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Genotypic and Phenotypic Variation of *Yersinia enterocolitica* Isolated from Different Sources

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Abstract

Yersinia enterocolitica is an enteropathogen that widely distributed in nature with high public health relevance and transmitted mostly by contaminated food. This study aimed to study the prevalence; phenotypic and genotypic characterization of the isolated *Y. enterocolitica* strains from 175 random samples of cow milk; beef; chicken meat; diarrheic cow and sheep faeces (35 for each), collected from different shops; dairy herds and sheep flocks, at Kaliobia Governorate Egypt, beside, investigation of the possible relationships among the isolated *Y. enterocolitica* strains. The obtained results revealed that 18 *Y. enterocolitica* isolates were recovered; they were isolated from cow milk samples (6/17.1%); chicken meat (5/14.3%); beef (4/11.4%) and cow faeces (3/8.6%), but failed to be isolated from sheep faeces. Four biotype groups (four 1A; seven 1B, three biotype 3 and four biotype4) were recognized biochemically. Moreover, many phenotypic virulence factors detected in some isolates as hemlysin , lecithinase, lipase ,proteinase and biofilm formation the isolated *Y. enterocolitica* were highly resistant for Penicillin G.; ampicillin; tetracycline; amoxicillin and streptomycin ,but they were highly sensitive to meropenem; norfloxacin; ciprofloxacin; gentamycin and Florfenicol. PCR appeared that, the *ail* gene was amplified in five and *yst* gene in four out of eight studied *Y. enterocolitica* strains. ERIC PCR showed many bands from 214 to 1228 and divided the 8 isolate to three genotypes. In conclusion, *Y. enterocolitica* strains with multiple antimicrobial resistances were isolated from milk, beef, chicken meat, cow faeces and could represent a public health concern. So, strict hygienic measures should be applied to minimize *Y. enterocolitica* contamination in milk and meat, also the antimicrobial resistances of *Y. enterocolitica* isolates should be monitored continuously to avoid public health hazards.

KEYWORDS
Yersinia enterocolitica, Genotypic variation, Phenotypic variation, Different sources.

INTRODUCTION

Yersinia (*Y.*) *enterocolitica* is a psychotropic zoonotic foodborne and waterborne enteropathogen transmitted by the fecal-oral route that can contaminate raw milk, meat and most food products (Ye *et al*., 2016; Penga *et al*., 2018). It is a Gram-negative, non-spore-forming, oxidase-negative, facultative anaerobic bacterium and the motility is temperature regulated; at 25°C, it is peritrichously flagellated, but at 37°C they are unflagellated and so non motile widely distributed in nature in addition to the psychotropic nature of *Y. enterocolitica*, which enable the bacterium to grow to large numbers at temperatures close to 0°C, it is characterized by temperature-dependent adaptations (Markey *et al*., 2013; Bancerz-Kisiel *et al*., 2018). It is highly heterogeneous and is represented by six biovars (1A, 1B, 2, 3, 4 and 5) depending on physio- and biochemical properties and additionally into approximately 70 serotypes depending on antigenic variation of the lipopolysaccharides (Ye *et al*., 2016; Peruzy *et al*., 2017).

Yersiniosis is a gastrointestinal infection caused by *Y. enterocolitica* and more than two-thirds of human yersiniosis infections manifest as acute diarrhoea lasting for 1-3 weeks, and extra-intestinal and post infectious manifestations, including reactive arthritis, mesenteric lymphadenitis, pseudo appendicitis and erythema nodosum, have been reported (Mastrodonato *et al*., 2018; Tavassoli *et al*., 2019). *Yersinia enterocolitica* was reported previously to be highly susceptible to most antibiotics and the level of resistance depends on the type of strains and temperature (Bonardi *et al*., 2018). However, the prevalence of drug-resistant *Y. enterocolitica* strains in food and the environment have been stated in recent years, due to overuse of antibiotics in animal and poultry farms and antimicrobial-resistance bacteria/genes transmission among different species (Musavian *et al*., 2014; Özdemir and Arslan, 2015; Ye *et al*., 2016). Moreover, the presence of antimicrobial resistance leads to treatment failures with the need for expensive and/ or toxic alternative drugs , on the other hand, the spread of drug resistance among *Y. enterocolitica* is also of concern for public health appraisal (Fàbrega *et al*., 2015).

