Evaluation of two real-time PCR methods to detect *Yersinia enterocolitica* in bivalve molluscs collected in Campania region

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ABSTRACT

Yersinia enterocolitica (*Ye*) is a foodborne pathogen isolated from humans, food, animals, and the environment. Yersiniosis is the third most frequently reported foodborne zoonosis in the European Union. *Ye* species are divided into six biotypes 1A, 1B, 2, 3, 4, and 5, based on biochemical reactions and about 70 serotypes. Biotype 1A is non-pathogenic, 1B is highly pathogenic, and biotypes 2–5 have moderate or low pathogenicity. The reference analysis method for detecting pathogenic *Ye* species underestimates the presence of the pathogen due to similarities between *Yersinia enterocolitica*-like species and other *Yersiniaceae* and/or *Enterobacteriaceae*, low concentrations of distribution pathogenic strains and the heterogeneity of *Yersinia enterocolitica* species.

In this study, the real-time PCR method ISO/TS 18867 to identify pathogenic biovars of *Ye* in bivalve molluscs was validated. The sensitivity, specificity and accuracy of the molecular method were evaluated using molluscs experimentally contaminated. The results fully agree with those obtained with the ISO 10273 method. Finally, we evaluated the presence of *Ye* in seventy commercial samples of bivalve molluscs collected in the Gulf of Naples using ISO/TS 18867. Only one sample tested resulted positive for the *ail* gene, which is considered the target gene for detection of pathogenic *Ye* according to ISO/TS 18867. Additionally, the presence of the *ystB* gene, used as target for *Ye* biotype 1A, was assessed in all samples using a real-time PCR SYBR Green platform. The results showed amplification *ystB* gene aim two samples.

1. Introduction

Yersinia enterocolitica (*Ye*) is a foodborne zoonotic pathogen from the *Yersiniaceae* family (Adeolu et al., 2016). In 2022, yersiniosis was the third most frequently reported foodborne zoonosis in the European Union (EU), with 7,919 cases of illness (EFSA, 2023).

The *Ye* species is highly heterogeneous, comprising six biotypes (BT) 1A, 1B, 2, 3, 4, 5, classified based on biochemical tests, and approximately 70 serotypes (Fredriksson-Ahomaa et al., 2017). These biotypes exhibit varying levels of pathogenicity: BT1A is generally regarded as non-pathogenic, biotypes 2–5 have mild pathogenicity, and BT1B is highly pathogenic (Reuter et al., 2014). Historically, pathogenic *Ye* strains have been characterized by the presence of a 70 kb virulence plasmid (pYV), which includes genes such as: *yadA* (adhesin A) and *virF*

(a transcriptional regulator gene). Additionally, they contain chromosomal virulence genes such as: *invA* (invasin), *ail* (attachment and invasion locus), *ystA* (stable *Yersinia* toxin A) and *myfA* (mucoid *Yersinia* factor A) (Rivas, Strydom, Paine, Wang, & Wright, 2021). The BT1A *Ye* strains are considered non-pathogenic due to the absence of the pYV plasmid and some chromosomal virulence genes like *ail*. Although BT1A strains typically lack the plasmid, they may possess alternative virulence factors, such as the thermostable toxin *ystB* and *hreP* (Ventola et al., 2023; Baghat and Virdi, 2011).

Ye can be classified into around 70 serotypes based on biochemical characteristics. Among these, the serotypes O:3, O:8, O:9, and O:5;27 are most commonly associated with human infections. In Europe, the most common biotype-serotype (bioserotype) combinations causing human yersiniosis are *Ye* 4/O:3 and 2/O:9 (Hassanzadeh et al., 2022; EFSA and

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ECDC European Food Safety Authority and European Centre for Disease Prevention and Control, 2023).

