PREVALENCE OF VIRULENCE GENES AND BIOTYPING OF *YERSINIA ENTEROCOLITICA* ISOLATED FROM CHICKEN MEAT IN SHAHREKORD, IRAN

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ABSTRACT

Yersinia enterocoliticabelongs to the family Enterobacteriaceae,which is known to be highly heterogenic andis divided into several biotypes and serotypes. Since the 1960s, this bacterium has increasingly been identified as an important foodborne pathogen. In this study, virulence factors such as yadA, inv, ail, ystAandvirF of 65 strains isolated from chicken meat samples were analysed by the multiplex polymerase chain reaction (PCR) method.Of 65 isolates of Y.enterocolitica. biotyping revealed biotypes 1A (35.38%), 1B(26.15%), 2 (6.15%), 3 (9.23%), 4 (6.15%) and unclassified (16.94%). Polymerase chain reaction tests showed that the prevalence of virulent genes was 44.74% for yadA, 100%forinv, 50% for ail, 51.85% for ystAand35.18%forvirF. This study highlighted the importance of chicken meat as a potential source of Y. enterocolitica infection in Iran.

Keywords: *Yersinia, meat, Shahrekord.*

Introduction

*Yersinia enterocolitica*was (*Y. enterocolitica*is) discovered more than 60 years ago but was not considered to be a human or veterinary pathogen until the late 1960s, when it became increasingly identified in foodborne gastrointestinal infections (Sabina et al., 2011).

*Y. enterocolitica*is a member of the genus *Yersinia*, which is composed of a heterogeneous collection of facultatively anaerobic bacteria that belong to the family *Enterobacteriaceae*. Of the 12 species in this genus, only three –*Y. pestis, Y. pseudotuberculosis,* and *Y. enterocolitica* – are regarded as pathogenic for humans, whereas *Y. ruckeri*is a fish pathogen and the *Y. enterocolitica*-like organisms *Y.krirtensenii*, *Y. intermedia, Y. mollaretii, Y. frederiksenii* and *Y. bercovieri*have not yet been identified as having a role in human disease (Simonova et al., 2007).

*Y. enterocolitica*causes human infections whose symptoms include diarrhoea, terminal ileitis, mesenteric lymphadenitis, arthritis and septicaemia. *Y. pseudotuberculosis* causes mesenteric lymphadenitis, diarrhoea and septicaemia in humans (Rahman et al., 2011).

The aim of this study is detection of pathogenic strains of *Yersinia* spp based phenotypic tests. Biotype 1A *Y.enterocolitica* is non-pathogenic, other pathogenic biotypes is 1B, 2, 3, 4 and 5. *Yersinia* spp can also be divided into serotypes based on predictive values for pathogenicity. Serological and biochemical classification (Thoerner et al., 2002).

Alternative phenotypic tests, such as calcium-dependent growth at 37 °C, or Congo red binding, and testing for pyrazinamidase, auto-agglutination and serum resistance, have limited predictive value for *Y. enterocolitica* pathogenicity (Simonova et al., 2007).

Test results are frequently ambiguous and their outcomes may be unreliable, since they depend on the presence and expression of plasmid-borne virulence genes, such as the virulence plasmid pYV, which can easily be lost during bacteriological isolation and enrichment procedures (Wren and Tabaqchali, 1990), depending on the culture conditions.

Therefore, pathogenic strain differentiation should not rely solely on the expression or detection of the virulence plasmid, but also on the detection of chromosomal virulence factors (Simonova et al., 2007).

The detection of pathogenic *Yersinia* species and laboratory diagnoses of *Y. enterocolitica* infections are based mainly on the isolation of bacteria from food and clinical specimens. Rapid methods for the detection of pathogenic *Yersinia* species in chicken meat, especially when it appears to be unhealthy, by polymerase chain reaction (PCR) techniqueshave been previously reported (Lambertz *et al.*, 2008).

