Retrospective study on the prevalence of *Yersinia enterocolitica* in food collected in Umbria region (central Italy)

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Abstract

Yersinia enterocolitica represents one of the main foodborne pathogens in Europe and the evaluation of possible sources of contamination and its prevalence in food is of considerable interest for risk analysis approach. The results of the search for *Yersinia enterocolitica* in food samples taken in Umbria region (central Italy) were evaluated during the years 2015-2018. Different types of foods were considered, both ready-to-eat (meat products, dairy products, and raw vegetables) and meat preparations to be eaten after cooking. Samples were assayed by molecular screening for the species indicator gene *ompF*. Screening positives were subjected to isolation and characterization by searching for specific virulence marker genes, including the *ail* gene responsible for invasiveness and the *ystB* gene for the production of enterotoxin. The total prevalence of positive samples for *Yersinia enterocolitica* was 16.86% with a higher percentage of positive samples in meat preparations (19.35%), followed by ready-to-eat vegetables (11.76%). Poultry meat samples had a higher prevalence than pork and beef samples. Neither positive samples were found in meat products and dairy, nor seasonality in positivity was observed. All isolated strains of *Yersinia enterocolitica* were biotype 1A, with absence of the *ail* virulence gene but presence of *ystB* gene. Since the strains isolated from human patients appear to be primarily biotypes that possess the *ail* marker, future investigations would be needed regarding the real role of biotype 1A in human disease. In this context, attention should certainly be paid to ready-to-eat vegetables and to careful cooking of meat preparations.

Introduction

Yersiniosis is a human disease, characterised by self-limiting enterocolitis with possible septicaemic, erythema nodosum, glomerulonephritis and reactive arthritis complications, mainly caused by *Yersinia enterocolitica* (Bolton *et al.*, 2013). The microorganism is present in the environment (*e.g.*, soil, water), in tissues and gastro-intestinal tract of different animals, and in foods (Le Guern *et al.*, 2016). Its relevance is highlighted in the ECDC/EFSA One Health 2020 Zoonoses Report as it is the 3rd zoonotic disease in EU countries with more than 5,600 confirmed cases and a notification rate of 1.8 per 100,000 population in 2020, with an increase of 5.9% compared to the rate on 2019 (European Food Safety Authority and European Center for Disease Prevention and Control, 2021). Moreover, this data is referred to a limited number of Member States (MS), as only 21 of them have a mandatory notification system of the disease and 5, including Italy, have a voluntary system. For this reason, in Italy, the reported number of cases were only 21, underestimating the real national epidemiological situation. The EU reported cases are not all referred to foodborne outbreaks (only 236 cases) and only in one of these there was a strong evidence of food involvement (European Food Safety Authority and European Center for Disease Prevention and Control, 2021). Even in other countries, such as the United States, *Y. enterocolitica* causes more than 100,000 illnesses and 60 deaths every year (Centers for Disease Control and Prevention, 2016) and was detected, worldwide, in 1.97% of the human patients with gastroenteritis (Rihai *et al.,* 2021). The disease is still considered underestimated since different diagnostic approaches and reporting systems are adopted (Van Cauteren *et al.*, 2017).

To complicate the situation, the diagnosis must consider either the presence of 70 serotypes of *Y. enterocolitica* or its 6 biotypes: biotype 1B highly pathogenic, biotypes 2, 3, 4, 5 weakly pathogenic and biotype 1A reported as non-pathogenic (Bottone, 2015). The pathogenicity is mainly related to the ability of the microorganism to penetrate the gastrointestinal mucus, to adhere to intestinal cells, to invade them and to produce *Yersinia*-stable toxins (YST) which is able to cause gastrointestinal disorders. Biotypes 1B, 2, 3, 4, 5 possess invasive factors (Bancerz-Kisiel *et al.*, 2018). The pathogenicity is considered primarily related to plasmid and chromosomally encoded proteins that allow adhesin formation facilitating the penetration of the microorganism in the host cells, to antiphagocytic activity and resistance to complement mediated conditions, and to toxins formation (Platt-Samoraj, 2022). Pathogenic factors are associated with genes, regulating these mechanisms, which have been recently considered as part of the diagnostic tools for the determination of infections and related biotypes (Bancerz-Kisiel *et al.,* 2018). Gene as *yadA* (Yersinia adhesin), *invA* (invasion), *ail* (attachment-invasion locus protein), *yops* (Yersinia outer membrane proteins) and *ystA, ystB, ystC* (Yersinia-stable toxins) are considered virulence marker to be investigated, confirming the presence of *Y. enterocolitica* and helping in defining its biotype in human, animal specimens and in foods (Bolton *et al.,* 2013; Bancerz-Kisiel *et al.,* 2018). There is still no consensus on the pathogenicity of the biotype 1A e as no plasmid factors are present but could produce YST (Guiller *et al.,* 2021).

