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Prevalence and antibiotic susceptibilities of pathogenic Yersinia enterocolitica strains in pigs slaughtered in northern Italy

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ABSTRACT

Yersinia enterocolitica is an important zoonotic pathogen causing yersiniosis in humans and animals. Human yersiniosis is the third most common enteric disease after campylobacteriosis and salmonellosis in many European countries.

Yersinia enterocolitica strains, belonging to bioserotypes associated with human disease, have frequently been isolated from tonsils and faecal samples of domestic pigs.

In this study, 46 of 354 tonsils from healthy slaughtered pigs resulted *Yersinia* spp.-positive (13.0%). The most common serotypes of *Y. enterocolitca* (42 strains) and *Y. pseudotuberculosis* (4 strains) were 4/O:3 (95.2%) and III, respectively.

Antimicrobial susceptibility testing of the 42 *Y. enterocolitica* isolates showed that all strains were resistant to the beta-lactam antibiotics tested and to erythromycin and novobiocin, while 90.5% and 67.7% were resistant to nalidixic acid and rifampicin, respectively. High rates of resistance towards tetracycline (50%) and chloramphenicol (38.1%) were found, while low frequencies were observed to gentamicin (2.4%), kanamycin (11.9%) and neomycin (21.4%). One strain was resistant to all the antibiotics tested.

Our results confirm the pigs as an important reservoir of pathogenic *Y. enterocolitica* and demonstrate the existence of a progressive increase of multi-drug resistance.

Indexing terms/Keywords

Yersinia enterocolitca, *Y. pseudotuberculosis*, pigs, tonsils, bioserotypes, antimicrobial susceptibilities

Academic Discipline And Sub-Disciplines

Microbiology

SUBJECT CLASSIFICATION

Bioserotyping and Antimicrobial susceptibility

TYPE (METHOD/APPROACH)

Analysis of the prevalence and distribution of *Yersinia* bioserotypes and determination of antimicrobial susceptibility pattern

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INTRODUCTION

Yersinia enterocolitica is an important zoonotic pathogen causing yersiniosis in humans and animals. Human yersiniosis is the third most common enteric disease after campylobacteriosis and salmonellosis in many European countries (1).

The predominant symptom in humans is gastroenteritis, typically affecting infants and young children (2). In older children and young adults, acute yersiniosis can be present as a pseudoappendicular syndrome, which is frequently confused with appendicitis. Sometimes extra intestinal manifestation, including reactive arthritis, erythema nodosum, uveitis, glomerulonephritis and myocarditis, may occur (3). Postinfection manifestations are mainly seen in young adults. Sepsis is a rare complication in case of *Y. enterocolitica* infection, except in patients who have a predisposing underlying disease or are in an iron-overloaded state. Sepsis can also occur during blood transfusion (2). Systemic and extra intestinal infections and enterocolitis in immuno-compromised patients require antibiotic therapy, and the agents used most commonly include chloramphenicol, gentamicin, tetracycline, cotrimoxazole and ciprofloxacin (3).

Approximately 90% of clinical yersiniosis cases are considered to be of foodborne origin (4).

Yersinia enterocolitica is widely distributed in nature in aquatic and animal reservoirs, with swine serving as a major reservoir for human pathogenic strains (4, 5). The potential sources of *Y. enterocolitica* in swine are numerous. Studies in Europe, Japan, and United States have reported the presence of this microorganism in rats and other rodents, while other studies have shown its presence in flies and water (3).

Numerous studies have been carried out to isolate *Y. enterocolitica* strains from a variety of animals. However, most of the strains isolated from animal sources differ under biochemical and serological profile from strains isolated from humans with yersiniosis. *Y. enterocolitica* strains, belonging to bioserotypes associated with human disease, have frequently been isolated from tonsils and faecal samples of slaughtered pigs (5).

Yersinia enterocolitica strains belonging to certain few specific bioserotypes can cause human disease. Most strains associated with yersiniosis, in fact, belong to the following bioserotypes: 1B/O:8; 2/O:5,27; 2/O:9; 3/O:3; 4/O:3. These bioserotypes have been shown to have different geographical distributions. Strains largely responsible for human yersiniosis in Europe, Japan, Canada and the USA belong to bioserotype 4/O:3 (2), that has been shown to be the predominant bioserotype in asymptomatic pigs. Occasionally, pathogenic *Y. enterocolitica* strains, mostly of bioserotype 4/O:3, have been isolated from dogs and cats (6).

