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Molecular Characterization of *Yersinia Enterocolitica* Isolated From Chicken Meat Samples

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Key words

Yersinia enterocolitica, chicken meat, PCR techniques, virulence genes.

ABSTRACT:

Identification and molecular analysis of *Yersinia enterocolitica* isolated from fresh raw chicken meat samples. A total of 114 chicken meat samples were collected randomly from different chicken shops in the Kafr El- Sheikh city and examined by culturing and PCR methods. Twenty isolates out of 114 fresh raw chicken meat samples (17.5%) were found to be positive for *Y. enterocolitica*. Out of 57 chicken breast muscle samples 12 were positive (10.5%) and in the same time only 8 positive samples detected in 57 chicken thigh muscle samples (7%) for isolation of *Y. enterocolitica*. A PCR-based assay was developed to detect the prevalence of *Y. enterocolitica* in chicken meat samples and to evaluate plasmid- and chromosome-borne virulence genes. The result of PCR examination revealed that, only 8 isolates out of 12 randomly selected isolates (66.6%) were positive by the specific gene of *Y. enterocolitica* (16S ribosomal RNA). The results of detection of the virulence genes (ail, ystA and virF) were 75%, 50% and 37.5% respectively. These virulence genes were not detected in 2 examined isolates. The yadA gene was not detected in any of the 8 examined isolates by multiplex PCR technique. This study highlighted the importance of chicken meat as potential sources of pathogenic *Y. enterocolitica*.

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1. INTRODUCTION

Poultry meat was indicated as a potential source of the pathogenic bacteria (Sharifi et al., 2011 and Aliyu *et al.*, 2012). Chicken meat was a specific food category with respect to yersiniosis risk assessment. The contact with chicken feces and lack of hygiene in chicken slaughterhouses are the two most frequent reasons in chicken meat contamination with *Yersinia enterocolitica*, which could easily spread to and cause yersiniosis in humans (Momtaz et al., 2013).

Y. enterocolitica is Gram-negative facultative anaerobic non-spore-forming straight rods or coccobacilli that belong to the family *Enterobacteriaceae* (Bottone et al., 2005).

Y. enterocolitica was classified as bacterial food poisoning according to the duration of onset slow within 16-48 hours similar to other bacteria e.g. *Salmonellae, Shigellae, Vibrio parahemolyticus, enteroinvasive Escherichia coli,* and *Clostridium botulinum*. *Y. enterocolitica* is the third most commonly reported zoonosis in Europe where 8,979 cases were reported in 2006 (EFSA, 2007a and Abu Elnaga *et al.* 2014).

Cefsulodin Irgasan Novobiocin (CIN) agar is the most widely used medium for *Yersinia* isolation in clinical pathology laboratories (Savin et al. ,2010). Moreover, the majority of *Y. enterocolitica* isolates recovered from food and environmental samples are nonpathogenic and culture-dependent methods can misidentify species and could not discriminate between pathogenic and non-pathogenic strains (Cocolin and Comi, 2005). Among bacteria with similar morphology to *Y. enterocolitica* colony on CIN agar, the strains belonging to the genus *Yersinia* can be identified by the biochemical tests including Kligler iron and Christensen urea. *Yersinia* is catalase positive and oxidase negative and ferment glucose, like other members of the family *Enterobacteriaceae* (EFSA, 2007b; Hallanvuo, 2009).

The Polymerase Chain Reaction is simultaneously used to detect *Y.enterocolitica* and to discriminate between pathogenic and nonpathogenic *Y. enterocolitica* isolates specifically and rapidly because phenotypic tests are time-consuming and are not always reliable .The methods are based on specific segments of the plasmid or the chromosomal DNA that have known virulence functions (Aarts et al. 2001). PCR method targeting 16S rRNA gene is used to identify presumptive *Y. enterocolitica* strains because of the problems of the identification systems of biochemical reactions (Neubauer et al. 2000 and Wannet et al. 2001).

The following chromosomal virulence genes were included in the present study: ail, the *Y.*

enterocolitica attachment invasion locus gene, was reported to be present in pathogenic strains only (Miller *et al.,* 1990); ystA, Yersinia heat-stable enterotoxins gene which was responsible for the production of a heat-stable enterotoxin in *Y. enterocolitica* (Delor *et al.,* 1990).The plasmid-borne virulence genes analyzed were yadA, Yersinia adhesin A gene ,the outer membrane protein was an important factor for the enteric route of *Y. enterocolitica* infection and presents as a febrile surface matrix extending from the outer membrane (El Tahir and Skurnik, 2001) and virF gene, virulence regulatory functions gene (called lcrF in *Y. pseudotuberculosis*), located on the virulence plasmid, for determination of the presence or absence of the plasmid (Thisted et al. 2000).