As, foods of animal origin, especially beef, chicken meat, milk and their products are recognized as important vehicles for the transmission of *Y. enterocolitica* with antimicrobial resistance to humans due to inadequate handling and cooking during preparation resulting in a significant health risk for consumers, especially in young children and infants (Bonardi *et al*., 2018). In addition, the importance of determining antimicrobial - resistance

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and detecting virulence genes as a consequence of chromosomal changes or the interchange of genetic material via plasmids and transposons have been previously highlighted (Li and Fanning, 2017), but there is currently a lack of information on Yersiniosis. Moreover, there is no regular and/or routine monitoring of *Y. enterocolitica* in animal products and human in Egypt. So, the present study was conducted to study the prevalence, phenotypic and genotypic characterization of *Y. enterocolitica* strains isolated from cow milk, beef, chicken meat, diarrheic cow and sheep faeces collected from different shops, dairy herds and sheep flocks, at Kaliobia Governorate Egypt. Besides, investigation of the possible relationships among *Y. enterocolitica* strains isolated from these sources using enterobacterial repetitive intergenic consensus (ERIC)-PCR, recognized as mobile DNA particles that used to determine the relationships between different isolates of *Y. enterocolitica* (Wojciech *et al*., 2004),

MATERIALS AND METHODS

Samples

A total of 175 random samples of cow milk, beef, chicken meat, diarrheic cow faeces and diarrheic sheep faeces, were collected from different shops, dairy herds and sheep flocks (35 of each), at Kaliobia governorate, Egypt. The samples were used for detection of the prevalence of *Yersinia enterocolitica* strains, beside the phenotypic and genotypic characterization, and investigation of the possible relationships among *Y. enterocolitica* strains isolated from these sources using enterobacterial repetitive intergenic consensus (ERIC)-PCR. In this study, there was no direct contact between the researchers and the living animals.

Preparation of samples

Aseptically; 10 mL of milk, 10 g of meat, two grams of faeces were obtained from each sample in Peptone sorbitol bile broth was prepared for bacteriological examination following Fredriksson and Korkeala (2003) and ISO 10273 (ISO, 2003).

Isolation and identification of Y. enterocolitica strains following Farmer et al. (1992); ISO 10273 (ISO, 2003) and Markey et al. (2013)

Typical *Y. enterocolitica* colonies (small colonies having deep red center with sharp border surrounded by clear colourless zone with entire edge "bull's eye" on *Yersinia* selective agar base with *Yersinia* selective supplement (CIN) medium and small (flat, pale pink colonies on MacConkey agar were picked up for identification morphologically by Gram stain; motility tests, by stabbing the bacterial isolate in the center of the semi-solid agar of two tubes and both tubes were incubated for 24 h, one at 25°C and the other at 35°C (*Y. enterocolitica* are motile at 25°C and non-motile at 35°C) and biochemically by Lysine Arginine Iron Agar (LAIA), urease test, catalase, oxidase, aesculin hydrolysis, Indole, Methyl red, Voges-Proskauer, citrate utilization, nitrate reduction, gelatin hydrolysis, Beta-D-Glucosidase, Pyrazinamidase and Sugar fermentation tests. In addition, the *Y. enterocolitica* isolates were bio-typed following to the scheme of Wauters (1981) (Table 1).

In vitro anti-microbial sensitivity test

The isolated *Y. enterocolitica* strains were subjected to the sensitivity test against 12 different antimicrobials belonged to different antimicrobial classes using Kirby–Bauer disk diffusion method on Mueller–Hinton agar (Oxoid) plates following CLSI (2018).

Phenotypic virulence of Yersinia enterocolitica

Proteolytic (caseinase); lipolytic and lecithinase activities for isolates were tested as described by Yang and Fang (2003). In brief, tryptic soy agar with 1% skimmed milk, tween 80, egg yolk was used to detect the proteolytic activities. The hemolytic activity was screened on 5% rabbit blood with 1% egg yolk (lecithin) on blood base agar according to Tsubokura (1979), and biofilm

Table 1. Biotype scheme for *Y. enterocolitica* isolates

Biochemical test	Reaction for biotypes (a)						
	1A	1B	2	3	4	5	6
Lipase	+	+	-	-	-	-	-
Esculin/salicin (24 h)	+/-	-	-	-	-	-	-
Indole	+	+	(+)	-	-	-	-
Xylose	+	+	+	+	-	V	+
Trehalose	+	+	+	+	+	-	+
Pyrazinamidase	+	-	-	-	-	-	+
β -D-Glucosidase	+	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+/-[(b)]	+	(+)	-

(a) = Delayed reaction; V = variable reactions. [b] Biotype of serotype O:3 found in Japan.

