



Virulence Characteristics of *Yersinia enterocolitica* Isolated from Dairy Products in the Northeast of Iran

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ARTICLE INFO

Article type:
Original article

Article history:
Received 6 April 2019
Revised 10 June 2019
Accepted 16 June 2019

DOI: 10.29252/jhehp.5.2.5

Keywords:
Yersinia enterocolitica
Virulence
Dairy products

ABSTRACT

Background: *Yersinia enterocolitica* (*Y. enterocolitica*) has a wide range of clinical, animal, food, and water sources. Most studies have indicated that food is the most common source of this organism. The present study aimed to evaluate the virulent genes of *Y. enterocolitica* isolated from dairy products in Iran.

Methods: The virulence of *Y. enterocolitica* biotypes was investigated, which was isolated from 38 cheese and 33 raw milk samples in the northeast of Iran. In total, six virulence-related genes were evaluated, including *ail*, *inv*, *yadA*, *myfA*, *ystA*, and *ystB* in 1A, 1B, and 5 *Y. enterocolitica* biotypes.

Results: In the isolates of the 1A biotype, *ystB* was the most frequent gene (86.95% and 38.46% in cheese and raw milk, respectively). In the 1B biotype, the most frequently isolated gene was *yadA* (92.30% and 66.66% in cheese and raw milk, respectively). In all the isolates, the least frequently isolated gene was *ail*, followed by *myfA*.

Conclusion: According to the results, the presence of virulence genes in the *Y. enterocolitica* strains isolated from dairy products suggested that these strains could pose significant risk to public health if dispersed in susceptible human population.

1. Introduction

Yersinia enterocolitica (*Y. enterocolitica*) has a wide range of clinical, animal, food, and water sources, which have been documented in the literature [1-3]. In general, the genus *Yersinia* consists of 18 species, 11 of which have been established, including *Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. bercovieri*, *Y. mollaretii*, *Y. rohdei*, *Y. aldovae*, and *Y. ruckeri*. Only the three species of *Y. Pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* are considered to be pathogenic for humans and animals [1].

According to a recent report by the European Food Safety Authority (EFSA), *Y. enterocolitica* is considered to be an important foodborne pathogen in Europe [4]. *Y. enterocolitica* causes various human infections, including

acute gastroenteritis and invasive syndromes such as mesenteric lymphadenitis, appendicitis, and septicemia [2, 4-6]. *Y. enterocolitica* is classified into six biotypes of 1A, 1B, 2, 3, 4, and 5, while it also has more than 70 different serotypes [1,7]. Based on their pathogenicity, *Y. enterocolitica* biotypes are classified as non-pathogenic biotype 1A and pathogenic biotypes of 1B, 2, 3, 4, and 5 [2, 6, 8]. Virulence in *Y. enterocolitica* could be categorized into two important structures, one of which is inside the chromosome and the other is on the plasmid of the *Yersinia* virulence (pYV) with the size of 64-75 kb [7]. All the six biotypes of *Y. enterocolitica* are able to attack the intestinal mucosa, while with only one plasmid, they could migrate to the digestive tract and internal organs [1, 2].

Biotype 1A strains are found in a wide range of environmental sources, such as soil, food, water, and sewage [3].

Biotype 1A of the *Y. enterocolitica* strains lack pYV, most of the chromosomal virulence genes (e.g., *ail*, *myfA*, *ystA*), and high pathogenicity island (HPI) [1-3]. Due to the lack of virulence genes, biotype 1A is often isolated from humans with gastrointestinal diseases [3]. For instance, some studies have shown that biotype 1A plays a pivotal role in diarrhea [9]. Biotype 1B of *Y. enterocolitica* is also highly pathogenic compared to the other five species and has two structure, including iron absorption and the HPI factor [5].

