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Molecular characterization of *Yersinia enterocolitica* strains to evaluate virulence associated genes

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Abstract

Introduction. *Yersinia enterocolitica* (*Ye*) species is divided into 6 biotypes (BT), 1A, 1B, 2, 3, 4, 5 classified based on biochemical reactions and about 70 serotypes, classified based on the structure of the lipopolysaccharide O-antigen. The BT1A is considered non-pathogenic, while the BT 1B-5 are considered pathogenic.

Methods. Evaluate the distribution of eleven chromosomal and plasmid virulence genes, *ail*, *ystA*, *ystB*, *myfA*, *hreP*, *fes*, *fepD*, *ymoA*, *sat*, *virF* and *yadA*, in 87 *Ye* strains isolated from food, animals and humans, using two SYBR Green real-time PCR platforms.

Results. The main results showed the presence of the *ail* and *ystA* genes in all the pathogenic bioserotypes analyzed. The *ystB*, on the other hand, was identified in all non-pathogenic strains biotype 1A. The target *fes*, *fepD*, *sat* and *hreP* were found in both pathogenic biotypes and in BT1A strains. The *myfA* gene was found in all pathogenic biotype and in some *Ye* BT1A strains. The *virF* and *yadA* plasmid genes were mainly detected in bioserotype 4/O:3 and 2/O:9, while *ymoA* was identified in all strains.

Conclusions. The two molecular platforms could be used to better define some specific molecular targets for the characterization and rapid detection of *Ye* in different sources which important implications for food safety and animal and human health.

Key words

- *Yersinia enterocolitica*
- SYBR Green real-time PCR
- virulence genes

INTRODUCTION

The genus *Yersinia* has been recently classified to *Yersiniaceae* family [1] and comprises of 28 species (https://lpsn.dsmz.de/genus/yersinia), three of which are pathogenic to humans *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis* [2]. *Yersinia enterocolitica* (*Ye*) is Gram-negative and psychrophilic enteropathogen [3]. In fact, *Ye* has an optimal growth temperature between 28-30 °C but is able to growth from 2 and 42 °C [4]. Yersiniosis is usually a self-limiting disease and gastrointestinal symptoms pre-dominate, but extra-intestinal disorders may also appear [5]. Children under 5 years of age, immunocompromised people, and elderly are more likely to get sick and to have a more serious illness [4].

In Europe, yersiniosis is the third most frequently reported foodborne zoonosis in humans with a stable trend in 2015-2019 [6]. In Italy the number of reported cases is very low compared with other EU/EEA member states, because the notification of yersiniosis is voluntary, and the number of cases is probably underestimated [7].

Ye is widely distributed in the environment and pigs are the main reservoir of pathogenic strains, in particular, the bacterium colonized tonsils, lymph nodes and intestines. *Ye* is also isolated from other animals, such

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as cattle, small ruminants, wild animals (e.g., deer) and pets (cats and dogs) [8, 9]. Humans become infected by ingesting undercooked pork contaminated with *Ye*, but also milk and dairy products, vegetables and untreated water [10, 11]. Although pork meat is the major source of pathogenic *Ye*, in recent years, fresh vegetables have been linked to several foodborne outbreaks, particularly spinach and salad [12, 13]. The *Ye* species is highly heterogeneous and is divided into 6 biotypes (BT), 1A, 1B, 2, 3, 4, 5, on the basis of biochemical tests and about 70 serotypes [14]. The biotypes show differing pathogenic potential, in particular: biotype 1A (BT1A) is considered non-pathogenic, biotypes 2-5 weakly pathogenic, and biotype 1B highly pathogenic [15]. Pathogenic *Ye* have historically been defined as carriers of a 70 kb virulence plasmid (pYV), which has genes encoding adhesin A (*yadA*) and a transcriptional regulator gene (*virF*); in addition, at the chromosomal level they harbour virulence genes as *invA* (invasin), *ail* (attachment and invasion locus), *ystA* (stable *Yersinia* toxin A), and *myfA* (mucoid *Yersinia* factor A) [16].

BT1A strains are considered non-pathogenic because they do not have the pYV and some chromosomal virulence genes such as *ail*. However, studies have shown that some biotype 1A strains, particularly those isolated from faeces during a gastrointestinal illness, could be pathogenic [17]. Although BT1A may lack the pYV plasmid, alternative virulence factors, including the thermostable toxin *ystB* and *hreP* [18] may be present. BT1A is the most heterogeneous of the six *Ye* biotypes and includes a wide range of serotypes of which O:5, O:6,30, O:6,31, O:7,8, O:10, as well as not-typable (NT) O strains, are most often reported [19]. The most common bioserotypes causing human yersiniosis in Europe are *Ye* 4/O:3 and 2/O:9 [6].

