Evaluation of virulence genes in *Yersinia enterocolitica* strains using SYBR Green real-time PCR

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**A B S T R A C T**

*Yersinia enterocolitica* comprises six biotypes 1A, 1B, 2, 3, 4, and 5. The virulence of the strains belonging to biotypes 1B and 2–5 depends on the presence of both chromosomal and plasmid-borne genes. Strains belonging to biotype 1A do not carry the virulence plasmid pYV. However, they carry other virulence genes, such as *ystB* and *hreP*. The aim of this study was to investigate the distribution of *yadA*, *virF*, *inv*, *ystA*, *ystB*, *myfA*, *hreP* and *ymoA* virulence genes in *Y. enterocolitica* strains in order to select the target genes that could be used for the development of a probe-specific real-time PCR to determine the presence of *Y. enterocolitica* in food samples. A total of 161 *Y. enterocolitica* strains isolated in eight countries and grouped into biotypes 1A, 2 (serotypes O3, O5 and O9), 3 (serotypes O3 and O9) and 4 (serotype O3) were examined for virulence genes. The most common virulence-associated gene in pathogenic *Y. enterocolitica* proved to be *ystA*, which can therefore be considered the best target gene to be amplified in order to evaluate the presence of pathogenic biotypes. By contrast, to identify *Y. enterocolitica* 1A strains, *ystB*, which codes for the enterotoxin YstB, can be proposed. This has been found in all non-pathogenic biotypes studied, but never in pathogenic biotypes.

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1. Introduction

*Yersinia enterocolitica* is a gram-negative bacteria belonging to the genus *Yersinia* and to the family of *Enterobacteriaceae*. Within the genus *Yersinia*, two species, in addition to *Y. enterocolitica*, are pathogenic for mammals, *Y. pestis* and *Y. pseudotuberculosis*, and one, *Y. ruckerii*, for fish (Ewing et al., 1978). Strains of *Y. enterocolitica* can be classified into six biotypes 1A, 1B, 2, 3, 4, 5 (Gupta et al., 2015) and at least 57 different serotypes (Robbins-Browne, 2007). The bio/serotypes mainly associated with human infection are 4/O:3, 2/O:9, 1B/O:8, 2/O:5, 27, 1B/O:20, 1B/O:13a and 1B/O:13b (Bottone, 1999; Kwaga et al., 1992). Most yersiniosis cases caused by *Y. enterocolitica* are reported as sporadic cases.

The biotype distribution of *Y. enterocolitica* is different worldwide. According to the EFSA (2015), 4/O:3 and 2/O:9 are the primary bio/serotypes in humans in Europe. In the USA, most human pathogenic strains belong to serotype O:8, followed by O:3, O:5,27, O:13a, 13b, O:20, and O:9 (Kwaga et al., 1992). In China, serotypes O:3, O:9 and O:8 are the most frequent (Wang et al., 2008).

The virulence of pathogenic biotypes depends on the presence of both chromosomal and plasmid-borne genes (Bottone, 1999; Cornelis et al., 1998). Chromosomal virulence genes include Attachment and invasion locus (*inv*), Invasin (*inv*), Mucoid Versinia factor (*myf*), Host-responsive element (*hreP*) and Versinia stable toxin (*yst*) (Young and Miller, 1997). Moreover, an important chromosomal gene is *ymoA* (*Yersinia*-modulating protein) encoding for the YmoA protein, which negatively regulates the expression of various genes. It inhibits particularly the expression of *inv* and *yst* (Platt-Samoraj et al., 2006). Moreover, there are several virulence plasmid genes (pYV). These include Adhesin A (*yadA*), whose product is involved in autoagglutination, serum resistance...
and adhesion (Skurnik and Wolf-Watz, 1989), and Transcriptional regulator (virF), which encodes transcriptional activators of the yop regulon (Cornelis et al., 1998) and is therefore fundamental for the type-III secretion system. To date, strains of biotype 1A have been considered to be non-pathogenic, since they do not have pYV plasmid and some chromosomal virulence genes, e.g. ystA and myfA (Tennant et al., 2003). Although inv is present, it seems to be non-functional in most 1A strains (Pierson and Falkow, 1990). Nevertheless, the 1A strains carry other virulence genes, such as ystB and hreP, and some biotype 1A strains have been isolated from humans with gastrointestinal infections (Bhagat and Virdi, 2006).

Although the source of infection and the transmission routes are still not fully known, it has been demonstrated that healthy pigs are the principal reservoir of Y. enterocolitica (Fredriksson-Ahomaa et al., 2006; Nesbakken, 1985) and contaminated raw pork is suspected to be the principal source of human yersiniosis cases (EFSA, 2015). Moreover, Y. enterocolitica can grow at 0 °C, and hence in foods stored at refrigerator temperatures without sign of spoilage.