The chromosomal virulence markers of *Y. enterocolitica* include *ail* (attachment-invasion locus [*ail*] protein), *inv* (invasin), *yst* (*Yersinia*-stable toxin), *myfA* (mucoid *Yersinia* factor), *ymoA* (*Yersinia* modulator). The plasmidial virulence markers of *Y. enterocolitica* include *yadA* (*Yersinia* adhesion), *yop* virulon (*Yersinia* outer membrane proteins), and *virF* (transcriptional activator of the *Yersinia* virulence regulon) [1,2,5,10]. The expression of several plasmid-encoded and chromosomal virulence genes is strongly regulated by temperature, calcium, iron ion concentrations, pH, and osmolality, which play a key role in the incidence of infections [9,10].

Adhesion and invasion factors are essential to the pathogenesis of bacteria. For the penetration of bacteria into the intestinal mucosa, three proteins absorb the process and bind to epithelial cells, including *inv*, *yadA*, and *ail* proteins [2,5]. *Inv* (invasin) is primarily involved in bacterial growth in epithelial cells. *Inv* uses β 1-integrins in the target host cell to strengthen bacterial internalization in the small intestine. *Inv* is found in all *Yersinia* species [5,10].

YadA plasmid protein is produced at the temperature of 37 °C, binds to collagen I, II, and IV and laminin, and protects bacterial colonies in the collagen gel [1,10]. Moreover, *YadA* causes an inflammatory reaction in epithelial cells to produce interleukin 8. It also stimulates cell adhesion and induction of host cell responses, such as cytokine production, auto agglutination, and serum resistance [3, 10]. After the inactivation of *inv*, the *YadA* protein attains invasion ability [5]. The size of *ail* protein is approximately 17 kDa, and it has eight membrane-spanning amphipathic β -strands and four extracellular loops, with the gene only expressed at the temperature of 37 °C and in pathogenic strains [11,12]. The chromosomal gene of *Yst* is considered to be an important factor in *Y. enterocolitica* virulence, which has three types of A, B, and C [1,12].

There are various indications of the *yst* gene. For instance, in laboratory animals, no diarrhea stool samples have been observed after *Y. enterocolitica* infection, while some strains carry the *yst* gene. However, these isolates are not able to produce an enterotoxin, and some of the studies in this regard have denoted that the ratio of enterotoxin bacteria is similar in the clinical and non-clinical isolation [1, 9]. Biotypes 1A and 1B, 2, 3, 4, and 5 of *Y. enterocolitica* produce *YstB* and *YstA* proteins, respectively [5,7].

In the present study, *myf* was the latest gene to be evaluated, which is a chromosomal gene that plays a pivotal role in the onset of infections [12,13], encoding several protein types, such as *myfA*, *myfB*, and *myfC*. However, the exact function of this gene in adhesion and pathogenesis remains unclear [1,5]. *MyfA* has been reported to be the most common gene in the pathogenesis of *Y. enterocolitica*, while it has also been observed in biotype 1A [13].

Molecular techniques have facilitated the identification and characterization of major virulence-associated genes

and proteins in *Y. enterocolitica*. Based on the studies regarding *Y. enterocolitica* and the associated virulence genes in Iran, this is the first study to compare these genes in the samples obtained from various food sources.

Since the ability to determine the pathogenic potential of *Y. enterocolitica* isolates is essential to restricting the spread of yersiniosis, the present study aimed to identify the pathogenic genes of *Y. enterocolitica* isolated from dairy products in a region in Iran.

2. Materials and Methods

2.1. Cheese and Raw Milk Samples

This study was conducted on 200 traditional cheese samples obtained from the northeast of Iran (100 samples from Khorasan Razavi and 100 samples from Golestan provinces) and 100 raw milk samples, which were randomly collected from Mashhad. In total, 38 *Y. enterocolitica* isolates were collected from the cheese samples, and 33 isolates were obtained from the raw milk samples in the northeast of Iran. The samples were evaluated in terms of the presence of six virulence genes.