Therefore, the present study was undertaken to determine the prevalence of *Y. enterocolitica* in chicken meat samples by culturing methods and PCR analysis and to describe the distribution of *Y. enterocolitica* virulence genes.

2. MATERIAL AND METHODS

Collection and preparation of Samples:

The current study was carried out during the period extended from December, 2013 to September, 2014. A total of 114 chicken meat samples were randomly collected from freshly slaughtered chicken sold in different chicken shops in the Kafr El- Sheikh city including 57 samples of breast muscles and another 57 samples of thigh muscles. The collected samples (about 5 g of sample were placed separately in clean sterile plastic bags under complete aseptic conditions) were delivered to the laboratory (Animal Health Research Institute, Kafr El-Sheikh branch) in an ice box at 4°C within 2hours. Collected samples were subjected to bacteriological examination according to the methods adapted by Rahimi et al., (2014).

About 5 g of sample were taken under aseptic conditions to sterile homogenizer flask containing 45 ml of sterile peptone water (0.1%). The contents were homogenized at 14000 rpm for 2.5 minutes. The mixture was allowed to stand for 10 minutes at room temperature, then 1ml of supernatant was added to 5 ml of trypticase soya broth (TSB) (enrichment broth) and incubated at 25 °C for 24 h. Transfer one loop-full of enrichment to 0.1 ml 0.5% KOH in 0.5% saline and mix for 2-3 s to suppress background flora after enrichment, then a loopful of of enrichment was streaked to MacConkey's plate and one loopful to (CIN) plate and incubated agar plates at 30°C for 1-2 days. The lactose negatives colonies on MacConkey's agar plates were selected and streaked onto CIN agar plates were incubated at 30°C for 1-2 days. One to five susceptible colonies of typical "bull's eye" appearance (small and smooth, with a red center and translucent rim) on the CIN agar plates were individually isolated and subculture on Nutrient agar for further studies.

Identification of bacterial isolates:-

The isolated organisms were identified biochemically according to Bercovier and Mollaret, (1984). The colonies were picked up and subjected to the following biochemical tests, catalase, oxidase, urease production, Simmon´s citrate, the behavior in TSI agar and sugar fermentation.

Identification of isolates of *Y. enterocolitica* by PCR*:-*

DNA extraction from bacterial cultures was applied according to Shah *et al.,* (2009)

PCR reaction kit (PCR Master Mix 2X, iNtRoN BiotoCHNology).

Programming of PCR to amplify *Y. enterocolitica* 16S ribosomal RNA gene was done according to Vázlerová and Steinhauserová, (2006).

Protocol of bacterial plasmid preparation was applied by bacterial plasmid extraction kit (iNtRoN BiotoCHNology).

Programming of PCR for detection of the virulence genes of *Y. enterocolitica* (ail, ystA, yadA and virF) was done according to Momtaz et al. (2013).

Table (1): Primers used for amplify the *Y. enterocolitica* 16S ribosomal RNA gene:

Gene	Primers name	Primer sequences (5′-3′)	Amplicon length, bp	Reference
Y. enterocolitica 16S ribosomal RNA gene	Y1	AATACCGCATAACGTCTTCG	330	(Wannet *et al.,* 2001)
	Y2	CTTCTTCTGCGAGTAACGTC		

Table (2): Primers used for detection of the virulence genes of *Y. enterocolitica*.

Gene	Primers name	Primer sequences (5′-3′)	Amplicon length, bp	Reference
ail	Ail1	ACTCGATGATAACTGGGGAG	170	(Nakajima *et al.*,1992)
	Ail2	CCCCCAGTAATCCATAAAGG		
ystA	Pr2a	AATGCTGTCTTCATTTGGAGCA	145	(Ibrahim *et al.*, 1997)
	Pr2c	ATCCCAATCACTACTGACTTC		
yadA	yadA1	CTTCAGATACTGGTGTCGCTGT	849	(Thoerner *et al.*, 2003)
	yadA2	ATGCCTGACTAGAGCGATATCC		
virF	virF1	TCATGGCAGAACAGCAGTCAG	590	(Wren and Tabaqchali, 1990)
	virF2	ACTCATCTTACCATTAAGAAG		

3. RESULTS and DISCUSSION

Our results revealed that, the contaminated raw chicken meat is one of the main sources of human infection caused by *Y. enterocolitica*. *Y. enterocolitica* is a common gram negative foodborne enteric pathogen and one of the most common causes of foodborne gastro-enteritis.