Yersiniosis occurs mostly in young children and is associated with a wide range of clinical and immunological manifestations, including diarrhea, at times bloody, fever, mesenteric lymphadenitis and terminal ileitis. Abdominal pain and fever can often be confused with appendicitis (EFSA, 2013). The disease is usually self-limiting. However, some intestinal and extra-intestinal complications, such as reactive arthritis or infected aneurysm may appear (Miller et al., 1989). Yersiniosis is the third most common bacterial enteric disease in Europe. In 2013, 6471 confirmed cases were reported and Y. enterocolitica was the predominant species among human cases. Unfortunately, no reliable incidence rates of yersiniosis are available in several countries (i.e. Belgium, France, Italy, Luxembourg and Spain) where notification is not compulsory, and no surveillance system exists in Greece, the Netherlands and Portugal. Therefore, the number of cases is probably underestimated. Moreover, the availability of reports on the prevalence of Y. enterocolitica in food and animals is also limited in EU member states (EFSA, 2015).

Current methods for the detection of pathogenic Y. enterocolitica bacteria in food samples are time-consuming and inefficient (Fredriksson-Ahomaa et al., 2008; Van Damme et al., 2013). Although the routine laboratory detection of bacteria is still commonly based upon traditional microbiological methods, alternative methods based on nucleic acid detection are now being increasingly adopted (Palomino-Camargo and González-Muñoz, 2014). By means of PCR, pathogenic Y. enterocolitica in samples can be detected rapidly and with high specificity and sensitivity (Fredriksson-Ahomaa and Korkeala, 2003).

The aim of this study was to evaluate the presence of virulence genes in Y. enterocolitica strains originating from eight different countries by using SYBR Green real-time PCR, including Tm analysis, to select target genes for the development of a probe-specific real-time PCR for the detection of pathogenic Y. enterocolitica biotypes in food samples.

2. Materials and methods

2.1. Strains

A total of 161 Y. enterocolitica strains isolated from 2003 to 2007 in eight countries were analyzed. Y. enterocolitica strains were grouped into four biotypes: 1A (9 strains); 2 (serotypes O3 [5 strains], O5 [5 strains] and O9 [9 strains]); 3 (serotypes O3 [2 strains] and O9 [1 strain]), and 4 (serotype O3 [130 strains]).

The samples were isolated from pig tonsils (134), human feces (19), pig pluck sets (5), pig feces (2) and lamb feces (1). The strains were grown in tryptone soy broth (Oxoid LTD, Basingstoke, Hampshire, United Kingdom) at 30 °C for 24 h.

2.2. Real-time PCR-based protocol

One mL of each broth culture was transferred into a clean microcentrifuge tube, and centrifuged for 10 min at 10,000 × g at 4 °C. The supernatant was carefully discarded and the pellet was re-suspended in 200 μL of 6% Chelex 100 (Biorad, Hercules, CA, USA) by vortexing, and incubated for 20 min at 56 °C and then for 8 min at 100 °C. The suspension was immediately chilled on ice for 1 min, and centrifuged for 5 min at 10,000 × g at 4 °C.

Two μL of the DNA extracted (DNA concentration = 15 ± 3.36 ng/μL) was used as a template for Y. enterocolitica Real-Time PCR evaluation assay (Josefsen et al., 2007) and added to 18 μL of mastermix (iQ™ SYBR Green Supermix; Bio-Rad, USA). The mastermix contains: 10 μL of iQ™ SYBR Green Supermix (2×) to 2 μL of forward and reverse primers and 6 μL of H2O. The fluorescence intensity of SYBR Green and the melting curve analysis were studied by means of the CFX96 system (Bio-Rad). A threshold cycle (Ct) under 35 and a specific melting temperature (Tm) indicated a positive result. Eight genes were investigated: two plasmid-borne genes, yadA and virF, and six virulence genes inv, ystA, ystB, myfA, hreP and ymoA located in the chromosome. The primers used in this study are presented in Table 1.