Ye is found in the gastrointestinal tracts of various mammals, predominantly in pigs, which are considered the primary reservoir for the bacteria. It is mainly transmitted to humans through consumption of contaminated foods (Arden et al., 2022). In humans, yersiniosis typically manifests as self-limiting diarrhea, occasionally bloody, in children under four years old. Other clinical and immunological manifestations include fever, mesenteric lymphadenitis, and terminal ileitis (Shoaib et al., 2019). In vulnerable groups like young children, the elderly, and immunocompromised persons some intestinal and extra-intestinal complications may occur (Peruzy et al., 2017; EN ISO, 2017).

The primary sources of human exposure are raw meat and meat products (Seakamela et al., 2022); however, the pathogen can survive in different foods, such as milk, dairy products, fish products, vegetables, fruits, tofu, and drinking water (Mancini et al., 2022). In the EU, during 2018–2021, raw meat and meat products and milk and milk products were the most contaminated food categories within ready-to-eat and non-ready-to-eat sampling units (EFSA and ECDC European Food Safety Authority and European Centre for Disease Prevention and Control, 2023). However, the prevalence of the pathogen is likely underestimated due to the several limitations of current isolation and identification methods (Peruzy et al., 2020).

The reference analytical method to detect *Ye* the ISO 10273:2017 (EN ISO, 2017; Hallanvuo et al., 2019) is based on culture technique: enrichment and isolation steps followed by identifying characteristic colonies and confirming pathogenic *Ye* isolate. This method takes several days, is laborious, and can hardly determine low levels of pathogens (Luciani et al., 2018).

According to an EFSA opinion from 2007 (EFSA, 2007), the detection of *Ye* in food, environmental, and clinical samples should utilize molecular testing methods. Techniques such as real-time PCR are highly sensitive and function by amplifying specific gene fragments, including *inv, ail, yadA, yst, yops,* or *virF*, as highlighted in research by Petsios et al. 2016.

To meet the requirement for faster analysis, ISO approved a standard method for rapid identification of pathogenic *Ye* in food, feed, and environmental samples, based on detection of the chromosome localized *ail* gene which is present in all pathogenic bioserotypes by real-time PCR (ISO/TS 18867:2015).

Recent research has focused on evaluating the effectiveness and limitations of various cultural and molecular methods for detecting *Ye* in raw meat, meat products, and vegetables (Peruzy et al., 2020; Cristiano et al., 2021; Petsios et al., 2016; Van Damme et al., 2013)). However, protocols for detecting *Ye* in molluscs have not yet been investigated and there remains limited knowledge regarding the prevalence of this pathogen in such food sources.

World aquaculture production of molluscs, primarily bivalves, reached 17.7 million tons (USD 29.8 billion) in 2020 (FAO, 2022). According to Precision Business Insights, the bivalve Market size was appreciated at USD 28,366.7 million in 2021 and will increase by 2.2 % from 2023 to 29 (Bivalve market, 2023). Regrettably, due to their role as filter feeders, bivalves can accumulate many harmful substances in water, including chemicals, pathogenic bacteria, and viruses. *Ye*, like other organisms, can reach the aquatic environment and contaminate bivalves through discharge from hospitals or livestock farming This is of particular concern since bivalves are often consumed raw or improperly cooked, therefore the presence of *Ye* in them could pose a significant threat to health (Crovato, Pinto, Arcangeli, Mascarello, & Ravarotto, 2017). Therefore, developing or validating sensitive and rapid methods for routine analyses of the most widespread pathogens, including *Ye*, in bivalve molluscs is necessary. In this study, the ISO/TS 18867:2015 real-time PCR method was validated for identifying pathogenic biovars of *Ye* in bivalve molluscs. Furthermore, the presence of *Ye* in commercial samples of bivalve molluscs collected from markets in southern Italy using the ISO/TS 18867:2015 and SYBR Green Real-Time PCR was

assessed, to detect respectively *ail* gene (found in pathogenic *Ye*) and *ystB* gene (found in non-pathogenic *Ye*).