Yersinia enterocolitica biotypes 2 and 3 and serotypes O:5,27 and O:9 have sporadically been isolated from slaughtered pigs, cows, sheep and goats; however, the reservoir of these bioserotypes is not clearly established (7, 8, 9). Even wild rodents and pigs have been shown to be reservoirs for *Y. enterocolitica* O:8 strains in Japan (10).

Raw pork products have been widely investigated because of the association between *Y. enterocolitica* and pigs. However, the isolation rates of pathogenic bioserotypes of *Y. enterocolitica* have been low in raw pork, except for in edible pig offal, with the most common type isolated bioserotype 4/O:3 (11). The major contamination source of edible offal may be the tonsils, which are frequently *Yersinia*-positive. The tonsils are removed along with the pluck set (tongue, oesophagus, trachea, lungs, heart, diaphragm, liver and kidneys) and then hung on a hook or placed on a conveyer belt. During slaughtering process the spread of pathogenic *Yersinia* from the tonsils to the pluck set is unavoidable.

Contaminated pork and offal are important transmission vehicles from retail shops to humans (12). Cross-contamination may occur directly or indirectly via equipment, air and food handlers in slaughterhouses, retail shops and residential kitchens (6, 12, 13, 14, 15, 16, 17).

This study was carried out to gain knowledge of the prevalence and distribution of different bioserotypes of *Yersinia* species in pig population, bred and slaughtered in northern Italy, and to determine the antimicrobial susceptibility pattern of the *Y. enterocolitica* isolates.

MATERIALS AND METHODS

Three thousands and fifty-four tonsils from healthy, freshly slaughtered pigs were analyzed for the presence of *Yersinia* species. The animals were from 4 different herds (named A, B, C and D) of Parma's province (northern Italy). Assays were performed within 2 hours from their collection.

The isolation of *Yersinia* species was carried out exclusively by direct smear onto CIN-agar plates, according to Schiemann (1979) (18) (Yersinia Selective Agar + Yersinia Supplement, Oxoid, Basingstoke, Hampshire, England).

In order to prevent the development of confluent colonies and to obtain the growth of isolated colonies, each tonsil was pressed on a quadrant of the plate, from which it was provided to crawl with the loop. After 36-48 hours of incubation at a temperature of 25°C, suspect colonies were collected, brought in pure culture and assayed for mobility, Gram, oxidase and catalase. The strains overcoming the initial screening, consisting of the planting onto TSI agar, on the research of urease and lysine decarboxylase, were then identified by API 20E assay (bioMérieux SA, Marcy-l'Etoile, France).

The enzymatic biochemical investigations, in order to confirm the strains of *Y. pseudotubercolulosis* and to identify biotypes of *Y. enterocolitica*, have been completed by the execution of the following tests: DNase, lipase (Tween 80), nitrate reduction, esculin hydrolysis, fermentation of lactose, xylose and trehalose.

The isolates were sent for biotyping confirmation and for the identification of serological and phage groups at the National Reference Center for *Yersinia* at the University La Sapienza of Rome (Italy).

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The antimicrobial susceptibility for all 42 *Y. enterocolitica* isolates was determined by the minimum inhibitory concentration test (MIC). Fifteen antimicrobials were analyzed: ampicillin, carbenicillin, cephalotin, methicillin, penicillin, erythromycin, novobiocin, nalidixic acid, rifampicin, tetracycline, chloramphenicol, streptomicin, gentamicin, kanamycin and neomycin.

MIC test was performed by a microdilution method, utilizing scalar dilutions in Mueller Hinton broth (MH) of each antibiotic. The concentration range of each antibiotic was from 256 to 0.125 µg or U.I./ml. The broth culture concentration of each *Y. enterocolitica* strain, in MH after an incubation at 28°C for 18 h, was $5x10^5$ UFC/ml. The microtiter plates were incubated at 28°C for 18 h in humid chamber.

RESULTS

Overall, 46 of 354 tonsils resulted *Yersinia* positive (13.0%): 42 strains (91.3%) corresponded to *Y. enterocolitica* and 4 strains (8.7%) were *Y. pseudotuberculosis*. The distribution of the two species within the 4 herds is summarized in Table 1.

