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Phenotypic and genotypic studies on antibiotic resistant *Yersinia enterocolitica* isolated from milk and milk products in Kaliobia Governorate, Egypt

Ashraf, A. Abd El Tawab¹, Ahmed, A. A. Maarouf², Fatma, I. El Hofy¹ and Ahmed, M. A. Mousa³

¹Bacteriology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Benha University, Egypt.

²Animal Health Research Institute "Benha branch" ARC, Egypt

³Veterinarian

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ABSTRACT

The study was applied on 175 random samples of milk and milk products (white cheese, kareish cheese, yoghurt and ice cream) collected from different shops (35 of each), at Kaliobia governorate, Egypt, for detection of *Y. enterocolitica* strains, beside the phenotypic characterization and detection of some antibiotic resistant virulence genes in the examined samples. The results revealed that 9(10.9%) *Y. enterocolitica* isolates, bio types 1A and 1B only were isolated from milk and Kareish cheese samples (5/14.3% for each), (4/11.4%) white cheese, (3/8.6%) ice cream, and (2/5.7%) from yoghurt samples. The antibiotic sensitivity profile showed that, the isolated *Y. enterocolitica* isolates were very high resistant for Penicillin-G followed by methicillin, ampicillin, oxytetracycline, amoxicillin, ampicillin, streptomycin and erythromycin. Meanwhile, they were highly sensitive to meropenem and norfloxacin followed by gentamycin, ciprofloxacin, cefotaxime and fluorophenol. PCR declared that *bla*TEM and *tet*A genes were detected in all eight studied *Y. enterocolitica* isolates. So, it was concluded that, the presence of antimicrobial resistant *Y. enterocolitica* strains in dairy products could be a public health concern for the consumers.

1. INTRODUCTION

Yersinia enterocolitica is a psychrotrophic, Gram-negative, facultative anaerobic zoonotic bacterium belonging to the family Enterobacteriaceae (Bancerz-Kisiel *et al.*, 2018). It is a biochemical and serologically heterogeneous species, it has six biotypes (1A, 1B, 2, 3, 4 and 5) with approximately 70 serotypes and some serotypes related to human diseases e.g. 0:3, 0:5, 27, 0:8 and 0:9 (Ye *et al.*, 2016 and Peruzzy *et al.*, 2017). *Y. enterocolitica* can be bio typed according to their pathogenic properties: the non-pathogenic biotype 1A has involvement in human gastroenteritis (Campioni and Falcão, 2014), less pathogenic the biotypes 2–5, and highly pathogenic biotype 1B associated with human infections (Bancerz- Kisiel *et al.*, 2018 and Tavassoli *et al.*, 2018). *Yersinia enterocolitica* can contaminate raw milk, pasteurized milk and milk products (Bernardino- Varo *et al.*, 2013 and Rahimi, *et al.*, 2014). Yersiniosis comes after Campylobacteriosis and Salmonellosis as a gastrointestinal infection (Bolton *et al.*, 2013 and Zadernowsks *et al.*, 2014) and manifested by acute diarrhea, arthritis, mesenteric lymphadenitis, pseudo appendicitis and erythema nodosum, (Huovinen *et al.*, 2010; Bernardino-Varo *et al.*, 2013 and Tavassoli *et al.*, 2018).

Uncontrolled usage of antibiotics for treating mastitis in dairy cows is considered as the main cause of multi-drug resistant strains of bacteria (Sadek *et al.*, 2014). *Yersinia enterocolitica* was susceptible to many antimicrobial agents except penicillin, ampicillin, amoxicillin-clavulanic acid,

and the first-generation cephalosporins (Bolton *et al.*, 2013 and Bonardi *et al.*, 2018). Though, the main cause of drug-resistant of *Y. enterocolitica* strains in food and the environment in recent years is usage of antibiotics in animal farms and transmission of antibiotic-resistance gene through dissimilar species of bacteria (Özdemir and Arslan, 2015 and Ye *et al.*, 2016). Moreover, the main cause of the treatment failures is the presence of antimicrobial resistance that lead to need for expensive and/or toxic alternative drugs which may be more expensive in most cases and this spreading of drug resistance for *Y. enterocolitica* is very important for public health appraisal (Pandove *et al.*, 2012). Resistant of microorganisms to an antimicrobial agents as a consequence of chromosomal changes or the inter change of genetic material via plasmids and transposons (Li and Fanning, 2017). The antibiotic resistance of *Y. enterocolitica* is mediated by the chromosomal genes *bla*A and *bla*B, which encode β -lactamases (Fabrega and Vila, 2012) and the prevalence of antimicrobial-resistant *Y. enterocolitica* strains, including multi drug-resistant *Y. enterocolitica* strains, has been progressively increasing (Fabrega and Vila 2012 and Bonardi *et al.*, 2018). However, the prevalence of antimicrobial-resistant *Y. enterocolitica* in dairy products in Egypt remains poorly characterized.