Pigs are considered the reservoir of the microorganism (Fredriksson-Ahomaa *et al.,*

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2006) but *Y. enterocolitica* is reported also in other animal species (ZhiChao *et al.,* 2019; Arden *et al.,* 2022). In foods *Y. enterocolitica* isolation is reported from meat, milk and dairy products and fresh vegetables (Bonardi *et al.,* 2018; Fois *et al.,* 2018; Espenhain *et al.,* 2019; Piras *et al.,* 2021; Mancini *et al.,* 2022). The microorganism can resist in food under different environmental situations, even in not culturable form, and can grow under refrigeration conditions (Fredriksson-Ahomaa, 2012).

The preventive monitoring of *Y. enterocolitica* in animals and food samples is even less harmonized, among MS, than the human monitoring, as it is not mandatory and related to the epidemiological situation of the disease, according to EC Directive 2003/99. Therefore, the EFSA-ECDC reports (European Food Safety Authority and European Center for Disease Prevention and Control, 2021) on *Y. enterocolitica* referred only to 5 MS and 4 MS in animals and in foods respectively. Therefore, the monitoring of the epidemiological situation in foods, defining prevalence and isolated biotypes, is of utmost importance to evaluate possible sources of contamination for human and a correct risk analysis approach.

The aim of this research is to define the prevalence of *Y. enterocolitica* in different foods received by the Istituto Zooprofilattico Sperimentale Umbria e Marche from local retailers (Umbria Region, central Italy) during a 4-year period. The sampling season was also considered to understand if this factor could influence the prevalence of the microorganism in food. The isolated strains were also characterised for the presence of selected virulence genes markers.

Materials and methods

The retrospective study was conducted in 261 food samples collected from the local market (supermarket) in Umbria Region (central Italy) between 2015 and 2018. The presence of *Y. enterocolitica* was investigated during routine sampling and under a specific research sampling program focused on meat preparations. The foods analysed were meat preparation intended to be cooked (n=217); ready-to-eat (RTE) vegetables (n=17); milk and cheeses (n=18) and cured and fermented meat products (n=9). Meat preparations were beef (n=127), pork (n=74) and poultry (n=16) samples. All the food tested were locally produced, with the exception to RTE vegetables that were produced also in other Italian regions.