ISO 10273 (International Organization for Standardization) standard cultural method for the detection and isolation of *Ye* from food samples are laborious and time-consuming to differentiate pathogenic and non-pathogenic strains [20, 21].

To meet the requirement for faster analysis, ISO approved a standard method for rapid identification of pathogenic *Ye* based on detection of the chromosome-localized *ail* gene which is present in all pathogenic bioserotype by real-time PCR [22]. However, recent studies have indicated that in some non-pathogenic 1A strains of *Ye*, the *ail* gene can be detected [23]. Therefore, it would be useful to consider other pathogenicity targets of the strains to have a more complete genomic characterisation. Recently, a study analyzed by real-time PCR the distribution of the *yadA*, *virF*, *inv*, *ystA*, *ystB*, *myfA*, *hreP* and *ymoA* genes in *Ye* strains in order to select useful target genes for assessing the presence of pathogenic *Ye* [18].

The aim of the present study is to extend the examination of the distribution of virulence genes in non-pathogenic and pathogenic *Ye* strains, isolated from animal, food and human samples. Additional molecular targets would allow for a more complete characterization of *Ye* strains, and the evaluation of the distribution of these genes which have virulence potential and are less investigated.

Thus, besides the virulence genes *ail*, *ystA*, *ystB*, *myfA*, *virF* and *yadA*, the other target genes analyzed are: *hreP* (host reactive element), *sat* (streptogramin acetyltransferase), *fepD* (enterochelin transporter ABC), *fes* (enterochelin esterase) and *ymoA* (*Yersinia* modulating protein) [24], using two molecular real-time PCR SYBR Green platforms.

MATERIALS AND METHODS

Identification and typing of bacterial strains

A total of 87 *Ye* strains, 82 isolates from 2005 to 2015 and 5 isolates from 1980 to 1985 in Italy, were identified at biochemical level and subsequently biotyped and serotyped. These strains were isolated from human (n=39), in particular stools (n=24), blood (n=3), appendix (n=1) and unknown (n=11), from animals (n=12), in particular faeces (n=7), amygdala (n=2) and unknown (n=3) and from food (n=36).

Identification of *Ye* species was performed using the API® 20E system (bioMérieux). *Ye* biotyping was carried out according to the ISO 10273 scheme, based on biochemical reactions, in particular: pyrazinamidase and lipase activity, production of indole, production of acids from xylose, trehalose and hydrolysis of esculin. Serotyping was performed using O-antisera for serogroups O:3, O:5, O:8, O:9 and O:27 purchased from Biolife (Biolife Italiana, Milan, Italy).

Genomic DNA extraction

The strains were grown in Tryptone Soy Broth (Biolife Italiana, Milan, Italy) at 30 °C for 24-48h. Two mL of each broth culture was subjected to DNA extraction according to the protocol of Peruzy *et al.* [18]. The extracted DNA was used as a template for real-time PCR.

Real-time PCR-based protocol

For the molecular characterization of *Ye* virulence genes, two SYBR Green real-time PCR platforms were developed with two different thermal profile. The first platform, a PCR reaction in singleplex, containing: 1X SsoAdvanced SYBR Green PCR Master Mix (Bio-Rad), one of the primer (*Table 1*) at different concentrations, of the virulence genes *ail* (250 nM), *ystA* (100 nM), *ystB* (150 nM), *myfA* (300 nM) and 3 µl of DNA in a final volume of 25 µl. 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. The second platform, a PCR reaction in singleplex, containing: 1X SsoAdvanced SYBR Green PCR Master Mix (Bio-Rad, USA), one of the primer (*Table 1*), at different concentrations, of the virulence genes *hreP* (300 nM), and 250 nM of the other virulence genes *virF*, *yadA*, *ymoA*, *fes*, *fepD*, and *sat* and 3 µl of DNA in a final volume of 25 µl. The thermal profile of the reaction was: 95 °C for 5 min, 35 cycles at 95 °C for 60 s, 60 °C for 60 s, 72 °C for 60 s, followed by a thermal cycle (65-95 °C) necessary for the analysis of the melting curve. The specificity of the reaction is given by the detection of the melting temperature (T_m) of the amplification products after the last reaction cycle. The melting curve was visualized with the software MxPro (Mx3005P v 4.00 - Agilent).