Y.enterocolitica can cause harmful effects in humans. Therefore, the present study was performed with the aim of determining the prevalence of virulence genes of *Y.enterocolitica* isolated from chicken meat in Shahreko.

Materials and Methods

In a previous study, from February 2012 to June 2012, a total of 300 fresh raw chicken meat specimens were collected randomly from chicken shops in Shahrekord cities. Using sterile scissors and tissue forceps, 25 g of the breast muscle was dissected separately into sterile containers and transported on ice to the Food Microbiology Laboratory at the Islamic Azad University of Shahrekord Branch and transferred to a plastic bag containing 225 ml of phosphate-buffered saline (PBS: 80 ml of 0.061MNa2HPO4, 120 ml of 0.061M KH2PO4, and 0.85%NaCl) at 4 °C for 14 days. After 14 days, 1 ml suspension was mixed with 9 ml KOH (25%) for 30 seconds, a loop of the suspension was then streaked on Cefsulodin-Irgasan-Novobiocin(CIN) agar and incubated at 25 °C for 48 hours (Saberianpour et al., 2012).

In this study, we used biochemical assays to detect biotypes of *Y.enterocolitica*. The samples were analysed for the presence of *Yersinia* spp. Following the examination of pure cultures, Preliminary tests such as oxidase, Urease production, glucose and lactose fermentation, hydrogen sulphide production, and gas formation from glucose were performed. Colonies with positive for urease test and glucose, negative for oxidase and lactose fermentation and hydrogen sulphide production and gas formation from glucose were then selected. In these colonies, biochemical confirmation tests such as lysine decarboxylase and ornithine decarboxylase, sucrose, rhamnose, xylose and trehalosefermentation and citrate were performed. Colonies with these characteristics (positive sucrose fermentation and ornithine decarboxylase, negative lysine decarboxylase and citrate and trehalose, rhamnose and xylose positive or negative) were tested for pathogenicity (esculin hydrolysis, pyrazinamidase activity and salicin). Biochemical identification of the strains was supplemented with biochemical reactions for *Y. enterocolitica* identification (indole, hydrogen sulphide, lysine, ornithine, urease, arginine, simmons citrate, malonate, phenylalanine, β-galactosidase, inositol, adonitol, cellobiose, sucrose, trehalose, mannitol, acetoin, esculin, sorbitol, rhamnose, melibiose, raffinose, dulcitol and glucose), which are part of the commercially available Enterotest 24 (Simonova *et al*., 2007).

The bacterial strains biochemically identified as *Y. enterocolitica* and cultivated on CIN agar medium were tested using the multiplex PCR method. Purification of DNA from bacterial colonies was achieved using a genomic DNA purification kit (Fermentas, Germany) according to the manufacturer's instructions. A Multiplex polymerase chain reaction (PCR) was used for the amplification of virulence genes in *Y.enterocolitica.* Primers specific for the *ail, ystA, yadA, virF* and *inv*genes of *Y. enterocolitica* were used.

The detail of the primers used in the study is given in Table 2. The PCR assay was carried out in a total volume of 50 µL of mixture containing 5 µL PCR buffer 10×, 3 mMof MgCl2, 0.2 mM concentrations of each of deoxynucleoside triphosphates, 1 µM of each the virulence gene-specific primers, 1.5U of *Taq*polymerase (Fermentas, Germany) and 5 µL of template DNA. 1 cycle of denaturation

at 95 °C for 4 min; 33 cycles of melting at 94 °C for 50 s; annealing at 56 °C for 60 s; and elongation at 72 °C for 70 s; and a final extension at 72 °C for 10 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis, after which the gel was stained with ethidium bromide and photographed.

Results

The microbiological method and PCR results showed that 65 *Y.enterocolitica* strains were isolated (representative of 65 different colony morphologies) (Saberianpour et al., 2012). The most common biotype found in the samples was 1A, which occurred in 23 samples (35.38%). Other common strains included 1B, 2, 3 and 4.