Initially, 10 grams of each type of cheese and raw milk were transferred to a stomacher bag containing 90 milliliters of Peptone-Sorbitol-Bile (PSB) broth (Sigma-Aldrich, USA) and homogenized for one minute. Afterwards, the samples were incubated at the temperature of 25 °C for 48 hours in a shaker incubator and cultured onto Cefsulodin-Irgasan Novobiocin agar plates (CIN- Merck, Darmstadt, Germany).

2.2. DNA Extraction of the Isolates

DNA extraction was performed using a DNA isolation kit (QIAGEN GmbH-Germany) in accordance with the instructions of the manufacturer.

2.3. Detection of the Virulence-related Genes Using PCR

Polymerase chain reaction (PCR) was performed (total volume: 25 μ L), and primer sequences were used in PCR in order to amplify six virulence genes (*ail*, *inv*, *yadA*, *MyfA*, *ystA*, and *ystB*) (Table 1). PCR reactions were carried out in a thermal cycler (Gradient Model T100, BioRad, USA). The cycling conditions were as follows: five minutes at the temperature of 94 °C; 30 cycles, including 45 seconds at the temperature of 94 °C, 45 seconds at the temperature of 45-61°C, and 45 seconds at the temperature of 72 °C, followed by the final extension for 15 minutes at the temperature of 72 °C or the optimal annealing temperature. At this stage, gradient PCR was performed, and the amplified products were analyzed via electrophoresis on 1.2% agarose gel.

3. Results and Discussion

3.1. *Y. enterocolitica* Virulence Gene Distribution

Tables 2 and 3 show the distribution of the virulence genes of *Y. enterocolitica*. Among the isolates of biotype 1A in the cheese samples, *ystB* was observed to be the most frequent gene (82.60%; 19/23), followed by *inv* (60.86%;

14/23). On the other hand, the *ystA* gene was only positive in two cases, and the other genes (*ail*, *yadA*, and *myfA*) were negative. Moreover, three isolates (14, 15, and 16) were negative for the mentioned genes.

In biotype 1B, *yadA* was the most commonly isolated gene with the prevalence of 92.30% (12/13), followed by *inv* (46.15%; 6/13), *ystA* (30.76%; 4/13), and *myfA* (23.07%; 3/13). On the other hand, *ystB* was negative in all the biotype 1B isolates, and only one isolate (24) was positive for the *ail* gene in biotype 1B. The *myfA* gene was positive in three isolates (26, 27, and 28), while biotype 5 was negative in all the genes, with the exception of one isolates (37), which was positive for *yadA*.

In the raw milk samples, 10 isolates in biotype 1A (1, 2, 8, 9, 10, 11, 12, 13, 24, and 25) were positive for the *ystB* gene. Among the studied isolates, five cases (1, 2, 13, 15, and 18) were positively harboring the *inv* gene. On the other hand, the *ail*, *myfA*, *ystA*, and *yadA* genes were negative in all the isolates of biotype 1A. In addition, 14 isolates of biotype 1A (3, 4, 5, 6, 7, 14, 16, 17, 19, 20, 21, 22, 23, and 26) were negative for all the genes. In biotype 1B, *yadA* was observed to be the most common gene (66.66%; 4/6), while *ail*, *myfA*, and *ystB* were negative in all the isolates of biotype 1B.

According to the results of the present study, all the investigated genes were negative in the isolates of five biotypes. Moreover, the *ail* and *myfA* genes were negative for all the samples that are isolated from raw milk. Figures 1 and 2 depict the distribution diagram of *Y. enterocolitica* biotypes and virulence genes isolated from cheese and raw milk.

In Iran, the pathogenesis of *Y. enterocolitica* and the associated public health concerns have not been addressed thoroughly [14]. *Y. enterocolitica* is mainly found in pigs, and several studies have been focused on its pathogenicity in food, especially dairy products [14-16]. In Iran, it is preferable to consume traditional food products rather than industrial products, especially in case of dairy products [14].