Table 1: Distribution of *Y. enterocolitica* and *Y. pseudotuberculosis* strains in the four herds analyzed

Herd	No. pigs analyzed	*Y. enterocolitica*		*Y. pseudotuberculosis*		*Yersinia spp.*
		No. pigs positive	% pigs positive	No. pigs positive	% pigs positive	% total pigs positive
A	38	1	2.6	4	10.5	13.2
B	42	2	4.8	-	-	4.8
C	58	4	6.9	-	-	6.9
D	216	35	16.2	-	-	16.2
Total	**354**	**42**	**11.9**	**4**	**1.1**	**13.0**

While *Y. entercolitica* was widespread in all herds, the presence of *Y. pseudotuberculosis* has been established in high percentage (10.5%) only in one of them (breeding A).

Although the percentage of *Y. enterocolitica* pig positive was globally consistent (11.9%), its distribution was not uniform, varying from 2.6 to 16.2%.

Noteworthy, in this regard, is the fact that the highest value has been observed in breeding with the largest number of animals in production.

Almost all of the strains (40/42 = 95.2%) of *Y. enterocolitica* belonged to 4/O:3/VIII type; their distribution, even in this case, was not homogeneous since this type has been found in all positive pigs of herd C (4/4) and D (35/35), in one animal (1/2) of herd B, but not from herd A, where 4 strains of *Y. pseudotuberculosis* (serotype III) and 1 strain 1A of *Y. enterocolitica* were isolated.

In B piggery, one strain 2/O:9/X_3 of *Y. enterocolitica* was also recovered.

The biotype/serotype/lysotype profile of isolates is shown in Table 2.

Table 2: Biological characteristics of *Yersinia* spp. isolates and their distribution in the four herds analyzed

Species isolated	Biotype	Serotype	Lysotype	No. strains	Herd
Y. enterocolitica	4	O:3	VIII	40	B-C-D
Y. enterocolitica	2	O:9	X_3	1	B
Y. enterocolitica	1A	-	-	1	A
Y. pseudotuberculosis	-	III	-	4	A

Concerning the antimicrobial susceptibility analysis, all *Y. enterocolitica* isolates were resistant to ampicillin, carbenicillin, cephalotin, methicillin, penicillin, erythromycin (MIC ≥256 µg or U.I./ml) and novobiocin (MIC ≥32 µg/ml).

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High resistance frequency was observed for nalidixic acid and rifampicin. Nalidixic acid resitance was 90.5%, with MIC levels particularly high (MIC_{90} = 256 µg/ml and MIC_{50} = 64 µg/ml). Rifampicin resistance was 66.7%, with relatively low levels of MIC_{90} (4 µg/ml) and MIC_{50} (2 µg/ml).

Unexpectedly, high and diffuse rates of strains resistant to tetracycline (50%) and chloramphenicol (38.1%) were found, though with MIC extremely low and often at the threshold limit: MIC_{90} equal to 16 and 32 µg/ml, respectively. All strains chloramphenicol-resistant were insensitive to tetracycline and all strains resistant to aminoglycoside were also resistant to streptomycin.

For the aminoglycosides tested - streptomycin, neomycin, kanamycin and gentamicin - the resistance was revealed in 47.6%, 21.4%, 11.9% and 2.4% of strains, respectively, with low MIC values: streptomycin, MIC_{90} of 32 µg/ml and MIC_{50} of 8 µg/ml; neomycin, MIC_{90} of 16 µg/ml and MIC_{50} of 8 µg/ml; gentamicin, MIC_{90} of 4 µg/ml and MIC_{50} of 2 µg/ml; kanamycin, MIC_{90} of 15.2 µg/ml and MIC_{50} of 4 µg/ml.

Concerning the distribution of multi-drug resistance, 11 different patterns were observed, in addition to the resistance spectrum common to all strains: AMP, CAR, KF, MET, P, E, NV. The most represented patterns have affected 8 (10 strains), 12 (7 strains) and 9 antibiotics (5 strains); the other patterns, resulting variously distributed and including also one strain resistant against all antibiotics tested, were less represented (see Table 3).