The obtained results tabulated in table (3) revealed that 20 out of 114 examined chicken meat samples were found positive for presence of *Y. enterocolitica* isolates with percentage of 17.5 %. This result was in consistent with Momtaz *et al.*, (2013) who examined 720 chicken meat samples collected randomly from abattoirs and found that 132 samples (18.33%) were positive for *Y. enterocolitica*. In addition, it was lower than that recorded by Bonardi *et al.*, (2010) who found that 26 out of 80 chicken meat samples (32.5%) were found to be positive for *Y. enterocolitica* while, it was higher than that recorded by Floccari *et al.*, (2000) who found that the detection rate of *Y. enterocolitica* was 4.3% in examined 70 raw chicken meat samples. On contrary, it disagreed with Pavlovic *et al.*, (2007); Mauro *et al.*, (2008) and Anju *et al.*, (2014) who could not isolate *Y. enterocolitica* in their examined fresh poultry meat samples. Isolation of *Y. enterocolitica* from examined raw chicken meat samples reflects contamination in working place, workers hands and cutting knife (Mahdavi *et al.*, 2012). The presented data in table (3) also showed that the isolation rate of *Y. enterocolitica* was higher in chicken meat samples collected from breast muscles than samples collected from thigh muscles although the samples were collected from each of thigh and breast simultaneously where 12 samples out of 57 chicken breast muscle samples were found to be positive for presence of *Y. enterocolitica* at a percentage of 10.5% while 8 samples out of 57 chicken thigh muscle samples were positive for isolation of *Y. enterocolitica* at a percentage of 7%. This difference may be explained as breast muscles were more susceptible than thigh muscles to the contamination and this may be attributed to the breast muscles always suffers from inflammation according to the habits of chickens of lying on the breast region. This result disagreed with the study of Purabi and Joshi, (2010) who found that the frequency of isolation of *Y. enterocolitica* was 70% in chicken breast and 69% from chicken thigh muscle samples but they did not give any explanation for this variation.

PCR is the accepted method for detecting nucleic acids in a variety of samples in the field of molecular diagnostics, however, an enrichment step prior to PCR is essential to increase the sensitivity and decrease the risk of false-positive results due to detection of dead cells (Fredriksson-Ahomaa and Korkeala, 2003a).

The result of PCR examination revealed that, only 8 isolates out of 12 randomly selected isolates (66.6%) were positive as *Y. enterocolitica* by the specific gene of *Y. enterocolitica* (16S ribosomal RNA). (Table 4), (Figure 1).

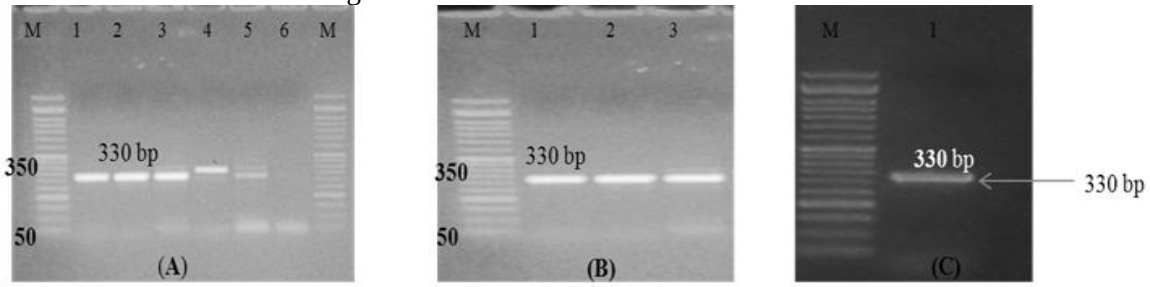

Figure 1. Agarose gel electrophoresis of PCR products for the *Y. enterocolitica* 16S ribosomal RNA gene of 330bp, M =50-bp DNA ladder.

In the present study, the difference between the results of identification of *Y. enterocolitica* by biochemical tests and PCR examination may be attributed to *Y. enterocolitica* gave similar morphology colony on (CIN) agar to other bacteria and like other members of the family *Enterobacteriaceae* in the biochemical reactions (EFSA,2007b and Hallanvuo, 2009).This difference agreed with the study of Neubauer *et al.*, (2000) where they attributed that to the problems in the identification systems found in biochemical reactions so, they used PCR technique to identify *Y. enterocolitica* based on specific targeting 16S rRNA gene, which previously biochemically identified as belonging to the *Yersinia* genus.

Several studies have been conducted to investigate the distribution of different virulence genes (ail, inv, yst, yadA, virF and yopT) among *Y. enterocolitica* strains by PCR (Thoerner *et al.*, 2003; Falcao *et al.*, 2004; Lee *et al.*, 2004; G¨urtler *et al.*, 2005 and Momtaz *et al.* ,2013).