2. Materials and methods

2.1. Sampling of bivalve molluscs at primary production and retail level

Live bivalve mollusc samples (n = 140) belonging to Mediterranean Mussel (*Mytilus galloprovincialis)* (N=35), rayed artemis (*Dosinia exolete)* (N=35), grooved razor shell (*Solen marginatus*) (N=35)*,* and European flat oyster (*Ostrea edulis*) (N=35), were collected from primary production and retail in Southern Italy from 2019 to 2020. All samples, weighing approximately 200–300 g, were transported to the laboratory at 4 °C within 1 h. Before inoculating with the *Ye* strain (as reported in paragraph 2.3), the samples were analyzed using the ISO 10273 method to ensure the absence of the pathogen. Seventy samples were experimentally contaminated (as reported in paragraph 2.3) and seventy samples were treated as reported in paragraph 2.4.

2.2. Bacteria strains, preparation, and standardization of broth cultures

Non-pathogenic *Ye* BT1A serotype O:5 (1A/O:5) and pathogenic *Ye* BT4 serotype O:3 (4/O:3) strains were obtained by the Pathogenic Enterobacteria Collection of the Istituto Superiore di Sanità (Rome, Italy).

The *Ye* strains were cultured in Triptone Soya Broth (Oxoid, Basingstoke, Hampshire, United Kingdom) at 30 °C for 24 h. Subsequently, ten-fold serial dilutions of the culture were prepared in TSB, and the cell concentration was measured by plating onto Triptone Soy Agar (Oxoid).

2.3. Inoculation of molluscs for method evaluation

Intravalvular liquid and the bodies of the molluscs (25 g) and Sorbitol Peptone Broth and Bile Salts (PSB; Kairosafe, Trieste, Italy; 225 ml) were homogenized in a blender (BagMixer400 P, Interscience, Saint Nom la Bretèche, France).

Specifically, 5 homogenates were not contaminated; 10 were contaminated with less than 1 log CFU/25 g cfu/25 g (colony-forming units per gram) of *Ye* 4/O:3, 10 with 2 log CFU/25 g /25 g of *Ye* 4/O:3, and 10 with *a* 200 cfu/25 g of *Ye* 4/O:3. Homogenates were subsequently incubated for 24 ± 3h at 25 ± 1 °C. The same experimentally contamination was applied using the *Ye* 1A/O:5 at the same concentrations.

Thetest repeatability was assessed on the experimentally contaminated samples for the presence of the *Ye* and checked three times.

2.4. Real samples

Seventy real samples of mollusc were analyzed as follow: 25 g of each sample were incubated with 225 ml of PSB at 25 °C for 24 h ± 3. Then, two ml of each enrichment were analyzed using ISO/TS 18876 to detect pathogenic *Ye* and SYBR Green real-time PCR to detect non-pathogenic *Ye* as reported in paragraphs 2.6 and 2.7.

2.5. Yersinia enterocolitica colonies isolation

The isolation of *Ye* was conducted following the ISO 10273 method. In summary, the process involved selective enrichment in Phosphate Buffered Saline (PBS), followed by inoculation on Petri plates with Agar Base-CIN (Cefsulodin–Irgasan–Novobiocin, Hi media, India; 14-cm diameter). The inoculated plates were then incubated under aerobic conditions at 30 °C for 24 h ± 2h.

2.6. DNA extraction

Two ml of each homogenate was transferred into a 2 ml tube and centrifuged for 10 min (10000 g; T=4 °C). The pellet was suspended in 200 μl of 6 % Chelex 100 (Eppendorf, Hamburg, Germany), vortexed, incubated (8 min at 100 °C), and centrifuged for 5 min (14000 g; T=4 °C). The DNA's dosage and qualities were determined by measuring the absorbance at λ260 and the A260/A280 ratio with a spectrophotometer (Eppendorf, Hamburg, Germany). Successively, DNA concentration was standardized to 35–50 ng/μl and stored at −20 °C.