Table 2. Primers sequences, target genes, amplicon sizes and cycling conditions.

		Primer sequence (5'-3')	Amplified segment (bp.)	Primary denaturation	Amplification (35 cycles)			Final extension	References
					Secondary denaturation	Annealing	Extension		
ail	F	TAATGTGTACGCTGCGAG	351 bp.	94˚C 30 sec.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 7 min	Koua *et al*. (2014)
	R	GACGTCTTACTTGCACTG							
yst	F	AATGCTGTCTTCATTTGGAGC	145 bp.	94˚C 30 sec.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min	
	R	ATCCCAATCACTACTGACTTC							
ERIC	F	ATG TAA GCT CCT GGG GAT TCA C	Variable	94˚C 30 sec.	94˚C 30 sec.	52˚C 1 min.	72˚C 1 min.	72˚C 7 min	Versalovic *et al*. (1991)
	R	AAG TAA GTG ACT GGG GTG AGC G							

formation of the isolates was screened using conge red agar method according to Pramodhini *et al.* (2012). All plates were incubated at 25°C for 48 h.

Molecular detection of virulence genes in some isolated Y. enterocolitica

Genotypic detection of two chromosomal virulence genes, adhesion invasion locus gene (*ail*); *Yersinia*-stable toxin gene (*yst*) in eight random *Y. enterocolitica* using polymerase chain reaction, following QIAamp® DNA Mini Kit instructions (Qiagen, Germany, GmbH), Emerald Amp GT PCR master mix (Takara, Japan) and 1.5% agarose gel electrophoreses (Sambrook *et al.*, 1989) using two pairs of primers supplied from Metabion (Germany); the Primers sequences, target genes, amplicons sizes and cycling conditions showed in Table 2.

ERIC fingerprinting data were transformed into a binary code depending on the presence or absence of each band. Dendrograms were generated by the unweighted pair group method with arithmetic average (UPGMA) and Ward's hierarchical clustering routine. Cluster analysis and dendrogram construction were performed with SPSS, version 22 (IBM 2013) (Hunter, 1990). Similarity index (Jaccard / Tanimoto Coefficient and number of intersecting elements) between all samples was calculated using the online tool (https://planetcalc.com/1664/).

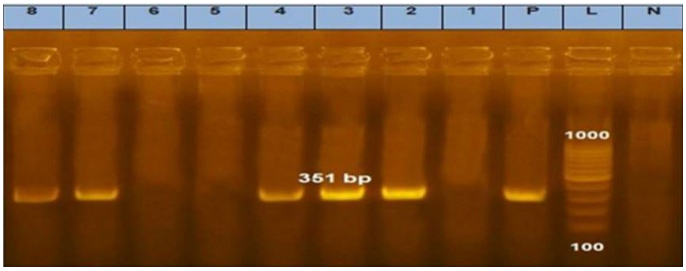

Fig. 1. Adhesion invasion locus (*ail*) gene. Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (E. coli AJ413986). Pos.: Positive control (*Y. enterocolitica* form Ahri. at 351 bp). Lanes 2, 3, 4, 7&8: *Y. enterocolitica* (Positive at 351 bp). Lanes 1,5&6 : *Y. enterocolitica* (Negative).

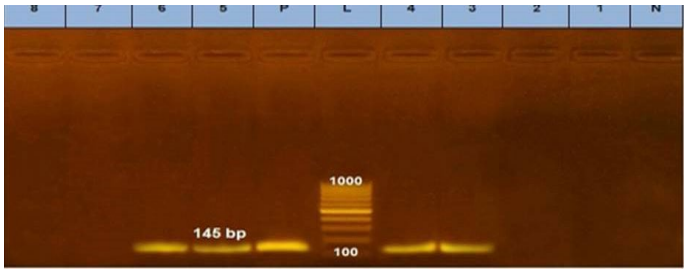

Fig. 2. *Yersinia*-stable toxin (*yst*) gene. Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (E. coli AJ413986). Pos.: Positive control (*Y. enterocolitica* form Ahri. at 145 bp). Lanes 3, 4, 5&6: *Y. enterocolitica* (Positive at 145 bp). Lanes 1, 2, 7&8: *Y. enterocolitica* (Negative)

RESULTS

The results of the bacteriological examination of examined samples, in vitro sensitivity testing, phenotypic and genotyping detection of virulence genes for the isolated *Y. enterocolitica* strains and (ERIC)-PCR results were tabulated in Tables 3-5, and Figures 1-4.