In the current research, 71 isolates of *Y. enterocolitica* were obtained and investigated from two dairy products, including cheese (38 isolates) and raw milk (33 isolates). The analysis of the results of six genes partially confirmed the virulence of *Y. enterocolitica*. As a result, six virulence genes were selected considering their key role in the pathogenesis of *Y. enterocolitica*. To date, several studies have been focused on the presence of these genes in *Y. enterocolitica* [1,2,5,7].

In the present study, most of the genes demonstrated in biotype 1A were also associated with *ystB*, which is consistent with the research by Bancercz Kisiel et al. (2018). In the mentioned study, more than 80% of biotype 1A contained the *ystB* gene. Similar findings have been reported in the studies by Schneeberger et al. (2015), Bonardi et al. (2014), and Peruzy et al. (2017), while lower rates of this gene have been denoted in the studies by Tadesse et al. (2013) and Zheng et al. (2008) [1, 2, 7, 8, 16-18]. In the current research, the *ystB* gene was only found in biotypes 1A, which is in line with the study by Lucero Estrada et al. (2012) conducted on meat products [7, 19]. It is notable that in the study by Peruzy et al. (2017), the gene was not detected in pathogenic strains.

According to the results of the present study, *Y. enterocolitica* isolated from the cheese samples responded better to the *ystB* gene compared to the isolates of raw milk. In addition, biotypes 1B and 5 in the isolates of both products lacked the *ystB* gene, which is in congruence with the results obtained by Peruzy et al. (2017) [7].

Biotype 1B of *Y. enterocolitica* produces *ystA* as a heat-stable enterotoxin, and the gene is often detected in biotype 1B. This is consistent with the findings of the current research, as well as the results obtained by Tadesse et al. (2013) and Ramamurthy et al. (1997) [17, 20]. In the present study, the *ystA* gene was observed in biotype 1A, which is consistent with the results of the previous studies in this regard [20,21]. In the research by Lucero Estrada et al. (2012), the biotype 1A isolated from meat products was reported to carry the *ystA* gene, while in the current research, the *ystA* gene was detected in the cheese isolates in biotype 1A, and the isolates of raw milk lacked the *ystA* gene [19]. Furthermore, more isolates of biotype 1B in the cheese samples showed the *ystA* gene more frequently than the raw milk samples although the rate was slightly lower compared to the previous studies in this regard [7,22].

The *Inv* chromosomal gene is responsible for the expression of the *INV* protein, which is involved in invasion mechanisms and is observed in most biotypes of *Y. enterocolitica* [1,2]. In the present study, the *inv* gene was isolated more from the biotype 1A of the cheese samples (60.83%), which is in congruence with the study by Peruzy et al. (2017) despite the lower rate [7]. However, in the study by Thoerner et al. (2003), the *inv* gene was not detected in biotype 1A [10].

Table 1: Primers sequences used for polymerase chain reaction (PCR) to amplify six virulence genes

Genes	Annealing temperature (°C)	Forward and Reverse	Primers sequences (5'→3')	Amplicon size (bp)	Reference
<i>ail</i>	57	F R	ACT CGA TGA TAA CTG GGG AG CCC CCA GTA ATC CAT AAA GG	170	[3]
<i>inv</i>	61	F R	TGCCTTGGTATGACTCTGCTTCA AGCCACCACTACTGGTGGTTAT	1144	[8]
<i>yadA</i>	60	F R	TAAGATCAGTGTCTCTCGGC TAGTTATTTGCGATCCCTAGCAC	747	[18]
<i>myfA</i>	60	F R	CAG ATA CAC CTG CCT TCC ATC T CTC GAC ATA TTC CTC AAC ACG C	272	[18]
<i>ystA</i>	60	F R	AAT GCT GTC TTC ATT TGG AGC A ATC CCA ATC ACT ACT GAC TTC	145	[35]
<i>ystB</i>	45	F R	GTA CAT TAG GCC AAG AGA CG GCA ACA TAC CTC ACA ACA CC	146	[35]

Table 2: Characteristics of virulence genes in *Y. enterocolitica* isolates from cheese