2.7. Real-time PCR

The presence of pathogenic *Ye* was evaluated according to ISO/TS 18867. The protocol was performed as follows: 2.5 μl of DNA of the sample and 1 μl of Internal Amplification Control (IAC) DNA were added to the 21.5 μl PCR master mix. The PCR master mix contained 12.5 μl of the TaqMan Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA) and 300 nmol/l of both primers (ye-ail-F2 5'–GGTTATGCACAAAGCCATGTAAA –3'; ye-ail-R25 '–AAACGAACCTATTACTCCCCAGTT–3 '), 125 nmol/l of ye-ail-tmp probe (5 '–FAM-AAC CTG AAG TAC CGT TAT GAA CTC GAT GA-BHQ1–3'), 250 nmol/l of both primers for the IAC pUC 18-F (5' –TGT CGT GCC AGC TGC ATT A-3') pUC 18-R (5' –GAG CGA GGA AGC GGA AGA g–3'), 100 nmol/l Tm-pUC18-probe (5 '–HEX – AAT CGG CCA ACG CGC GG –BHQ1–3') and H₂O to adjust the volume to 25 μl. The reaction was run at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s and 60 °C for 30 s. Amplification reactions were performed with Bio-Rad CFX96 platform (Biorad, Hercules (CA), USA), using a 96-well PCR multiplate (Biorad, Hercules (CA), USA).

The presence of non-pathogenic *Ye* was assessed using SYBR Green real-time PCR and *ystB* gene was used as a target following the procedure of Peruzy et al. (2017). Three μL of DNA were added to 22 μL of PCR mix containing 12.5 μL of 1X SsoAdvanced-SYBR Green PCR Mastermix (Biorad, Hercules (CA), USA), 150 nM of each primer (ye-ystB-F, 5'-GTACATIAGGCCAAGAGACG-3'; ye-ystB-R, 5'-GCAACATACCTCA-CAACACC-3') and nuclease-free water as required.The fluorescence intensity of SYBR Green and the melting curve analysis were studied using the CFX96 system (Bio-Rad). The thermal profile was: 95 °C for 5 min, 35 cycles at 95 °C for 10 s, and 60 °C for 30 s, followed by a thermal cycle (65–95 °C) necessary for the analysis of the melting curve. A threshold cycle (Ct) under 35 and a specific melting temperature (Tm) indicated a positive result.

2.8. Biochemical confirmation

The suspected colonies with typical traits and red bull eye on CIN agar were used. API 20E (BioMerieux, France) confirmed *Yersinia* strains. All experiments were carried out according to the manufacturer's instruction (incubation time: 24–48 h; temperature = 25 ± 1 °C).

2.9. Statistical analysis

Statistical analyses, to evaluateaccuracy, sensitivity and specificity were conducted using EpiInfo 7 software package (Centers for Diseases Control and Prevention; Atlanta, USA).

3. Results

The results of real-time PCR method validation have demonstrated a full concordance between ISO 10273 and ISO/TS 18867. In fact, all samples contaminated at different concentrations with pathogenic *Ye* 4/O:3 resulted positive while all not experimentally contaminated samples resulted negative, showing high specificity (high probability of a negative test result = [true negatives/(true negatives + false positives)] x 100.

true positives/(true positives + false negatives)] x 100), and sensitivity (high probability of a positive test result = [true positives/(true positives + false negatives)] x 100) in detecting *Ye* in bivalve molluscs.

The *Ye* strains were added at three different concentrations (2, 20, 200 cfu/25 g) into samples to assess the performance of the molecular methods.

The results of real-time PCR method validation have demonstrated a full concordance between the ISO 10273 and the SYBR Green real-time PCR. In fact, all samples contaminated at different concentrations with pathogenic *Ye* 4/O:3 and non-pathogenic *Ye* 1A/O:5 resulted positive and all negative samples were negative, showing high specificity and sensitivity in detecting non-pathogenic *Ye* in bivalve molluscs. The bacterial genomic DNA replicated three times at each concentration was stably detected. The method sspecificity, sensitivity and accuracy were 100 %. Furthermore, SYBR Green and TaqMan duplex real-time PCR methods showed that the *Ye* strains artificially inoculated into bivalve molluscs were detectable at 2 cfu/25 g with a probability of 95 %. No real-time PCR amplification was observed in samples not contaminated with *Ye*. Table 1 and Table 2 report Cₜ values in relation to the different concentrations.