Table 1
Primers used for SYBR Green real-time PCR

Gene	Primer sequence (5'->3')	Amplicon size (bp)	Reference
ail	ACTCGATGATAACTGGGGAG	170	[25]
	CCCCCAGTAATCCATAAAGG		
ystA	ATCGACACCAATAACCGCTGAG	79	[26]
	CCAATCACTACTGACTTCGGCT		
ystB	GTACATTAGGCCAAGAGACG	146	[26]
	GCAACATACCTCACAACACC		
myfA	CAGATACACCTGCCTTCCATCT	272	[27]
	CTCGACATATTCCTCAACACGC		
hreP	GCCGCTATGGTGCCTCTGGTGTG	757	[24]
	CCCGCATTGACTCGCCCGTATC		
virF	GGCAGAACAGCAGTCAGACATA	591	[25]
	GGTGAGCATAGAGAATACGTCG		
yadA	TAAGATCAGTGTCTCTGCGGC	747	[28]
	TAGTTATTTGCGATCCCTAGCAC		
ymoA	GACTTTTCTCAGGGGAATAC	330	[29]
	GCTCAACGTTGTGTGTCT		
fes	GCCGGCAGGCACAGCGTAAT	561	[30]
	GGCCAACCCACCCAAAACTT		
fepD	GTGTGATTGCCTTACTATTG	381	[30]
	CGGTCATCCTTTTATTACGG		
sat	CCGATGGTGGGGTTTTCTCAAG	456	[24]
	GGGATTACCGCCGACCACACTA		

RESULTS

The results obtained of serotyping and biotyping of the 87 *Ye* strains have identified four biotypes and several serotype: 1A/O:5 (n=15), 1A/O:8 (n=9), 1A/NT (n=25), 2/O:9 (n=5); 3/O:5,27 (n=1); 4/O:3 (n=32). The results obtained analysing these strains through SYBR Green real-time PCR showed a close association between the *ail* gene, considered an exclusive indicator of pathogenicity, and the *ystA* gene as they were found in all strains belonging to the pathogenic biotypes (*Table 2*). In contrast, *ystB* was found exclusively in all non-pathogenic biotype 1A strains. The presence of the *virF* and *yadA* plasmid genes appears to be exclusive to pathogenic biotypes, found in 42.1% (n=16) of the total of the pathogenic strains (*Table 2*).

The *myfA* gene was detected in all pathogenic strains (n=38), but also in 3 strains of BT1A strains (6.1%). The *fes* and *fepD* genes, which encode for factors capable of capturing and utilizing host iron, were found in synergy with each other in both pathogenic and non-pathogenic biotypes. The *hreP* and sat genes appear present in high percentages in all biotypes analyzed, as is the *ymoA* gene, which is present in 100% of strains (*Table 2*). The distribution of bioserotypes showed the prevalence of 4/O:3 and 2/O:9 mainly in human samples, and 4 of 32 strains of *Ye* 4/O:3 were found in pigs, whereas BT1A was found mainly in a wide variety of food samples, but also, less frequently, in animals and humans (*Table 3*).

DISCUSSION

The results showed a non-uniform distribution of the different target genes in the various *Ye* strains. Chromosomal virulence genes are very important elements that determine the pathogenic capabilities of *Ye*. These include the attachment invasion locus (*ail*), which encodes the outer membrane proteins responsible for adhesion, and is considered the target gene for detection of pathogenic *Ye* according to ISO/TS 18867:2015.

Furthermore, *Ye* has the ability to produce three types of YstI toxins (YstA, YstB and YstC) encoded by *ystA*, *ystB* and *ystC* genes, respectively, which play a crucial role in the origin of diarrhea [31]. As expected, all strains belonging to pathogenic biotypes, analyzed in this work, contain the *ail* gene together with the *ystA* gene. *Ye* BT1A mainly produce the enterotoxin YstB and rarely YstC. The ability of some BT1A strains to cause illness gives indirect evidence that YstB plays an important role in yersiniosis, as suggested in some studies [31, 32]. Indeed, in the present study the *ystB* was detected in 100% of BT1A strains, results in agreement with literature data [24, 33].

The pYV plasmid undoubtedly plays an important role in pathogenicity and carry virulence factors, such as *virF*, a regulatory gene, which encodes the transcriptional activator of several genes, including the plasmid virulence gene *yadA* involved in the *Yersinia* invasion process. However, plasmids are unstable structures, and *Ye* BT1A generally do not harbour plasmids [31, 34].