In Egypt, Yersiniosis have a lack of information on the prevalence of food borne diseases. Moreover, there is no regular and routine monitoring of this bacterium in animal products, limiting the information regarding its occurrence,

* Corresponding author: ahmed_abdelwhap@yahoo.com

characterization and prevalence. Therefore, this study was conducted to throw light over the prevalence of *Y. enterocolitica* in milk and some milk products (white cheese, kareish cheese, yoghurt and ice cream) at Kaliobia Governorate beside the phenotypic characterization of the isolates and determination their antibacterial resistant genes.

2. MATERIAL AND METHODS

2.1 Samples:

A total of 175 random samples of milk and milk products (white cheese, kareish cheese, yoghurt and ice cream) were collected from different shops (35 of each), at Kaliobia governorate, Egypt, for detection the prevalence of *Yersinia enterocolitica* strains in these samples, beside the phenotypic characterization and detection of some antibiotic resistant virulence genes in them.

2.2 Bacteriological examination:

Aseptically (10 mL of milk and 10 g of milk products) from each sample were prepared using Peptone sorbitol bile broth for bacteriological examination according to FDA (2017).

2.2.1. Isolation and identification of Y. enterocolitica strains following Markey et al. (2013) and FDA(2017): Typical *Y. enterocolitica* colonies (small colonies having deep red center with sharp border surrounded by clear colorless zone with entire edge “bull’s eye” on Yersinia selective agar base with Yersinia selective supplement (CIN) and on MacConkey agar the colonies appears medium and small (flat, colorless, or pale pink colonies) then were picked up for identification morphologically by Gram stain then biochemical tests following Farmer et al. (1992), and Markey et al. (2013).

2.2.2. Biotyping of Y. enterocolitica isolates

Biotyping of *Y. enterocolitica* isolates was determined according to the scheme of Wauters (1981) as shown in Table (1).

2.2.3. In-Vitro anti-microbial sensitivity test:

The isolated *Y. enterocolitica* strains were subjected to the sensitivity test against different antibiotics using the disc method of CLSI (2018).

2.2.4. Detection of antibiotic resistant genes of Y. enterocolitica by PCR:

Genotypic detection of two antibiotic resistant genes, β -lactamase ampicillin resistance gene (*bla*TEM) and tetracycline resistant A gene(*tetA*) in eight random isolated *Y. enterocolitica* that showed antibiotic resistant by disk diffusion method to the same studied isolates using polymerase chain reaction, following QIAamp® DNA Mini Kit instructions (Qiagen, Germany, GmbH), Emerald Amp GT PCR mastermix (Takara,Japan) and 1. 5% agarose gel electrophoreses (Sambrook et al., 1989) using the Primers sequences, target genes, amplicons sizes and cycling conditions showed in Table (2).

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, V = variable

^b Biotype of O:3. serotype

Table (2): Primers sequences, target genes, amplicons sizes and cycling conditions

Target gene	Primer sequence (5'-3')	Amplified segment (bp.)	Primary denaturation	Amplification (35 cycles)			Final extension	References
				Secondary denaturation	Annealing	Extension		
<i>bla</i> TEM	F ATCAGCAATAA ACCAGC	516 bp.	94°C 5 min.	94°C 30 sec.	54°C 40 sec.	72°C 45 sec.	72°C 10 min.	<i>Colom et al., 2003</i>
	R CCCGAAGAAC GTTTTTC							
<i>tetA</i>	F GGTTCACTCGA ACGACGTCA	576 bp.	94°C 5 min.	94°C 30 sec.	54°C 40 sec.	72°C 45 sec.	72°C 10 min.	<i>Randall et al.,2004</i>
	R CTGTCCGACAA GTTGCATGA							

3. RESULTS

The results of bacteriological examination of examined samples, In-vitro sensitivity test, genotyping detection of antibiotic resistant genes for the isolated *Y. enterocolitica* strains were tabulated in Tables (3-4) and Figures (1-2).

The results of biochemical identification showed that, all isolates had characteristic biochemical features as that of *Y. enterocolitica*, where, all the 19 isolates were positive for Indole, catalase, Methyl red, Voges-Proskauer and Urease tests. Meanwhile, they were negative for oxidase, citrate utilization, nitrate reduction, gelatin hydrolysis and Lysine

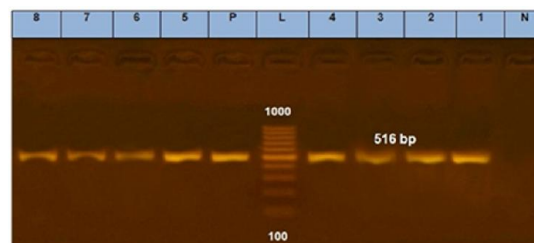
Arginine Iron Agar (LAI) tests. Moreover, the biotype 1A is positive for Pyrazinamidase test and Beta-D-Glucosidase tests while biotypes1B is negative for these tests.