The phenotypic characters of *Y. enterocolitica* isolates showed Gram- negative coccobacilli with rounded edges or short rods. Their colonies on *Yersinia* selective agar base with *Yersinia* selective supplement (CIN) medium had a very offensive odour and appeared small mucoid with convex and deep red center with sharp border surrounded by clear colourless zone and entire edge "bull's eye", onto MacConkey agar they appeared as small, non-lactose fermenter, flat, pale pink colonies. All isolated *Y. enterocolitica* showed 100% motilities at 25°C but not at 35°C.

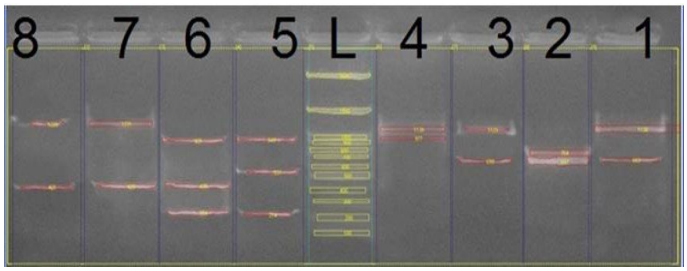

Fig. 3. DNA fingerprints of *Yersinia enterocolitica* isolates generated by ERIC-PCR and separation in agarose gel. Ladder (100 to 2000 bp), in the middle 1 to 8 tested isolates.

About 8/18 isolates produce licithinase and hemolysin activity, 6/18 produced lipase enzyme, 12/18 produced protease enzyme and 15/18 produced biofilm (2 strong (metallic black colonies), 6 moderate (pink colonies), 7 weak) as in Table 4.

Table 3. Prevalence of *Yersinia enterocolitica* strains isolated from examined samples

Samples	Number of samples	Negative samples		Positive samples	
		No.	%	No.	%
Cow milk	35	29	82.9	6	17.1
Beef	35	31	88.6	4	11.4
Chicken meat	35	30	85.7	5	14.3
Cow faeces	35	32	91.4	3	8.6
Sheep faeces	35	35	100	0	0
Total	175	157	89.7	18	10.3

Table 4. Phenotypic virulence of *Yersinia enterocolitica*

Samples	Hemolysin	Lecithinase	Lipase	Protease	Biofilm formation
Cow milk (n.=6)	2\6	2\6	2\6	4\6	2M, 3 W
Beef (n.= 4)	2\4	2\4	1\4	2\4	1S,1M,1W
Cow faeces (n.= 3)	1\3	1\3	1\3	2\3	1S, 1W
Chicken meat (n.=5)	3\5	3\5	2\5	4\5	3M, 2W

S: Strong; M: Moderate; W: Weak

Table 5. In vitro anti-microbial Sensitivity test for the isolated *Y. enterocolitica*

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		AA
		No.	%	No.	%	No.	%	
Penicillin G.	10units	0	0	0	0	18	100	R
Ampicillin	20 µg	0	0	1	5.6	17	94.4	R
Tetracycline	30 µg	1	5.6	2	11.1	15	83.3	R
Amoxicillin	25µg	0	0	4	22.2	14	77.8	R
Streptomycin	S/10	2	11.1	4	22.2	12	66.7	R
Doxycycline	30 µg	3	16.7	11	61.1	4	22.2	IS
Co- Trimoxazole	(1.25/23.75) mcg	3	16.7	10	55.5	5	27.8	IS
Meropenem	10 µg	16	88.9	2	11.1	0	0	S
Norfloxacin	10 µg	15	83.3	2	11.1	1	5.6	S
Ciprofloxacin	5 µg	13	72.2	3	16.7	2	11.1	S
Gentamicin	30 µg	12	66.7	4	22.2	2	11.1	S
Florfenicol	30 µg	11	61.1	4	22.2	3	16.7	S

No.: Number of isolates; AA: Antibiogram activity; %: Percentage in relation to total number of studied *Y. enterocolitica* (18)

The results of gene virulence factors were shown in Table 6 and Figures 1-4.