Table 2
The distribution of genes in *Ye* strains grouped by biotype/serotype

Biotype/serotype (n)	*ail*	*ystA*	*ystB*	*myfA*	*hreP*	*virF*	*yadA*	*fepD*	*fes*	*ymoA*	*sat*
4/O:3 (32)	100%	100%	-	100%	100%	40.1%	40.1%	43.8%	43.8%	100%	96.8%
2/O:9 (5)	100%	100%	-	100%	100%	60.0%	60.0%	100%	100%	100%	100%
3/O:5.27 (1)	100%	100%	-	100%	100%	-	-	100%	100%	100%	100%
1A/NT (25)	-	-	100%	12.0%	72.0%	-	-	96.0%	96.0%	100%	84.0%
1A/O:5 (15)	-	-	100%	-	93.3%	-	-	100%	100%	100%	100%
1A/O:8 (9)	-	-	100%	-	100%	-	-	100%	100%	100%	100%

Table 3
The distribution of different sources (food, animals and human) in *Yersinia enterocolitica* biotypes (BT)

Source (n)	Specific source	BT1A	BT2	BT3	BT4
Animals (12)	Bear	1			
	Bovine	1			
	Sheep	1		1	
	Swine	4			4
Food (36)	Beef	1			
	Chicken meat	4			
	Fish	2			
	Fresh vegetables	2			
	Milk	1			
	Minced meat of beef and pork	3			
	Pork meat	8			
	Ready to eat pork meat	2			
	Ready to eat vegetables	12			
	Sheep meat	1			
Humans (39)	Humans	6	5		28

The results reported in our study confirm that plasmid virulence genes are not present in any strain belonging to the non-pathogenic biotype (BT1A), whereas they are found in about 42.1% of the total pathogenic biotypes, with a different distribution between bioserotype 4/O:3 where plasmid genes are present in 40.1% and bioserotype 2/O:9 where *virF* and *yadA* are present in 60%.

MyfA, plays an important role at the beginning of infection by promoting adhesion to enterocytes. The *myfA* gene has been found in *Ye* strains of bioserotype 4/O:3 isolated from human and pigs [33] and has also been detected in some *Ye* strains of BT1A [24].

These results are confirmed in this study, and we also found *myfA* in all strains of bioserotype 2/O:9 isolated from human and in the only strain belonging to bioserotype 3/O:5,27 isolated from animal.

The *ymoA* gene was present in all *Ye* isolates studied, as reported in several studies [18, 24, 33]. According to recent studies, the *ymoA* gene is the main regulator of *yst* gene expression and other virulence genes [34].

The *hreP* gene, which encodes the bacterial subtilisin/Kexin-like protease, and the *sat* gene, which encodes the acetyltransferase streptogramin, are both present in high percentage in all *Ye* strains. These results are in agreement with data from some other Authors [18, 24], but seem to be in contrast with data reported by Morka *et al.* [33]. Few studies have described the *hreP* gene, encoding for a bacterial protease, relevant for full virulence of *Ye* [35]. Finally, the *fes* and *fepD* genes, involved in iron capture and utilization, contribute to the growth of microorganism [30].

The genes *fepD* and *fes* were found in 100% or percentage near 100% in all isolates of all bioserotypes except for BT4/O:3 where the prevalence of these genes was about 44%. BT1A strains possessing the genes *ystB*, *hreP*, *sat*, *fes*, *fepD* and *myfA* genes may have a virulence potential with respect to causing infections in humans and animals [24, 36]. Although in the study of Campioni and Falcão [36], *myfA* appears to have a higher prevalence in the BT1A strains (55%) than in our study.

Furthermore, the lack of identification of BT1B strains in this work, appears to be in line with what has been reported in the literature regarding the limited presence or absence of this biotype in Europe [4, 6].

Overall, the study seems to confirm what other authors have already pointed out regarding the distribution of these virulence genes in *Ye* strains.

CONCLUSIONS

The isolation and biotyping of *Ye* are currently difficult and time-consuming, but biotyping remains important as a basis for assessing the pathogenicity of isolated strains. The detection of this microorganism by means of molecular biology tools as a real-time PCR allows the quick detection of pathogenic *Ye* in food, animal and human and can be a valid support to classical microbiology techniques. Although, the *ail* gene remains the main virulence marker as reported by ISO/TS 18867:2015, other virulence genes are important in evaluating the pathogenicity of *Ye*, such as the *ystB* gene to identify strains of biotype 1A. Therefore, the use of two SYBR Green real-time PCR platforms in this study, allowed the rapid detection of the eleven virulence genes, in pathogenic biotypes and in BT1A of *Ye*. It also allowed to highlight a diversity in the distribution of virulence genes in *Ye* strains isolated from different sources, which has valuable implications in terms of food safety and animal and human health from a One Health perspective.

Authors' contributions

Conceptualization: ED and EV; methodology: ED; investigation: ED, EV, SF, SL, GF, SB and SO; re-

sources: ED, SF, SL, GF, SB and SO; writing-original draft preparation: EV and ED; writing-review and editing: ED, SF, SL, GF, SB; supervision: ED. All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

The Authors declare no conflict of interest.

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