The results of biotyping tests for isolated *Y. enterocolitica* appeared that, all of them were biotype 1A and biotype 1B only. Seven biotype1A were isolated from raw milk, Yoghurt (two for each sample), white cheese, Kareish cheese, Ice cream (one for each sample) and 12 biotype 1B were isolated from Kareish cheese, raw milk, white cheese, Ice cream with total numbers of 4,3,3,2, respectively but failed to be detected from yoghurt samples.

Table (3): Incidence of isolated *Y. enterocolitica* strains

Samples	Number of samples	Negative samples		Positive samples	
		No.	%	No.	%
Raw milk	35	30	85.7	5	14.3
White cheese	35	31	88.6	4	11.4
Kareish cheese	35	30	85.7	5	14.3
Yoghurt	35	33	94.3	2	5.7
Ice cream	35	32	91.4	3	8.6
Total	175	156	89.1	19	10.9

Percentage in relation to total number of each sample in each row (35 for each sample and 175 for total).

Fig. (1): β -lactamase ampicillin resistance (*bla*TEM) gene

- Lane L: 100-1000 bp. DNA Ladder.
- Neg.: Negative control (*E. coli* AJ413986)
- Pos.: Positive control (*Y. enterocolitica* form Ahri. at 516 bp.)
- Lanes 1-8: *Y. enterocolitica* (Positive at 516 bp.)

Table (4): In-Vitro anti-microbial Sensitivity test for isolated *Y. enterocolitica*

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		AA
		No.	%	No.	%	No.	%	
Penicillin G.	10 units	0	0.0	0	0.0	19	100.0	R
Methicillin	5 µg	2	10.5	0	0.0	17	89.5	R
Oxytetracycline	30 µg	1	5.2	3	15.8	15	79.0	R
Ampicillin	20µg	2	10.5	3	15.8	14	73.7	R
Amoxicillin	25µg	1	5.3	4	21.0	14	73.7	R
Streptomycin	S/10	0	0.0	5	26.3	14	73.7	R
Erythromycin	15 µg	2	10.5	5	26.3	12	63.2	R
Doxycycline	30 µg	3	15.8	12	63.2	4	21.0	IS
Meropenem	10 µg	15	79.0	4	21.0	0	0.0	S
Norfloracin	10 µg	15	79.0	3	15.8	1	5.2	S
Gentamicin	10 µg	13	68.4	3	15.8	3	15.8	S
Ciprofloxacin	5 µg	12	63.2	5	26.3	2	10.5	S
Cefotaxime	30µg	12	63.2	4	21.0	3	15.8	S
Florphenicol	30 µg	10	52.6	6	31.6	3	15.8	S

No.: Number of isolates AA: Antibiogram activity % Percentage in relation to total number of isolated *Y. enterocolitica* (19)

4. DISCUSSION

Yersinia enterocolitica is considered as one of food poisoning pathogen associated with milk and its products and if present with high levels indicates a potential risk of producing yersiniosis infection in human and animals (Jamali *et al.*, 2015).

The results declared that, 19 *Y. enterocolitica* isolates (10.9%) were recovered from the examined samples; from raw milk and Kareish cheese samples (5/14.3% for each) followed by white cheese (4/11.4%), ice cream (3/8.6%); and yoghurt samples (2/5.7%)Table(3). The results of *Y. enterocolitica* isolation from milk and its products were nearly similar to those recorded by Harakeh *et al.* (2012); Ali and Al-Samarai (2020); who reported that *Y. enterocolitica* isolates in milk and milk products were 9.75%; 12%, respectively. But disagreed with those obtained by Ali *et al.* (2015); Jamali *et al.* (2015); Ozdemir

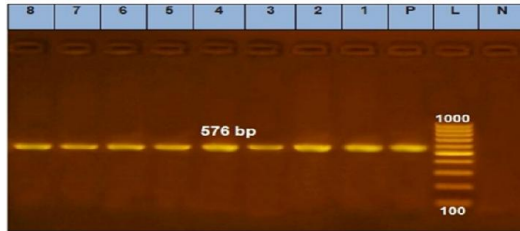
and Arslan (2015) who isolated *Y. enterocolitica* from milk and milk products with lower incidence 5.78%,5.3%,3.3%,respectively; Darwish *et al.* (2015), Ahmed *et al.*(2019) recorded higher incidence 46%, 22%, respectively,while Zeinhom and Abdel-Latef(2014)failed to isolate it from milk and milk products samples.