A 25 g aliquot of each sample was put into sterile Stomacher bags (Blender bag Plain, 400 mL, Sterile VWR®, Milano, Italy) with 225 mL of Peptone-Sorbitol-Bile broth (Biolife Italiana s.r.l., Milan, Italy) and homogenized (Stomacher 400 circulator, Seward Ltd., Norfolk, UK). After 48 hours of incubation at 25°C, 1 mL of each broth culture was used for DNA extraction, using 6% Chelex-100 sodium form (Sigma-Aldrich, Inc., St. Louis, MO, USA), according to the manufacturer's instruction. A screening end-point polymerase chain reaction (PCR) was carried out for the species indicator gene *ompF*, coding the outer membrane porin F protein (Stenkova *et al.,* 2008). The PCR reaction was performed in 50 µL of a final mixture containing: 2 µL of DNA, 0,2mM deoxyribonucleotides triphosphate (GE Healthcare Chicago, Illinois, USA), 10 µL of PCR buffer 5X, 1.5mM MgCl2, 1U GoTaq® flexi DNA Polymerase (Promega Corporation, Madison, USA) and 0.2mM of each primer. The conditions used were: initial denaturation at 95°C for 5 minutes, 35 cycles at 95°C for 30 seconds, 55°C for 60 seconds, 65°C for 30 seconds and a final extension step at 68°C for 15 minutes. For the culture broth that tested positive at screening PCR, the incubation was prolonged up to 5 days and isolation was carried out. An amount of 0.5 mL of the enriched samples were mixed with 4.5 mL of potassium hydroxide (KOH) 0.25% for 20 seconds and streaked onto Cefsulodin-Irgasan-Novobiocin agar (Biolife Italiana s.r.l., Milan, Italy) plates (Sirghani *et al.,* 2018). After 24–48 hours of incubation at 30°C, suspected colonies (characteristic *bull's-eye* colourless colony, with red centers) were presumptively identified based on Gram staining (Gram-negative, bacillus-shaped bacterium) and biochemical tests including catalase, oxidase, and urease (oxidase-negative, catalase-positive and urease-positive) (Sirghani *et al.,* 2018). A multiplex end-point PCR assay was performed to confirm the species and to check pathogenicity, targeting the specie-specific frame of *ompF* (Stenkova *et al.,* 2008), and *ail*, which encodes an outer membrane protein that promotes attachment and invasion (Wannet *et al.,* 2001). A second end-point PCR investigated *ystB*, an enterotoxin usually carried by 1A biotype strains (Garzetti *et al.,* 2014). The first multiplex PCR reaction (*ompF* and *ail*) was performed following the same conditions described for the screening PCR, while the second PCR (*ystB*) was carried out in 50 µL of final mix containing: 2 µL of DNA, 25 µL of HotStarTaqMM 2X (Qiagen, Hilden, Germany), 2mM MgCl2 (Qiagen, Hilden, Germany), 0,4 µM of each primer. The reaction was performed under the following conditions: initial denaturation at 95°C for 15 minutes, 30 cycles at 95°C for 40 seconds, 58°C for 40 seconds, 72°C for 60 seconds and a final extension step at 72°C for 8 minutes. All the PCR reaction were performed on Eppendorf Mastercycler instrument (Eppendorf s.r.l., Milan, Italy) using primers described in the literature and reported in Table 1. For size determination of the detected fragments, the PCR products were uploaded in the QIAxcel System Instrument (Qiagen, Hilden, Germany), an automated capillary electrophoresis device and analysed by the QIAxcelScreengel 1.4.0 software (Qiagen, Hilden, Germany) (Licciardi *et al.,* 2021).

Data of all the positive samples were registered in an excel datasheet. The prevalence for each food category was calculated and confidence intervals (CI) (CI 95%, CI + and CI -) were determined (Veronesi *et al.,* 2011). Chi^2 test was then performed (Microsoft Excel statistical package) to define differences between the prevalence detected in the food types; between beef, pork and poultry meat preparations; and

Table 1. Primer sequences and amplicon sizes of the two performed PCR assays for *Yersinia enterocolitica* identification.

Target	Primers	Primer sequences (5′→3′)	Amplicon size (bp)	Reference
ompF	227FMod YE2R	GTC TGG GCT TTG CTG GTC ATC TTG GTT ATC GCC ATT CG	660	(Stenkova *et al.*, 2008)
ail	A1 A2	TTA ATG TGT ACG CTG GGA GTG GGA GTA TTC ATA TGA AGC GTC	425	(Wannet *et al.*., 2001)
ystB	ystB_FW ystB_RV	ACC TTT TTG GAC ACC GCA CAG GTC TGA GTA TCG CAC GCT	208	(Garzetti *et al.*., 2014)

according to the season of sampling. Prevalence values were considered statistically different when the P value obtained was below 0.05.

Results

A total of 44 out of 261 food samples were found positive for *Y. enterocolitica* (overall prevalence=16.86%, 13.05-22.15 95% CI). Meat preparation samples registered an isolation prevalence of 19.35% (14.14-24.66 95% CI) and RTE vegetables of 11.76% (0.01-27.13 95% CI) with no difference between these two categories. No *Y. enterocolitica* was detected in processed/cured meat, milk and dairy products (Figure 1).

The results according to the meat type of the preparation are reported in Figure 2. The positive samples were 12 out of 127 in beef (prevalence=9.45%, 4.40-14.60 95% CI), 21 out of 74 in pork (prevalence=28.38%, 18.13–38.67 95% CI) and 9 out of 16 poultry preparations (prevalence=56.25%, 31.94–80.55 95% CI). The prevalence in poultry preparations was statistically higher than in pork samples (P<0.001) and the prevalence in pork was higher than in beef (P<0.001).