For gene detection, *ail*, *yadA*, *inv*, *ystA*, and *virF* were PCR-amplified and individual amplified fragments were subjected to agarose gel electrophoresis.

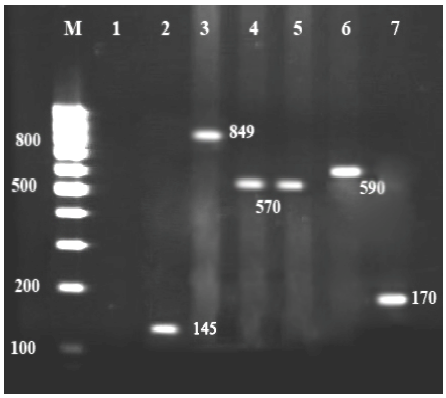

Figure 1. Agarose gel electrophoresis of polymerase chain reaction products (genes from *Yersinia enterocolitica*) amplified with the multiplex polymerase chain reaction method. Gene biotypes: *ail*, 170 bp; *yadA*, 849 bp; *inv*, 570 bp; *ystA*, 145 bp; *virF*, 590 bp.

The virulence genes *ail*, *yadA*, *inv*, *ystA*, *virF* were detected in isolates of *Y. enterocolitica* strains.

Discussion

*Y. enterocolitica*is a common Gram-negative, foodborne enteric pathogen found in water, dairy products, and meats. It is one of the most common causes of foodborne gastroenteritis in western and northern Europe. The incidence is also increasing in the United States and Canada, although this may be a result of improved surveillance and detection methods (Weynants *et al.*,1996; Lamps *et al.*, 2006).

Bacteriological examination and bio- and serotyping, commonly applied in diagnostics of infections with *Y. enterocolitica*, are time consuming and labour intensive, while at the same time they do not clearly identify pathogenic strains. Molecular methods, such as the multiplex PCR, which is particularly useful in showing the presence or absence of fragments of several genes, were used in this study for determining the prevalence of virulence genes in *Y. enterocolitica*. The virulence of the pathogenic biotypes, namely 1B and 2–5, is attributed to the presence of a highly conserved 70-kb virulence plasmid, termed pYV/pCD, and certain chromosomal genes (Weynants *et al.*,1996).

This study was performed using 65 samples of chicken meat positive for *Y. enterocolitica* (Saberianpour *et al*., 2012). The prevalence of *Y. enterocolitica* biotypes isolated from samples was as follows: 1A (35.38%), 1B 26.15%), 2 (6.15%), 3 (9.23%), 4 (6.15%) and unclassified (16.94%). When the bacteria are stored for a longer period, subjected to numerous passages or grown at temperatures above 37°C, The plasmid pYV might be lost easily. So, it is therefore better to use chromosomal genes (*ail*, *rfbC*, and *yst*) (Simonova *et al.*, 2007).

In this study, multiplex PCR assay results showed that chromosomal virulence genes included *inv* (100%), *ail* (50%) and*ystA* (51.85%), and plasmid-encoded virulence factors included *yadA* (44.74%) and *virF* (35.18%).In an Indian study, 81 strains of *Y. enterocolitica* biotype 1A were isolated from diarrhoeic human stools (51 strains), wastewater (18 strains), pig throats (seven strains) and pork (five strains). Virulence-associated genes, including *ail*, *virF*, *inv*, *myfA*, *ystA*, *ystB*, *ystC*, *tccC*, *hreP*, *fepA*, *fepD*, *fes*, *ymoA* and *sat* were detected in 81 clinical and non-clinical strains of *Y.*