The variations between findings of numerous authors and of this study would possibly be due to many factors such as distinction in ways of sampling of analyzed samples, ways of analyzing, sources of samples, season, sanitary measures and geographical location. These factors might cause a rise or decrease in the incidence of *Yersinia* spp. infection. Moreover, the diagnosis of diarrhea and gastroenteritis in clinical laboratories in Egypt is solely based on the detection of *Salmonella*, *Shigella*, *E. coli* and *Entamoeba*. Thus, diarrhea caused by other pathogens, *Campylobacter* and *Y. enterocolitica* may not be reported and usually if cultures were negative for the sought-after

pathogens, then the diarrhea case could be reported as “unknown etiology” and/or “viral infection”. So, detection of *Y. enterocolitica* must be considered in these cases.

The colonial appearance (characteristic bull's eye appearance on CIN media) and the biochemical profile of *Y. enterocolitica* isolated was similar to those previously reported by Wanger (2007); Markey *et al.* (2013) and FDA (2015) and Drake *et al.* (2018).

Fig. (2): Tetracycline resistant A (tetA) gene



- Lane L: 100-1000 bp. DNA Ladder.
- Neg.: Negative control (*E. coli* AJ413986)
- Pos.: Positive control (*Y. enterocolitica* form Ahri. at 576 bp.)
- Lanes 1-8: *Y. enterocolitica* (Positive at 576 bp.)

In addition, the biotyping tests for isolated *Y. enterocolitica* showed that, all of them were biotype 1A and biotype 1B only. Seven biotype 1A were isolated from raw milk, Yoghurt (two for each); white cheese, Kareish cheese, Ice cream (one for each) and 12 biotype 1B were isolated from Kareish cheese, raw milk, white cheese, Ice cream with total numbers of 4,3,3,2, respectively but failed to be detected from yoghurt samples. Similar biotypes were isolated from milk and its products by Gamal- Eldin(2008); Bernardino-Varo *et al.*(2013); Ali *et al.*(2015) and Tavassoli *et al.* (2019)

The results of in- vitro sensitivity tests for isolated *Y. enterocolitica* (Table 4) revealed that, the isolated *Y. enterocolitica* were highly resistant for Penicillin G. (100.0%); then methicillin (89.5%); oxytetracycline (79.0%); amoxicillin; ampicillin and streptomycin (73.7% for each) and erythromycin (63.2%). Meanwhile, they were intermediate sensitive to doxycycline (63.2%). Moreover, they were highly sensitive to meropenem and norfloxacin (79.0% for each) followed by gentamycin (68.4%); ciprofloxacin and cefotaxime (63.2 % for each) and florphenicol (52.6%). Nearly similar results were recorded by Pugazhenthii *et al.* (2013); Bharathy *et al.* (2015); Jamali *et al.* (2015); Darwish *et al.* (2015) and Ahmed *et al.* (2019).

Moreover, the recorded results proved that multiple antibiotic resistances are widely spread among isolated strains of *Y. enterocolitica* and the improper use of antibiotics in Egypt may leads to the high resistance rate in local *Yersinia* isolates.

The PCR technique is capable of identifying the antibiotic resistant genes in pathogenic bacterial strains but there is no enough studies on *Y. enterocolitica* resistance, high lighting on the presence of genes concerning to the formation of β -lactamases and tetracycline resistant genes is required(Bent and Young, 2010).So, the present study was directed for recognizing two antibiotic resistant genes on eight random isolated *Y. enterocolitica* that showed antibiotic resistant by disk diffusion method, which may play a role in pathogenicity of these isolates by using one of the recent developments molecular biological techniques

(PCR).These genes were β -lactamase ampicillin resistance gene (*bla*TEM) and tetracycline resistant A gene(*tetA*).The results of PCR cleared that, *bla*TEM and *tetA* virulence genes were detected in all eight studied *Y. enterocolitica* isolates, where *bla*TEM gene was amplified at 516 bp. (Fig., 1) and *tetA* gene was amplified at 576 bp. (Fig., 2). Similar detection of these genes in *Y. enterocolitica* strains isolated from food and milk products by Bradford (2001); Fabrega and Vila (2012); Bonardi *et al.* (2013); Ye *et al.* (2015); Zamzam (2017) and Younis *et al.* (2019).So, there were positive correlation between the presence of *bla*TEM and *tetA* genes with the phenotypic resistance to ampicillin and oxytetracycline.

Finally, the results proved that multiple antibiotic resistances are widely spread among isolated strains of *Y. enterocolitica* and decided the fact of Jamali *et al.* (2015) that application of antibiotics in animal food to control and treat infectious diseases in dairy farms can be the main cause of transmission of resistant bacteria for many antibiotics from the animal to human populations. Therefore, it was concluded that; the presence of antimicrobial resistant *Y. enterocolitica* strains in milk and milk products could be a public health concern for the consumers.

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