References of the isolates (No)	Biotypes	Genotypic virulence genes					
		<i>ail</i>	<i>inv</i>	<i>yadA</i>	<i>myfA</i>	<i>ystA</i>	<i>ysfB</i>
1		-	+	-	-	-	+
2		-	+	-	-	-	+
3		-	+	-	-	-	+
4		-	+	-	-	-	+
5		-	(+)	-	-	-	+
6		-	+	-	-	-	+
7		-	-	-	-	-	+
8		-	+	-	-	-	+
9		-	-	-	-	-	+
10	1A	-	+	-	-	-	+
11		-	-	-	-	-	+
12		-	-	-	-	(+)	+
13		-	-	-	-	(+)	+
14		-	-	-	-	-	-
15		-	-	-	-	-	-
16		-	-	-	-	-	-
17		-	-	-	-	-	(+)
18		-	+	-	-	-	-
19		-	+	-	-	-	+
20		-	+	-	-	-	+
21		-	+	-	-	-	+
22		-	+	-	-	-	+
23		-	+	-	-	-	+
24		+	-	-	-	+	-
25		-	+	+	-	-	-
26		-	+	(+)	(+)	(+)	-
27		-	+	(+)	(+)	(+)	-
28		-	+	+	(+)	+	-
29		-	+	+	-	-	-
30	1B	-	(+)	+	-	-	-
31		-	-	+	-	-	-
32		-	-	+	-	-	-
33		-	-	+	-	-	-
34		-	-	+	-	-	-
35		-	-	+	-	-	-
36		-	-	+	-	-	-
37	5	-	-	(+)	-	-	-
38		-	-	(+)	-	-	-

* Negative, ** Positive, *** Weak positive

The *inv* gene was also detected in biotype 1B of both products in the studies by Momtaz et al. (2013), Schneeberger et al. (2015), and Peruzy et al. (2017), while this finding is inconsistent with the research by Thoerner et al. (2003) [2,7,8,10, 23]. In the study by Bonardi et al. (2018), all the biotypes 1A isolated from raw milk carried the *inv* gene, while in the current research, the biotypes 1A isolated from raw milk carried a lower percentage of the *inv* gene (17%) [1].

The *yadA* gene is plasmid and absent in biotype 1A (pYV) [1]. According to the results of the present study, biotype 1A isolates showed no *yadA* gene in neither of the studied dairy products; this finding is in line with the study by Peruzy et al. (2017) in various food and human food samples. In contrast, only one case was observed in the study by Thoerner et al. (2003), and the difference could be due to the fact that the strains were isolated from human specimens, as well as the difference in geographical regions [2,7,10]. On the other hand, the biotype 1B isolated from both products contained the *yadA* gene, and the *yadA* gene was positive in the biotype 5 isolates of the cheese samples, while it was negative in the isolates of the raw milk samples.

According to the results of the present study, all the biotypes 1A isolated from both products were negative in terms of the *myfA* gene, which is similar to the biotypes 1A isolated from meat products in the study by Lucero Estrada

et al. (2012). On the other hand, in the study by Bonardi et al. (2018), two isolates of the biotype 1A of raw milk carried the *myfA* gene [15, 19]. The *myfA* gene is most often found in pathogenic strains, while in the studies by Bhagat et al. (2007) and Peruzy et al. (2017), biotype 1A was reported to be positive [7,13]. In another study by Kot et al. (2017), biotype 1A isolated from the fecal samples collected from children harbored the *myfA* gene [24], and the gene was positive in biotype 1B of cheese (20.07%), while it was not detected in the isolates of raw milk or any of the biotype 5. Other studies have confirmed that the clinical sources of *Y. enterocolitica* contain more of the *myfA* gene compared to the non-clinical sources [25]. In the present study, only three out of 71 isolates of *Y. enterocolitica* were positive for the *myfA* gene.