Table 1. Biotyping scheme of *Yersinia enterocolitica.*

Biochemical test	**Biotypes**						
	1A	**1B**	**2**	**3**	**4**	**5**	**6**
Lipase	+	+	-	-	-	-	-
Esculin/salicin(24 h)	+/-	-	-	-	-	-	-
Indole	+	+	(+)	-	-	-	-
Xylose	+	+	+	+	-	V	+
Trehalose	+	+	+	+	+	-	+
Pyrazinamidase	+	-	-	-	-	-	+
β–D-Glucosidase	+	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+/-	+	(+)	-

Table 2. Primers used for detection of the various genes of *Yersinia enterocolitica.*

Reference	**Amplicon length (bp)**	**Gene**	**Primer name**	**Primer sequence (5'-3')**
Pepe and Miller, 1993	849	*yadA*	yadA1	CTTCAGATACTGGTGTCGCTGT
			yadA2	ATGCCTGACTAGAGCGATATCC
Rahman*et al.*, 2011	570	*inv*	YC1	CTGTGGGGAGAGTGGGGAAGTTTGG
			YC2	GAACTGCTTGAATCCCTGAAAACCG
Ibrahim *et al.*. 1997	170	*ail*	Ail1	ACTCGATGATAACTGGGGAG
			Ail2	CCCCCAGTAATCCATAAAGG
Blais and Philippe, 1995	145	*ystA*	Pr2a	AATGCTGTCTTCATTTGGAGCA

Table 3. Biotyping of *Yersinia enterocolitica.*

Biotype	**Number**	**Percentage (%)**
1A	23	35.38
1B	17	26.15
2	4	6.15
3	6	9.23
4	4	6.15

Table 4. Percentage of *Yersinia enterocolitica* virulence genes isolated from chicken meat by multiplex PCR.

Biotype		**Total**	***inv***		***Ail***		***ystA***		***yadA***		***virF***
		number	%	number	%	number	%	number	%	Number	**%**
1A	**23**	23	100	1	4.37	2	8.69	-	-	1	**4.37**
1B	**17**	17	100	15	88.23	16	94.11	13	76.47	10	**58.82**
2	**4**	4	100	3	75	3	75	2	50	2	**50**
3	**6**	6	100	5	83.33	4	66.66	5	83.33	4	**66.66**
4	**4**	4	100	3	75	3	75	2	50	2	**50**
Total	**54**	54	100	27	50	28	51.85	22	40.74	19	**35.18**

enterocolitica biotype 1A by PCR amplification. All strains lacked *ail*,*virF*,*ystA* and *ystC* genes. The distribution of other genes with respect to clonal groups revealed that four genes (*ystB*, *hreP*, *myfA* and *sat*) were associated exclusively with strains belonging to clonal group A (Bhagat and Virdi, 2007).

In another study, Blais and Phillippe (1995) found the *ail* gene in 100% of pathogenic *Y. enterocolitica*strains and the *yadA* gene in only 86% of pathogenic *Y. enterocolitica* strains, but they found neither of the genes in non-pathogenic strains of *Yersinia* spp. This study found that all 65 isolates of strains of *Y. enterocolitica* from chicken meat in Shahrekord investigated for the virulence genes *inv*, *ail*, *ystA*, *yadA* and *virF* were *inv*positive and biotype 1A had the highest percentage compared with the other biotypes. "Some biotype 1A strains,despite lacking virulence plasmid (pYV) and traditional chromosomal virulence genes, can caused gastrointestinal diseases. The pathogenic biotypes of *Y. enterocolitica* have evolved two major properties: the ability to infeluence the intestinal wall, which is thought to be controlled by plasmid genes, and the production of heat-stable enterotoxin, which is controlled by chromosomal genes." (Sabina *et al.*, 2011).

In summary, the importance of chicken meat in the transmission of *Y. enterocolitica* is that it may cause yersiniosis in humans. Strict observance of good storage principles and consumption of chicken meat only in a healthy state are recommended. According to the authors' knowledge, contact of meat with chicken faeces and lack of hygiene in chicken slaughterhouses and the meat transportation system maybe the most frequent reasons for contamination of chicken meat with *Y. enterocolitica.*

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