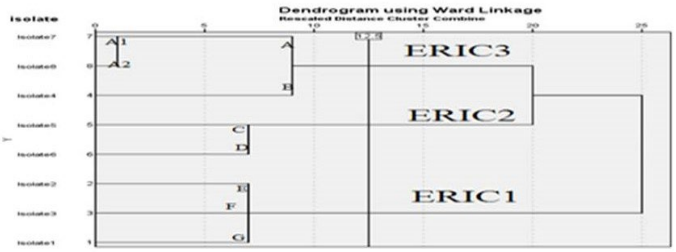

Fig 4. Dendrogram genetic relationships between *Y. enterocolitica* isolates based on ERIC PCR analysis.

Detection of adhesion invasion locus (*ail*) gene was shown in Fig. 1 which revealed that 5 samples were positive out of 8, while findings in Figure 2 showed *Yersinia*-stable toxin (*yst*) gene, as 4 samples were positive for this gene, while the results of ERIC PCR in Figures 3, 4, were found to be clustered by Dendrogram genetic relationships, 3 isolates from chicken meat (1, 2, 3) has two bands from 650 to 1138, two isolates from milk (5, 6) had three bands from 214 to 977 bp), two isolates from beef meat (7, and 8) had two bands from (448, and 1228) and one isolate from cow feces (4) had two bands (977, and 1228).

DISCUSSION

Yersinia enterocolitica is one of the most important food poisoning pathogens associated with milk; meat and their products and the presence of them with high levels indicates a potential risk of producing yersiniosis infection in human and animals (Jamali *et al*., 2015; Penga *et al*., 2018).

Regarding to the colonial appearance (characteristic bull's eye appearance on CIN media) and the biochemical profile of *Y. enterocolitica* isolated, it was similar to those previously reported by Soltan *et al*. (2020) and Sen *et al*. (2021). The results of biochemical biotyping tests for 18 isolated *Y. enterocolitica* appeared that four biotype groups: four 1A (two from cow milk, and one form each sample of beef, chicken meat samples); seven 1B (four cow milk and one form each sample of beef, chicken meat and cow faeces); three biotype 3 (two from chicken meat and one from beef) and four biotype 4 (two cow faeces and one from each sample of beef and chicken meat) were recognized. These biotypes were compatible with previous studies (Falcao *et al*., 2003; Rusak *et al*., 2014; Bancerz-Kisiel *et al*., 2018; Tavassoli *et al*., 2019; Abd El Tawab *et al*., 2021).

In the present study, the recorded results in Table 3, cleared that, 18 *Y. enterocolitica* isolates (10.3%) were obtained from 175 samples represented as 6/17.1% from cow milk samples, and it came in harmony with those of Aliand Al-Samarai (2020) and Abd El Tawab *et al*. (2021), but disagree with those obtained by Khalid and Abbas (2019); Soltan *et al*. (2020) who isolated *Y. enterocolitica* from milk with lower incidence 4%, 0.83%, respectively; and disagree also with those of Darwish *et al*. (2015), who recorded higher incidence (46%). Meanwhile, the prevalence in chicken meat was 5/14.3% that was similar to that of Penga *et al*. (2018) and Younis *et al*. (2021) but lower than those of Soltan *et al*. (2010), who isolated *Y. enterocolitica* from chicken meat with higher incidence (21.6%). The prevalence in beef was 4/11.4% and it is consistent with Younis *et al*. (2021), whereas it is lower than that reported by Sen *et al*. (2021) with incidence of 76.5%. Moreover, the prevalence of *Y. enterocolitica* in faeces of the dairy herd cows was (3/8.6%), which came in harmony with those of Falcao *et al*. (2003) and McNally *et al*. (2004), but disagree with those obtained by Bharathy *et al*. (2015); who isolated *Y. enterocolitica* from faeces of dairy herds with higher incidence (38.37%). In addition, *Y. enterocolitica* failed to be isolated from sheep faeces. These results disagreed with those of Joutsen *et al*. (2016) and Stanger (2017) who isolated *Y. enterocolitica* from faeces of sheep flocks.

