical. Virulence in *Yersinia enterocolitica* is mediated by both chromosomal and plasmid-

borne genes. While chromosomal determinants are stable, plasmids containing virulence genes may be lost during culture and conformational procedures. Temperatures above 30 °C are known to cause a loss of virulence plasmids in pathogenic *Y. enterocolitica*, but plasmids loss may also occur as a result of other less defined circumstances (Johnson, 1998).

Our results generally agree with those of others with regard *in vitro* to susceptibilities of ciprofloxacin, kanamycin, amikacin, chloramphenicol, trimethoprim/sulfamethoxazole, imipenem, ceftriaxone, ceftazidime and tetracycline (Franzin et al., 1984; Ahmedy et al., 1985). The results of antimicrobial resistance showed that the strains of *Y. intermedia* presented multiple antibiotic resistances as seen with ampicillin, amoxicillin, amoxicillin/clavulanic acid, cephalotin, penicillin and ticarcillin. The resistance profile of the strains analyzed here was similar to those of others studies (Franzin et al., 1984; Tzelepi et al., 1999; Stock and Wiedemann, 2003). Resistance to ampicillin and numerous cephalosporins (cephalotin) was common to the strains of *Y. enterocolitica* (Falao et al., 2004). These and related drugs are used in veterinary medicine to treat swine (Prescott and Baggot, 1988). Therefore, the administration of antimicrobial agents for the treatment of bacterial infections in both veterinary and human medicine poses a potential risk because it leads to the selection of strains resistant to antibiotics. A high incidence of resistant bacteria has particularly been reported from developing countries, where antibiotics are freely available and their use is not subjected to any regulation (Levy, 1998). The main reason for the increase in resistant bacteria population is apparently in the application of antibiotics as prophylactics and growth stimulants in animals (Aarestrup, 1999; Teuber, 2001).

The results of our study showed that 4% of pig tonsils from different pig farms tested at slaughterhouse were contaminated with *Y. intermedia*. The present study supports

that swine represent a potential reservoir of *Yersinia intermedia* strains which are possibly pathogenic for humans. To our knowledge, no report of *Y. intermedia* isolation from Côte d'Ivoire was made before. So, this study is the first which showed the existence of *Yersinia intermedia* in our country.

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