The *ail* gene is often diagnosed in biotype 1A [1,26]. According to the findings of the current research, none of the biotypes 1A were positive for the *ail* gene, which is in contrast to the studies by Cheyne et al. (2009) and Lucero Estrada et al. (2012) [19, 26]. Similarly, Fois et al. (2018) reported that *Y. enterocolitica* isolated from raw milk did not contain the *ail* gene [4]. In contrast, some studies have denoted that most isolates harbored this gene [10,27, 28]. In another research conducted in the northwest of Iran, the isolates of *Y. enterocolitica* from raw milk and cheese samples contained 2.26% and 4% *ail* gene, respectively [14].

The results of the present study indicated that the *myfA* and *ail* genes were negative in all the raw milk samples, which indicated the possible similarity of these genes. In the study by Bhagat and Viridi (2007), a correlation was reported between the *myfA* and *ystB* genes, and this finding is inconsistent with the current research [13]. In the present study, the *ystB* gene had the highest isolation rate, while the *ail* gene had the lowest isolation rate since the *Y. enterocolitica* strains of biotype 1A have *ystB* and biotypes 1B, 2, 3, 4, and 5 have *ystA* [1].

The *ystA* gene is found in pathogenic biotypes and was only detected in two cases of the biotype 1A of the cheese

samples in the present study. This finding is in line with the studies by Ramamurthy et al. (1997) and Huovinen et al. (2010), which were conducted on cheese and milk products, respectively. This gene has various mechanisms of epithelial cell invasion; as such, the biotypes are considered to be pathologic [1, 20, 29]. In the current research, the *yadA* gene was not detected in any of the biotype 1A isolates, which is in congruence with the findings of Mantle and Husar (1993) in milk samples [30]. Therefore, it could be concluded that pathogenic diseases cannot be determined based on the serotype alone, and further examinations are required regarding the pathogenic genes of *Y. enterocolitica*.

Table 3: Characteristics of *Y. enterocolitica* isolates from raw milk

References of the isolates (No)	Biotypes	Genotypic virulence genes					
		<i>ail</i>	<i>inv</i>	<i>yadA</i>	<i>myfA</i>	<i>ystA</i>	<i>ystB</i>
1		-*	+**	-	-	-	+
2		-	+	-	-	-	+
3		-	-	-	-	-	-
4		-	-	-	-	-	-
5		-	-	-	-	-	-
6		-	-	-	-	-	-
7		-	-	-	-	-	-
8		-	-	-	-	-	+
9		-	-	-	-	-	+
10	1A	-	-	-	-	-	+
11		-	-	-	-	-	+
12		-	-	-	-	-	+
13		-	+	-	-	-	+
14		-	-	-	-	-	-
15		-	+	-	-	-	-
16		-	-	-	-	-	-
17		-	-	-	-	-	-
18		-	+	-	-	-	-
19		-	-	-	-	-	-
20		-	-	-	-	-	-
21		-	-	-	-	-	-
22		-	-	-	-	-	-
23		-	-	-	-	-	-
24		-	-	-	-	-	+
25		-	-	-	-	-	+
26		-	-	-	-	-	-
27		-	-	-	-	-	-
28		-	+	+	-	-	-
29	1B	-	+	+	-	(+) ^{***}	-
30		-	-	(+)	-	(+)	-
31		-	-	+	-	-	-
32		-	-	-	-	-	-
33	5	-	-	-	-	-	-