In the present study, the results of detection of the virulence genes (ail, ystA and virF) were 75%, 50% and 37.5% respectively. These virulence genes could not be detected in 2 examined isolates out of 8 *Y. enterocolitica* isolates. The yadA gene could not be detected in any of the 8 examined isolates by multiplex PCR technique. (Table 5), (Figure 2).

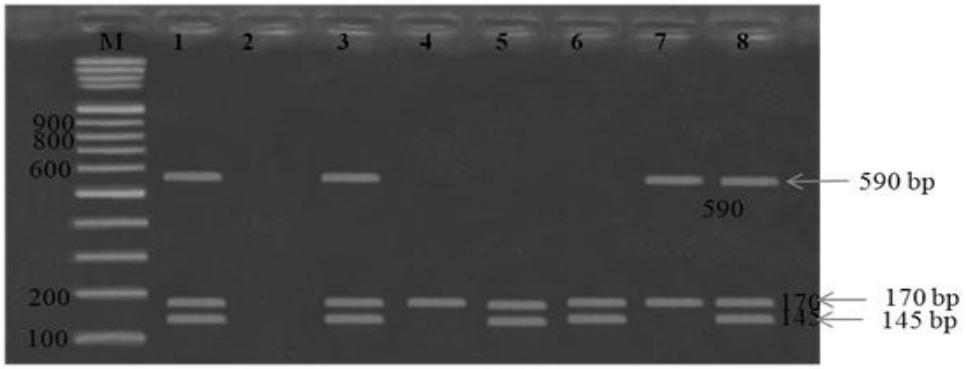

Fig. 2. Agarose gel electrophoresis of multiplex PCR of ystA (145 bp), ail (170 bp), virF (590 bp) and yadA (849 bp) virulence genes of *Y. enterocolitica* isolates, M =100-bp DNA ladder, lane1: Control positive for ystA, ail and virF genes, lane 2: Control negative for virulence genes of *Y. enterocolitica*, lanes 3 & 8: Positive isolates for \ystA, ail and virF genes, lane 4: Positive isolates for ail gene, lane 5 & 6: Positive isolates for yst A and ail genes and lane 7: Positive isolates for ail and virF genes. Negative *Y. enterocolitica* isolates for yadA virulence gene.

Table (3): Rate of isolation of *Y. enterocolitica* from examined chicken meat samples.

Chicken meat samples	No. of examined samples	Positive samples	
		No.	%
breast muscles	57	12	10.5%
thigh muscles	57	8	7%
Total	114	20	17.5

Table (4): Results of identification of *Y. enterocolitica* by detection of specific 16S rRNA gene by using PCR technique:

Type of examined isolates	No. of examined isolates	Positive isolates	
		No.	%
Chicken breast	6	5	83.3%
Chicken thigh	6	3	50%
Total	12	8	66.7%

Table (5): Results of detection of the potential chromosomal and plasmid-encoded virulence genes of *Y. enterocolitica* isolates.

No. of *Yersinia enterocolitica isolates*	Positive isolates							
	ail gene		ystA gene		yadA gene		virF gene	
8	No.	%	No.	%	No.	%	No.	%
	6	75	4	50	0	0	3	37.5

The results present in table (5) agreed with those of Zheng *et al.,* (2008) where they reported that all pathogenic *Y. enterocolitica* did not necessarily carry all traditional virulence genes in both chromosomes and plasmids to cause illness. Perhaps, some of them, lacking some traditional virulence genes, contain other unknown virulence markers that interact with each other and play an important role in the diverse pathogenesis of pathogenic *Y. enterocolitica*.

However Fredriksson-Ahomaa *et al.,* (2006) which reported that no pathogenic *Y. enterocolitica* has been detected in fish and chicken samples in Finland, which disagreed with the results in the present study.

In the present study the yadA gene could not detect in any of the 8 examined isolates and that agreed with those of Blais and Phillippe, (1995) where they stated that, the plasmid harboring the yadA gene can be lost under certain cultivation conditions in the laboratory.

Also, In the present study, pathogenic yst-positive *Y. enterocolitica* isolates detected in 4 examined samples (50%) and this result disagreed with this of Fredriksson- Ahomaa and Korkeala, (2003b) and Estrada *et al.*,(2012) where they could not detected pathogenic yst-positive *Y. enterocolitica* isolates in chicken meat samples.

Based on the obtained results in the current study, it was concluded that chicken meat was a possible source of gastroenteritis caused by pathogenic *Y. enterocolitica* after detection of the virulence genes (ail, ystA, yadA and virF).

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