The results according to the seasonal evaluation of the samples are reported in Table 2. No significant difference was detected between the overall prevalence values among the sampling seasons considered. A comparison was also possible for beef and pork preparations, where no difference was moreover registered (P>0.05; beef prevalence values ranging from 12.82% in spring to 7.31% in summer; pork prevalence values ranging from 40.00% in winter to 21.73% in autumn). The limited number of samples collected for poultry (only one sample collected in winter) and RTE vegetables (no collected samples in summer) did not allow a specific statistical evaluation of seasonal effect.

All the *Y. enterocolitica* strains isolated from food samples were negative for the presence of *ail* gene and positive for the *ystB* gene, denoting a 100% prevalence of Biotype 1A.

Discussion

The results obtained highlight a high prevalence of *Y. enterocolitica* in raw pork and poultry meat. Pig can harbour the pathogen in the intestine and other tissues (*e.g.*, tonsils) and could contaminate the carcass during slaughtering (Zdolec and Kiš, 2021). Thus, a high prevalence of *Y. enterocolitica* in ground pork could be expected: *e.g.,* 22.6% in minced pork (Ferl *et al.,* 2020); 24.0% in retail pork (Terentjeva *et al.,* 2022). Nonetheless, the prevalence detected is not consistent with other authors that report lower values in pork cuts [15.2% (Bonardi *et al.,* 2010); 2.33% (Wang *et al.*, 2021)]. The same consideration could be done for poultry meat with some authors that report prevalence equivalent to those found in the present survey, 55%, (Capita *et al.,* 2002) and others much lower than those recorded [12.3%, (Momtaz *et al.*, 2013); 4.8% (Peng *et al.*,

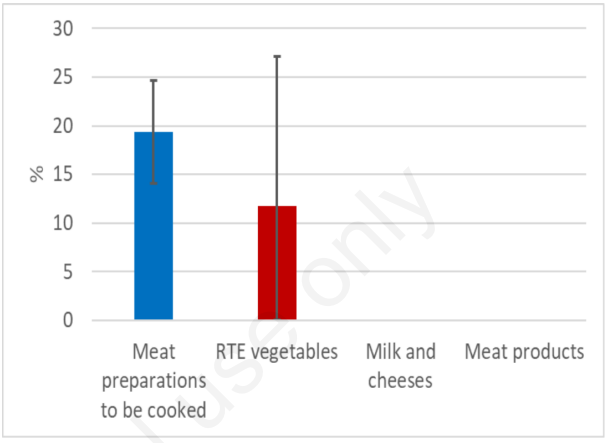

Figure 1. Prevalence (%) and Confidence Intervals (95% bars) of *Yersinia enterocolitica* in foods.

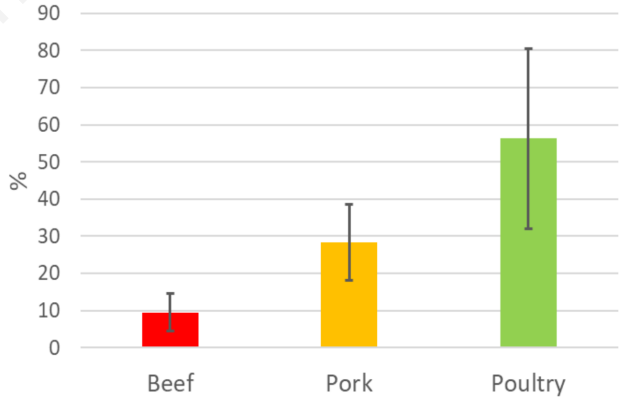

Figure 2. Prevalence (%) and Confidence Intervals (95% bars) of *Yersinia enterocolitica* in meat preparation to be cooked.

Table 2. Prevalence of *Yersinia enterocolitica* in foods according to seasonal sampling.

Season	Number positive/total samples	Prevalence (%)	CI-/ CI+
Spring	17/90	18.88	10.80 / 26.98
Summer	16/81	19.75	11.08 / 28.42
Autumn	8/69	11.59	4.03 / 19.14
Winter	3/21	14.29	0.00 / 29.25

CI, confidence interval.