The presence of *Ye* was successfully confirmed using the reference method ISO 10273 and the confirmation of isolated colonies was performed using ISO/TS 18867 and API 20E.

Finally, ISO/TS 18867 and SYBR Green real-time PCR were used to evaluate *ail* and *ystB* genes, in 70 samples taken from primary production and retail level of bivalve molluscs.

Real-time PCR showed the amplification of the *ail* gene in only one sample (1.4 %) and the amplification of the *ystB* gene in two samples (2.9 %). All other samples resulted negative by both real-time PCR methods.

The presence of *Ye* was not confirmed using the ISO 10273 for all 70 mollusc samples tested. The amplification of IAC in all samples made it possible to exclude false negative results.

4. Discussion

The prevalence of *Yersinia enterocolitica* (*Ye*) is generally underestimated due to the low concentration of *Ye* in the samples and the diverse and abundant accompanying microorganisms in the enrichment phase that overgrow the *Ye* complicating their detection and leading to false negative outcomes (Cristiano et al., 2021). In the first part of the experimental work, the ISO/TS 18867 method was validated to verify the applicability of the molecular methods in the detection of *Ye* in live lamellibranch edible molluscs. Samples were seeded with three different levels of *Ye* (2, 20, and 200 cfu/25 g). The bacterial concentration was not tested after seeding; however, the pathogen was always detected in inoculated samples, even in samples seeded with less than 1 log CFU/25 g. Results are of particular interest because the infective dose of

Table 1

Report ct values in relation to the different concentrations (2, 20, 200 cfu/25 g).

	Mollusc − *ail* gene		
	200 ufc/25 g	20 ufc/25 g	2 ufc/25 g
	Cₜ	Cₜ	Cₜ
	27,95	31,36	33,09
	26,77	30,69	32,16
	26,83	29,8	32,36
	27,27	30,71	31,7
	27,36	30,88	31,73
	27,51	30,24	32,84
	27,1	30,84	32,01
	26,79	30,69	31,93
	26,9	31,16	31,73
	24,14	30,5	33,13
Average	26,86	30,69	32,27
Standard Deviation	1,03	0,44	0,56

Table 2
Ct values and melting temperature in relation to the different concentrations (2, 20, 200 cfu/25 g).

	Mollusc- *ystB* gene					
	200 ufc/25 g	20 ufc/25 g	2 ufc/25 g	200 ufc/25 g	20 ufc/25 g	2 ufc/25 g
	C_t	C_t	C_t	Tm °C	Tm °C	Tm °C
	21,46	24,2	28,37	81,5	81,5	81,5
	20,95	23,9	28,6	81,5	81,5	81,5
	21,08	23,71	28,42	81,5	81,5	81,5
	20,93	23,93	28,62	81,5	81,5	81,5
	20,66	23,92	28,42	81,5	81,5	81,5
	20,97	23,92	28,38	81,5	81,5	81,5
	20,94	24,16	28,81	81,5	81,5	81,5
	20,85	24,02	28,41	81,5	81,5	81,5
	20,75	23,83	28,34	81,5	81,5	81,5
	21,08	23,8	28,33	81,5	81,5	81,5
Average	20,97	23,94	28,47	81,5	81,5	81,5
Standard Deviation	0,22	0,15	0,16	0,00	0,00	0,00

pathogenic *Ye* is 10^8-10^9 cells (Chlebicz & Śliżewska, 2018), higher than the detection limit of the present work.

The presence of colonies referable to the species *Ye* obtained by molecular test was confirmed by presumptive biochemical tests and verified by API 20E. The real-time PCR and API showed concordant results (agreement 100%). To our knowledge, this is the first study that provides a method for the detection of *Ye* in bivalve.