3. Results

All 161 strains tested for the ymoA gene were positive (100%). However, the most common virulence-associated gene in

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used for PCR.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Primer sequence (5′ → 3′)</td>
</tr>
<tr>
<td>inv</td>
<td>TCCGTGATGACTCGTCTTCA</td>
</tr>
<tr>
<td>myfA</td>
<td>GGGACCATCTCGTGTGTTAT</td>
</tr>
<tr>
<td>ystA</td>
<td>CAATACACCCATCCTTACCT</td>
</tr>
<tr>
<td>ystB</td>
<td>GTTACATTAGCCAAAGACC</td>
</tr>
<tr>
<td>ymoA</td>
<td>GAATTTCCAGGGGATAC</td>
</tr>
<tr>
<td>hreP</td>
<td>GCCCTATGTCGCCGCTTCT</td>
</tr>
<tr>
<td>yadA</td>
<td>TAATAGCTGTCTCGCGCC</td>
</tr>
<tr>
<td>virF</td>
<td>TGGTGGCATAAGAACACG</td>
</tr>
</tbody>
</table>
pathogenic serotypes was ystA. The yersinia stable toxin A gene was found in 99% of 4/O:3 strains tested and in all (100%) the strains belonging to the other pathogenic biotypes analyzed. By contrast, ystB was present in all non-pathogenic biotype 1A strains, but was never detected in isolates belonging to pathogenic bio/serotypes (Table 2).

Although belonging to the same plasmid, the single plasmid-genes displayed different prevalence. Moreover, in 4/O:3 strains, the yadA gene amplicon was found in a higher prevalence than that of virF (p value < 0.05).

Differences were observed in the prevalence of the inv and hreP genes in 4/O:3 isolates from the eight countries (Table 3). The invasin gene (inv) was found in all Spanish, Finnish and Nigerian strains, and in 97% of Italian, 93% of Belgian, 91% of Russian and English, and 87% of Estonian strains. hreP was found in all Spanish, Russian, Estonian, Nigerian and Belgian strains; 97% of Italian samples, 96% of Finnish strains and 91% of English strains were positive to hreP gene amplification.

The presence of virulence-associated genes in 4/O:3 isolates from different sources is reported in Table 4. Although the number of the samples was limited, the same virulence-associated gene pattern was found in pluck sets and feces of pigs. Strains isolated from human feces were all positive to ystA, myfA, inv, hreP and ymoA, whereas yadA and virF genes were absent in six and five strains, respectively.

4. Discussion

The results showed that not all of the strains analyzed were positive to all the virulence genes tested. It is possible that not all of these genes are necessary for the virulence of *Yersinia enterocolitica*.

<table>
<thead>
<tr>
<th>Country (n)</th>
<th>ystA</th>
<th>ystB</th>
<th>myfA</th>
<th>inv</th>
<th>hreP</th>
<th>yadA</th>
<th>virF</th>
<th>ymoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Italy (33)</td>
<td>100%</td>
<td>0</td>
<td>93.93%</td>
<td>96.96%</td>
<td>96.96%</td>
<td>87.87%</td>
<td>78.78%</td>
<td>100%</td>
</tr>
<tr>
<td>Finland (24)</td>
<td>100%</td>
<td>0</td>
<td>100%</td>
<td>100%</td>
<td>95.83%</td>
<td>95.83%</td>
<td>91.66%</td>
<td>100%</td>
</tr>
<tr>
<td>Russia (22)</td>
<td>100%</td>
<td>0</td>
<td>100%</td>
<td>90.9%</td>
<td>100%</td>
<td>100%</td>
<td>81.81%</td>
<td>100%</td>
</tr>
<tr>
<td>Estonia (15)</td>
<td>100%</td>
<td>0</td>
<td>100%</td>
<td>86.66%</td>
<td>100%</td>
<td>93.33%</td>
<td>80%</td>
<td>100%</td>
</tr>
<tr>
<td>Belgium (14)</td>
<td>100%</td>
<td>0</td>
<td>100%</td>
<td>92.85%</td>
<td>100%</td>
<td>85.71%</td>
<td>71.42%</td>
<td>100%</td>
</tr>
<tr>
<td>England (11)</td>
<td>100%</td>
<td>0</td>
<td>100%</td>
<td>90.9%</td>
<td>100%</td>
<td>90.9%</td>
<td>90.9%</td>
<td>90.9%</td>
</tr>
<tr>
<td>Spain (10)</td>
<td>90%</td>
<td>0</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>70%</td>
<td>60%</td>
<td>100%</td>
</tr>
<tr>
<td>Nigeria (1)</td>
<td>100%</td>
<td>0</td>
<td>100%</td>
<td>100%</td>
<td>0</td>
<td>0</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

Alternatively, other, as yet unknown, markers may exist that could play an important role in the pathogenesis of pathogenic *Y. enterocolitica* (Zheng et al., 2008).