2018)]. Beef is generally registered to be less contaminated by *Y. enterocolitica* than pork, with prevalence values of 13% (Terentjeva *et al*., 2022). The variability encountered in the abovementioned literature could be due to different hygiene levels and practices during slaughtering, sectioning, and preparation. Also, different molecular and cultural diagnostic approaches were adopted. Furthermore, another aspect to consider when discussing the multifarious results in the literature is the type of market considered for sampling (Wang *et al*., 2021). In the present survey this evaluation was not performed as only supermarket was investigated. Despite meat is considered one of the main sources of yersiniosis in humans, the recent European outbreaks are mainly related to RTE vegetable consumption (European Center for Disease Prevention and Control, 2019; European Center for Disease Prevention and Control, 2021) or mixed dishes such as pasta meal in Denmark, (European Food Safety Authority and European Center for Disease Prevention and Control, 2021). The presence of *Y. enterocolitica* in these foods, that are not cooked before eating, should be carefully considered in the monitoring programmes, using proper diagnostic methods (Cristiano 2021). Other authors confirm the same prevalence in RTE vegetables registered in the present survey [20%, (Mengal *et al.,* 2019); 11.2%, (Verbikova *et al.,* 2018)].

The absence of positive samples for milk and cheese could be due to the sampling of pasteurized or UHT treated milk and cheese obtained with pasteurized milk. Unpasteurized or improperly pasteurized milk and fresh raw milk cheeses could harbour *Yersinia spp*. during their shelf life (Longenberger *et al.,* 2014; Özdemir and Arslan, 2015; Gruber *et al.*, 2021) but no positive samples are considered detectable in hard-ripened cheeses (Bonardi *et al.,* 2018). Similar conclusions could be posed for cured or fermented meat products that are subjected to hurdle technology contributing to the elimination of *Y. enterocolitica* in the final product (Peruzy *et al.*, 2020).

Regarding the impact of seasonal variation in the presence of *Y. enterocolitica* in foods, the reported results suggest that this aspect does not influence the presence of this microorganism in foodstuff. The result is corroborated by other authors who report the negligible effect of seasonal variation for human yersiniosis, than for other foodborne enteric diseases (Rosner *et al.,* 2010). Other authors (Rastawicki *et al.,* 2013) report an increase in human disease prevalence from May to August related to climate, to the extent of pork consumption or pig slaughter. In these cases, the possible effects of exposure to specific animal sources could be strongly considered (Le Guern *et al.,* 2016).

Under these considerations, the relationship between foods and human disease is still strongly debated as the "non-pathogenic" biotype 1A is frequently isolated from foods (Le Guern *et al.,* 2016; Guiller *et al.,* 2021). Even in the present research all the isolated strains were negative for the detection of *ail* gene as reported by other authors (Mancini *et al.,* 2022). Furthermore, all the *Y. enterocolitica* strains isolated from diseased people in Umbria region were positive for *ail* gene detection (Primavilla *et al.,* 2017) even in the same period of observation (data not reported). This finding could lead to the conclusion that food could not be considered a source of infection for human local cases, but the epidemiological situation needs deeper investigation as unreported disease cases could not be excluded. Furthermore, according to the findings, the *ail* gene detection could be considered the gold standard for *Y. enterocolitica* diagnosis in human samples but not for its association to food. Indeed, several sporadic human cases referred to biotype 1A are reported with difficulties in determining the sources of contamination (Guiller *et al.,* 2021). In these situations, the detection of the *ystB* gene virulent marker on foods should be considered even when no *ail* gene is detected (Platt-Samoraj, 2022). The absence of mandatory notification in Italy hinders the proper definition of the real disease prevalence and therefore its sources.

Conclusions

The present survey confirms that *Y. enterocolitica* is frequently isolated from foods, but it generally belongs to biotype 1A. The relationship between this biotype and human disease needs further studies, even to define the exact role of specific food as a source of the disease. Therefore, national monitoring programmes for this foodborne pathogen need to be structured and implemented to obtain data for exposure assessment and risk characterization. Furthermore, these programs should be harmonized in EU member states to obtain more relevant and useful data. Considering the possible pathogenic role of 1A biotype, the screening methods based only on *ail*-specific gene detection need to be carefully considered.

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