Concerning biotype 4/O:3, higher detection limits were observed by Peruzy et al., (2020) in experimentally inoculated pork meat samples ($>10^2$ cfu/g) and by Cristiano et al., (Cristiano et al., 2021) in experimentally inoculated leaf green samples (>10 cfu/g) by using the real-time PCR. Instead, concerning biotype 1A the results of the present work are in line with those of Peruzy et al., (2020) and (Cristiano et al., 2021) who were always able to detect this biotype in meat and vegetable samples.

An IAC was included in the real-time PCR to avoid false-negative results due to inhibitors in food samples (Thisted Lambertz et al., 2008). IAC is a non-target DNA sequence amplified simultaneously with the target sequence. An IAC indicates the presence of DNA polymerase inhibitors, errors caused by PCR components, or malfunction of the thermal cycler. Even though no target sequence exists, a control signal is produced in a PCR with an IAC. The use of IAC in food molecular microbiology diagnostics is becoming mandatory (Hoorfar et al., 2004). The positive IAC signal in all samples analyzed demonstrated the absence of false negatives, showing that the molluscs matrix has no inhibition during the PCR reaction, thus confirming the truthfulness of the negative results. The results of the analysis of experimentally contaminated molluscs samples showed a 100 % concordance between ISO/TS 18867 and ISO 10273. In fact, the presence of amplification plots referable to the *ail* gene obtained by molecular test was confirmed by the same molecular method, considered by ISO 10273 as "alternative confirmation pathway", on colonies isolated on CIN agar and using biochemical tests API 20E.

In the second part of the experimental work, the occurrence of the *ail* (target gene present in pathogenic *Ye*) and the *ystB* gene (usually present in non-pathogenic *Ye* strains) were evaluated in 70 samples of bivalve molluscs taken from the production primary and retail level in Southern Italy. Although the number of analyzed samples was limited, to the best of our knowledge, it is the first study giving data on the prevalence of *Ye* in bivalve molluscs in Italy. Only one sample of bivalves (*M. galloprovincialis*), taken from primary production, highlighted the presence of the *ail* gene. This result shows a low occurrence of *Ye* in this matrix in primary production. However, the discovery of a positive sample highlights the importance of assessing the presence of *Ye* along the shellfish chain. The results demonstrated the ability of ISO/TS 18867

to detect *Ye* in shellfish from primary production to retail. This confirms the importance of adopting this method to rapidly assess the presence of *Ye* and further ensure product safety. The proposed analytical approach proposed for detecting *Ye* in bivalve molluscs serves as a rapid monitoring tool capable of analyzing many samples simultaneously. In the event of a positive result, it prevents the sale of hazardous foods.

However, adequate good hygiene practices throughout the supply chain and heat treatments before consumption constitute a valid tool for guaranteeing food safety even in the case of products at risk.

5. Conclusions

The research validated a real-time PCR method using to detect pathogenic *Yersinia enterocolitica (Ye)* in bivalve molluscs rapidly. Molecular platforms revealed the presence of pathogenic strains of *Yersinia enterocolitica* in 24 h. This analytical protocol may represent a methodological reference for official laboratories and facilitate surveillance activities to estimate the microbiological risk associated with *Ye* concretely. If adopted in routine analyses, such diagnostic systems could significantly impact food safety, bringing significant advantages in the scientific and technological sectors. The analytical approach proposed here could also be tested for diagnostics in the clinical field, both human and veterinary since current methods for *Ye* detection in different matrices are still considered inadequate and time-consuming. Based on the results of the present work, molluscs can be a source of human infection. However, good hygiene practices and good manufacturing practices throughout the supply chain, followed by adequate heat treatments before consumption, constitute a valid tool for guaranteeing food safety even in the case of products at risk.

CRediT authorship contribution statement

Andrea Mancusi: Methodology, Conceptualization. **Elisabetta Delibato:** Investigation. **Maria Francesca Peruzy:** Investigation. **Santa Girardi:** Investigation. **Orlandina Di Maro:** Formal analysis. **Daniela Cristiano:** Formal analysis. **Eleonora Ventola:** Formal analysis. **Irene Dini:** Writing – original draft. **Yolande Thérèse Rose Proroga:** .

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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