The results of in- vitro sensitivity tests for isolated *Y. enterocolitica* (Table, 5) revealed that, they were highly resistant for Penicillin G. (100.0%); then ampicillin (94.4%); tetracycline (83.3%); amoxicillin (77.8%) and streptomycin (66.7%). Meanwhile, they were intermediate sensitive to doxycycline (61.1%) and Co- Trimoxazole (55.5%). Moreover, they were highly sensitive to meropenem (88.9%) followed by norfloxacin (83.3%); ciprofloxacin (72.2%); gentamycin (66.7%) and Florfenicol (61.1%). Nearly similar results were recorded by Özdemir and Arslan (2015); Bharathy *et al*. (2015); Ye *et al*. (2016); Penga *et al*. (2018); Abd El Tawab *et al*. (2021) and Younis *et al*. (2021). The recorded results proved that, phenotypic multiple antibiotic resistances (MDR) are widely spread among 21 isolated *Y. enterocolitica* and, drug-resistance of *Y. enterocolitica* was neither related to the source origin nor their biotypes. Similar results were observed by Penga *et al*. (2018) and Younis *et al*. (2019) where the improper use of antibiotics in Egypt and other countries may be the main cause of high resistance rate in these isolates. Also the isolates have many phenotypic virulence factors as lecithinase, hemolysin , lipase , protease enzyme and biofilm formation which share in pathogenicity of *Y. enterocolitica* many authors also detect these virulence factors as Tsubokura *et al*. (1979) who detected hemolysin and lipase enzyme in *Y. enterocolitica*. Dowidar and Khalifa (2023) detected

biofilm formation of *Y. enterocolitica* isolated from milk and dairy product, and Anna and Wioleta (2017) showed a moderate ability of *Y. enterocolitica* to form a biofilm. Utomo and Rahardja (2019) recorded that *Yersinia enterocolitica* Produced the protease enzyme. The PCR technique is capable of identifying the pathogenic bacterial strains but only a few studies on virulent *Y. enterocolitica* strains have been described in the literature, focusing on the presence of chromosomal virulence genes (Zheng *et al*., 2008). The (*ail* and *yst*) proteins are important factors for *Y. enterocolitica* virulence. Hence, the existence of the adhesion invasion locus gene (*ail*) and *Yersinia*-stable toxin (*yst*) genes, which encode these proteins, are used as appropriate pathogenicity markers of *Y. enterocolitica* isolates (Thoerner *et al*., 2003). The result of PCR amplification of adhesion invasion locus (*ail*) gene in *Y. enterocolitica* isolates (Fig. 1) showed that, the *ail* gene was amplified in five out of eight studied *Y. enterocolitica* strains giving product of 351 bp. The isolates were recovered from two chicken meat, cow feces, and beef samples. The results came in harmony with those of Thoerner *et al*. (2003); Darwish *et al*. (2015); Jamali *et al*. (2015); Imori *et al*. (2017); Bancerz-Kisiel *et al*. (2018); Ali and Al-Samarai (2020) and Soltan *et al*. (2020). In addition, the result of PCR amplification of *Yersinia*-stable toxin (*yst*) gene in *Y. enterocolitica* isolates (Fig., 2) showed that, the *yst* gene was amplified in four out of eight studied *Y. enterocolitica* strains giving product of 145 bp. The isolates were recovered from one chicken meat, cow feces, and milk samples. These results came in accordance with those recorded by Jamali *et al*. (2015); Peruzy *et al*. (2017); Bancerz Kisiel *et al*. (2018); Tavassoli *et al*. (2019); Younis *et al*. (2019) and Ali and Al-Samarai (2020). The pathogenicity of *Y. enterocolitica* associated closely with *ail* and *yst* virulence genes, biotypes, and serotypes (Peruzy *et al*., 2017). The diversity in the pathogenicity of *Y. enterocolitica* isolates may be depended on the geographical area of isolation.

Finally, the results proved that *Y. enterocolitica* isolates with virulent toxigenic genes and multiple antimicrobial resistances are widely spread among cow milk; beef; chicken meat and cow faeces and decided the fact of McDermott *et al*. (2002) and Jamali *et al*. (2015) that application of antibiotics in animal food to control and treat infectious diseases in dairy and poultry farms can be considered as the main route of transmission of antimicrobial resistant bacteria between the animal and human populations.

CONCLUSION

Therefore, it was concluded that; *Y. enterocolitica* strains with multiple antimicrobial resistances are food-borne pathogens and their presence in milk, beef, chicken meat, cow faeces could be a public health concern for the consumers. So, strict hygienic measures should be applied to minimize *Y. enterocolitica* contamination in milk and meat, also the antimicrobial resistances of *Y. enterocolitica* isolates should be monitored continuously to avoid public health hazards.

CONFLICT OF INTEREST

The Authors declare that they have no conflict of interest.

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