The ymoA gene was present in all the *Y. enterocolitica* isolates studied, as reported by Bancerz-Kisiel et al. (2012). The percentage of ystA detection was higher than those reported by Tadesse et al. (2013, 62%) and Ramamurthy et al. (1997, 78%), but similar to those reported by Montaz (2013, 93%) and Bancerz-Kisiel et al. (2012, 100%). Inv gene prevalence was lower than in Montaz (2013, 100%), while Schneeberger et al. (2015) reported a 100% prevalence of the myfA gene. Thus, we hypothesized that there was no association between the occurrence of the ymoA and ystA genes, as reported by Bancerz-Kisiel et al. (2012), and no association was identified between the ymoA and inv genes.

The prevalence of the plasmid-borne genes ystA and virF seems to differ somewhat among the countries; however, this result may arise from the fact that the plasmids are lost during laboratory cultivation (Farmer et al., 1992). Moreover, as reported in other studies, there is a conflicting occurrence of ystA and virF, while both belong to the pYV plasmid, they are not always present together in the same bacteria (Montaz, 2013; Schneeberger et al., 2015; Zheng et al., 2008). It is possible that the plasmids are lost and that beneficial genes are integrated into the bacterial chromosome (Harisson and Brockhurst, 2012); another possibility is the deletion of sections of the plasmid genome, and hence the loss of some genes. Indeed, selection may involve genes encoding core functions (Heuer et al., 2007).

The distribution of a small group of virulence-associated genes in isolated strains from eight countries revealed that inv and hreP, which are important in the infection of *Y. enterocolitica*, show different prevalence among the countries. Few studies have described the prevalence of hreP in the *Y. enterocolitica* genome; hreP is a species-specific gene for *Y. enterocolitica*, and has never been reported in other *Yersinia* pathogens (Young and Miller, 1997). The bacterial subtilisin/Kexin-like protease codified by this gene is required for the full virulence of *Y. enterocolitica*, as observed in a mouse model of infection; indeed, hreP mutant strains display a reduction in both 50% lethal dose and in vivo survival assay (Young and Miller, 1997).

The role of hreP and myfA in the virulence of the non-pathogenic *Y. enterocolitica* strains is not fully understood (Bhagat and Virdi, 2011). Campioni and Falcão (2013) described a correlation between hreP and ystB genes, and Bhagat and Virdi (2006) found a correlation between myfA and ystB. In the present study, however,
no association between the occurrence of these genes was observed. The prevalence of the *myfA* gene in the study by Bhagat and Virdi (44%) was higher than that observed by us. The percentage of *yshT* detection in the present study was similar to those reported by Schneeberger et al. (2015) and Bonardi et al. (2014). However, this gene was detected in 26% of strains analyzed by Tadesse et al. (2013) and in 5% of strains in a study by Zheng et al. (2008). Moreover, more recently than Bhagat and Virdi (2006), Campioni and Falcao (2013) also demonstrated that *myfA*, *hrep* and *ystB* were not present in all 1A isolates, but only in strains belonging to a specific clonal group, identified by repetitive extragenic palindrome (REP) and enterobacterial repetitive intergenic consensus (ERIC).

The detection rates in the strains isolated from pig tonsils - 94% of *inv* positive and 100% of *myfA* positive – is in line with the observations made by Schneeberger et al. (2015). However, in the 4/12 O:3 strains, they reported a prevalence of 100% for the *ystA* and *myfA* genes. These results conflict with the findings of our study, in which *ystA* and *myfA* were found in 99% and 98% of the strains, respectively.

In conclusion, *Y. enterocolitica* isolation is currently difficult and requires long incubation. The detection of this bacteria by means of molecular biology tools can be rapid, with high specificity and sensitivity; thus, a real-time PCR technique able to evaluate the presence of pathogenic *Y. enterocolitica* in foods in a rapid and sensitive way is needed. Not all pathogenic *Y. enterocolitica* carry all the traditional virulence genes. On the basis of the isolated strains analyzed, *ystA*, one of the important virulence markers, can be considered the best target gene to amplify in order to evaluate the presence of pathogenic serotypes in food, water or clinical material. If this gene alone were tested, 99% of the pathogenic strains would be characterized correctly. To identify the biotype 1A strains, the *ystB* gene, which codes for the enterotoxin YstB, can be proposed, as it is widely distributed in this biotype. Indeed, it has been found in all non-pathogenic strains (100%), but never in pathogenic serotype strains isolated. Unfortunately, epidemiological data on the prevalence of *Y. enterocolitica* in foods, animals and the environment are still lacking in many countries. The development of more sensitive methods for the detection of *Y. enterocolitica* is important in order to obtain more information and to prevent the spread of this bacteria. Additional research is clearly needed in order to investigate the loss or the lack of chromosomal virulence genes.

**Conflict of interest**

The authors have no conflict of interest to declare.

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