Table 3: Antimicrobial resistance pattern in *Yersinia enterocolitica* strains

No. strain	No. antibiotic	AMP	CAR	KF	MET	P	E	NV	AN	RD	TE	S	N	C	K	GN
4	7	■	■	■	■	■	■	■								
10	8	■	■	■	■	■	■	■	■							
5	9	■	■	■	■	■	■	■	■	■						
3	11	■	■	■	■	■	■	■	■	■	■	■				
2	11	■	■	■	■	■	■	■	■	■		■	■			
3	11	■	■	■	■	■	■	■	■	■	■		■	■		
2	12	■	■	■	■	■	■	■	■	■	■	■	■			
7	12	■	■	■	■	■	■	■	■	■	■	■		■		
1	13	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
1	13	■	■	■	■	■	■	■	■	■	■	■		■	■	
3	14	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
1	15	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

AMP: ampicillin, CAR: carbenicillin, KF: cephalotin, MET: methicillin, P: penicillin, E: erythromycin, NV: novobiocin, AN: nalidixic acid, RD: rifampicin, TE: tetracycline, S: streptomycin, N: neomycin, C: chloramphenicol, K: kanamycin,

GN: gentamicin.

DISCUSSION

In this study, tonsil samples were chosen because tonsils are more effective for the recovery of *Y. enterocolitica* strains than are tongues and faeces, and tissue samples yield higher isolation rates than do swabs (19). In the study of Nesbakken et al. (2003) (20), the proportion of pathogenic *Yersiniae* detected in tonsils compared to faeces was six to one. These proportions are similar to Frederiksson-Ahomaa et al. (2007) (1) and Gürtler et al. (2005) (21) reports; they, in fact, demonstrated that the frequency of isolated pathogenic *Yersiniae* was significantly greater from tonsils than that obtained from faeces at slaughter. Published data indicate that infected pig tonsils generally contain a large number of *Y. enterocolitica* (19).

Recently it has been shown, that direct plating resulted in a similar percentage of positive pig tonsils as obtained by the use of ISO 10273 (22). For this reasons, we performed the direct smear, as isolation technique, and the percentage of animals carrying the *Yersinia* genus (13.0%) was remarkable: *Y. enterocolitica* in 11.9% and *Y. pseudotuberculosis* in 1.1% of tonsil samples.

The applied direct method is simple to perform and results in low cost analysis and a shorter analysis time compared to the enrichment cold. In return, it has required considerable manual skill in the smear of the surface footprint of the tonsil on the plate and especially willingness to follow the growth of the colonies in order to pick them up before that their typical dark-red hue tacked in consequence of the concomitant and often numerous development of other colonies.

Our results confirm that pigs are also in northern Italy an important reservoir of pathogenic *Yersinia enterocolitica*. In fact, as many as 100% of swine herds had at least one infected animal. Although this prevalence rate is higher than reported in

earlier studies, it is in accordance with studies which have identified swine as a fundamental reservoir of this bacterium (4, 23).

Serotyping of *Y. enterocolitica* isolates recovered in this work revealed that 95.2% (40/42) of them belonged to 4/O:3/VIII serotype. This is in agreement with previous data reporting that bioserotype 4/O:3 was predominant in slaughtered pigs in other European countries: Denmark, Estonia, Greece, Italy, Finland, Germany, Latvia, Norway, Russia, Sweden, Switzerland, and Poland (24).

Christensen (1980) (25) in Denmark indicates the presence of serotype O:3 in 6 of the 10 farms monitored, with percentages varying from 6.7% to 49%. In Switzerland, Fredriksson-Ahomaa et al. (2007) (1) reported an overall isolation rate of pathogenic *Y. enterocolitica* in pig tonsils of 34% when direct plating, overnight enrichment and selective enrichment were used. It was clearly lower than the isolation rate of 60% obtained in Germany from pig tonsils (1).

This same serotype has also been shown to be the most prevalent in isolates of human origin in most European countries (1, 23, 24).

The broad findings of serotype O:3, on the one hand, emphasizes the spread between our swine population and, on the other, confirms pig as the most likely vehicle of transmission to man either directly or indirectly through meat.