* Negative, ** Positive, *** Weak positive

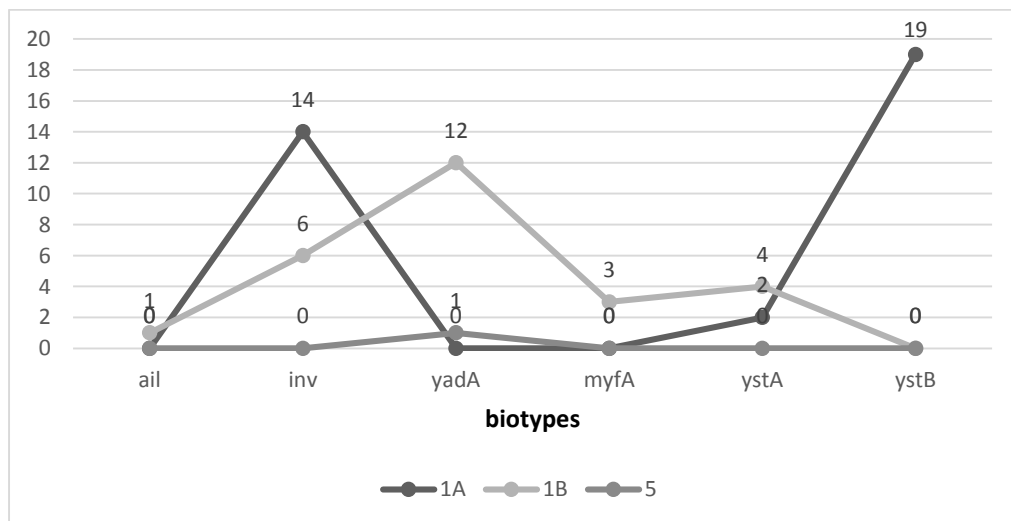


Figure 1: Strains sorted by biotype and genes in cheese

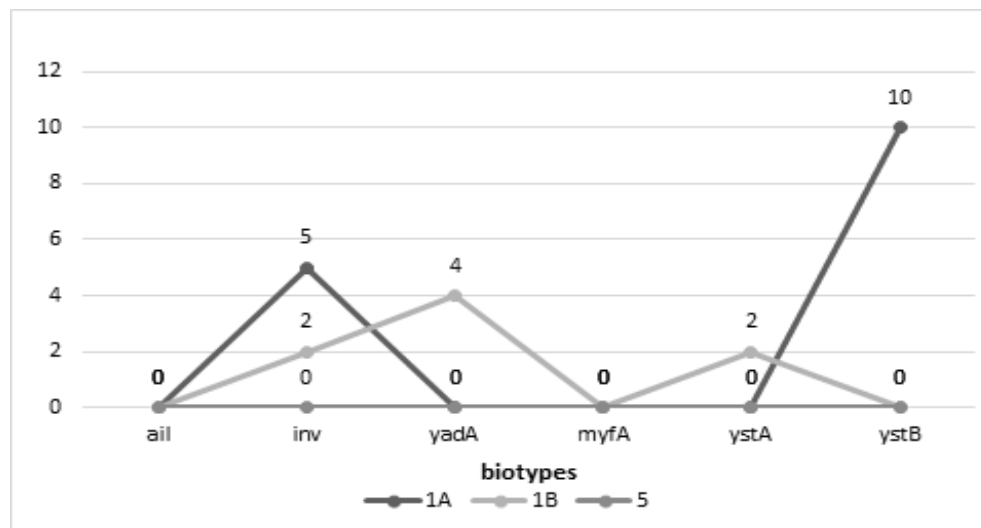


Figure 2: Strains sorted by biotype and genes in raw milk

4. Conclusion

According to the results, the prevalence of *Y. enterocolitica* virulence genes was relatively high and had the potential to be transmitted to humans through cheese and other dairy products, especially non-pasteurized milk. Moreover, *Y. enterocolitica* isolates had higher gene variability in cheese. In all the isolates, the lowest rate was observed in the *ail* gene, followed by the *myfA* gene. On the other hand, the *ystA* gene was detected in both biotypes and could be a good indicator for the detection of *Y. enterocolitica* virulence in these regions.

Authors' Contributions

M.T., and F.M., designed and conducted the study; M.T., drafted the manuscript; A.A., and A.J., supervised data analysis and edited the manuscript.

Conflict of Interest

None declared.

Acknowledgments

Hereby, we extend our gratitude to Mashhad University of Medical Sciences in Mashhad, Iran for the financial support of this research project. The study protocol was approved by Mashhad University of Medical Sciences. (Project No. 970968).

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