The isolation of one strain 2/O:9/X_3, also pathogen but spread mainly in Holland and Finland, indicate the circulation of other pathogenic serotypes in our swine herds, probably as result of the animal importation. *Y. enterocolitica* strains of biotype 2 and serotype O:9 have sporadically been isolated from slaughter pigs, cows, sheep and goats; serotype O:9 has sporadically been isolated from German (0.3%) and Italian pigs (4%) (24, 26). The possible reservoir for this type is not clear but it has thought to be ruminants (1).

Finally the last strain identified as 1A was not serologically typed or identified as phage group, since this biotype has any epidemiological relevance, being considered as “environmental”.

With regard to the 4 strains of *Y. pseudotuberculosis* belonging to serotype III, although falling in the three serotypes responsible in world-wide environment of infections in humans (I, II, III), they don’t seem to be of significant epidemiological importance, in consideration of the fact that the serotype I is isolated in 90% of clinical cases. The prevalence of *Y. pseudotuberculosis* has been at similar levels on pig farms in Estonia (1%), Finland (4%), Italy (0.3%), Germany (6%), Latvia (5%), Russia (7%), and the Netherlands (4%) (24).

Concerning the supsceptibility analysis, antimicrobial agents commonly used in either treatment of pig disease or as growth promoters or those used in the treatment of human clinical disease were selected for testing.

All 42 strains of *Y. enterocolitica* were found to be uniformly resistant to the β-lactam agents (ampicillin, carbenicillin, cephalotin, methicillin, penicillin) and to novobiocin. These results agree with results of other Authors indipendently from the strain origin and the susceptibility test performed (3, 27).

References reported in literature were discordant with our high resistance frequency observed for nalidixic acid (28, 29).

Concerning tetracycline, only sporadic findings of resistant isolates are reported in literature, performed on dogs (30, 31). Relatively to chloramphenicol, resistance segnalations are more frequent (29, 30, 31, 32) and concern in particular strains 4/O:3.

Although streptomycin resistance is a phenomenon diffusely reported (1, 27, 29, 30, 31, 32), with frequent findings for serotype O:3, the resistance to others aminoglycosides - it was always associated to streptomycin - is not reported from other Authors.

The first and most obvious observation refers to the unusual and extended antibiotic-resistance that, though generally reported in literature and characterized by more contained rates, in certain cases (neomycin, kanamycin and gentamicin) represents an unexpected phenomenon.

The classic pattern of sensitivity of *Y. entericolitica* serotype O:3, as reported by Pérez Trallero et al. (1988) (32), has got to be modified by the insertion of variability for some aminoglycosides and significant resistance to tetracycline, streptomycin and chloramphenicol.

The analysis of the resistance spectra has highlighted a very heterogeneous situation, with 12 different patterns and multi-drug resistance also extended to all the antibiotics tested.

The results obtained demonstrate the existence of a progressive increase of multi-drug resistance in strains of animal origin and can confirm the hypothesis that the use of antibiotics in veterinary medicine represents the first cause of the resistance observed in human (32).

In fact, nalidixic acid, tetracycline, erythromycin, neomycin and - less frequently - gentamicin and streptomycin are antibiotics commonly used in swine therapy. Similar considerations are valid for chloramphenicol, whose misuse in mass therapy - now made more difficult by the current legislation - can not be excluded in previous years.

In conclusion, pigs harbor habitually *Y. enterocolitica* in the oral pharyngeal hollow and to a lesser extent in the intestine. This microorganism can grow actively at refrigeration temperatures and in the conditions of low pH values that occur during refrigeration, so meat, chicken, milk, contaminated with this bacterium could become a significant health risk for consumers (3).

In addition to the hypothetical risk for the operators of the pig sector, slaughterers and consumers, should not be overlooked the aspect of environmental contamination by pathogenic serotypes present in swine faeces. The most wide distribution in nature of these strains could help in changing the epidemiology of yersiniosis, involving water, fish, shellfish and vegetables, products from which non-pathogenic serotypes are usually isolated (23, 33).

Moreover it is necessary monitoring *Y. enterocolitica* strains isolated from animal sources and improving techniques and hygiene at slaughterhouse, on the one hand, to reduce the potential transmission of enteropathogenic *Yersinia* to humans and, on the other one, can result fundamental to avoid the diffusion of multi-drug resistance to humans via food chain, since using of antimicrobial agent in veterinary as growth promotion, treatment or prophylactic could develop, by a selection process, antibiotic resistance in food of animal origin.

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