

Prevalence, Detection Methods and Antimicrobial Susceptibility of *Listeria monocytogenes* Isolated from Milk and Soft Cheeses and its Zoonotic Importance

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Abstract: The present study was carried out to detect *Listeria monocytogenes* prevalence by conventional isolation, colony PCR and enrichment PCR methods in 200 dairy product samples (50 each of market raw milk, bulk tank milk, Damietta cheese and kareish cheese), as well as 120 human specimens. While conventional biochemical tests revealed that 36% (72/200) of the dairy products were positive for *L. monocytogenes*, only 14% (28/200) of these products were positive for *L. monocytogenes* using colony PCR method. Enrichment PCR method, however, indicated that 23% (46/200) of samples tested were confirmed as *L. monocytogenes*. Only 5% (2/40) of stool specimens from apparently healthy dairy handlers were positive for *L. monocytogene* by enrichment PCR method which is proven to be more precise and fast. The fifty four *L. monocytogenes* strains detected by colony PCR method were multidrug resistant. Pasteurization should be applied to milk and milk used for cheeses manufacture. There is a need of continuous surveillance and more prudent use of antibiotics in human and dairy farms.

Key words: *Listeria monocytogenes* • Hly_a Gene • Antimicrobial Susceptibility

INTRODUCTION

Food safety is a complex issue that has an impact on all segments of the society, from the public to government, industry and academia. Diseases caused by foodborne pathogens constitute a worldwide public health problem. Listeriosis; a foodborne disease, has been considered to be an emerging zoonotic disease worldwide.

Listeriosis caused by *Listeria monocytogenes* that is a ubiquitous bacterium, widely distributed in many natural and man-made environments where it can survive for long periods of time [1]. It has been isolated from a wide variety of ready to eat (RTE) foods, dairy products specially soft cheeses and unpasteurized milk, which are typically foods associated with *L. monocytogenes* contamination [2- 5]. Outbreaks of *L. monocytogenes* infection associated with consumption of milk and dairy products have been reported [6-8].

Since the first reported case of Listeriosis in 1953 after a stillbirth was linked to mothers' consumption of raw milk from a cow suffering from mastitis [1], it is known that aged people, pregnant women or immunocompromise people are considered risk groups [9, 10]. Listeriosis commonly associated with febrile gastroenteritis in immunocompromise groups [11- 13]. While, healthy people and non-pregnant women are rarely suffer from life threatening clinical illness as a result of exposure to *L. monocytogenes* in food [14]. Such clinical illness is characterized by aches, fatigue, fever, chills, nausea, vomiting and diarrhea, it is generally self-limiting.

In terms of microbiological criteria, food safety requires the reliable detection of pathogens such as *L. monocytogenes* along the food chain by appropriate analytical methods. Microbiological specifications for food items including milk and dairy products often stipulate absence of *L. monocytogenes* in 25 g of food

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sample [15]. Subsequently, pre-enrichment and enrichment steps are therefore necessary to determine stressed with low detection level organism [15]. Numerous enrichment and isolation media have been developed for isolation of *L. monocytogenes*, Oxford agar is a specified plating medium in the FDA/BAM isolation procedure [16]. Standard methods for detection of *L. monocytogenes* rely on cultivation, nevertheless presumptive *Listeria* colonies take 3-4 weeks before species identification is possible [17] thus rapid analytical methods other than conventional one should be applied.

In Egypt, lack of simple, rapid and sensitive isolation and identification procedure for *L. monocytogenes* make many questions regarding the epidemiology of the disease, the extent of food contamination and the importance of food borne route of transmission remain unanswered. Also, due to the increased multidrug resistance in the foodborne pathogen due to increase the use of antimicrobials in feeds for the control and treatment of diseases in animals, this work was carried out to study the prevalence and the antimicrobial susceptibility of *L. monocytogenes* isolated from milk, soft cheeses, stool specimens of diarrheic patients, as well as stool and hand swabs of apparently healthy dairy handlers.

MATERIALS AND METHODS

Collection of Samples

Milk and Soft Cheese Samples: A total of 200 samples (50 each of market raw milk, bulk tank milk, Damietta cheese and Kareish cheese) were collected from dairy farms, groceries, retail outlets, different shops and supermarkets in Mansoura city, Egypt. All samples were aseptically collected and transferred into individual sterile bags or flasks then transported to the laboratory in insulated coolers containing cold packs and were analyzed immediately.

Human Samples: A total of 120 human samples representing 40 each of stool specimens from diarrheic patients (attending the outpatient clinic of Gastrointestinal System Diseases Medical Centre, Faculty of Medicine, Mansoura University, Mansoura, Egypt), apparently healthy dairy handlers and hand swabs from dairy handlers from different dairy farms and shops in Mansoura city. All specimens from human sources were immediately transferred into sterile buffered peptone water (BPW) tubes under aseptic condition. The tubes were labeled then ice packed and transferred immediately to the laboratory.

Preparation of Samples: Twenty five ml or g of each dairy sample were aseptically added to 225 ml of 0.1% bacteriological peptone and mixed well. In the case of soft cheese, samples were homogenized in a blender as required for thorough mixing, the homogenate was then incubated at 30°C for 24 h. Collected swabs of human samples were incubated in BPW 30°C for 24 h.

Conventional Method for Isolation and Identification of *L. Monocytogenes*:

The isolation of *L. monocytogenes* is adopted according to Roberts *et al.* [15]. Ten ml of the incubated homogenate were added to 90 ml of *Listeria* enrichment broth base (CM862, Oxoid) with *Listeria* selective enrichment supplement (Nalidixic acid, cycloheximide, Acriflavine hydrochloride) (SR141, Oxoid) and incubated for 48 h at 30°C. Thereafter, the homogenate was streaked onto Oxford agar plates (CM856, Oxoid) supplemented with *Listeria* selective supplement (SR140, Oxoid) and incubated for 48 h at 37°C. Grey-green colonies surrounded by black zones of aesculin hydrolysis were presumed to be *Listeria*.

At most, five presumptive *Listeria* colonies were picked from each selective agar plate. These colonies were purified using Tryptone soya agar (CM131, oxoid) and subjected to biochemical identification. Non-spore forming, Gram positive coccobacilli isolates were tested for catalase and umbrella growth in motility test medium at 25°C. Isolates positive for these tests were further examined for hemolysin production using Blood agar base (CM854, Oxoid) supplemented with 5% sheep blood; the isolated were also tested for fermentation of rhamnose, xylose and mannitol, CAMP test (synergistic lyses of red blood cells) against *S. aureus* [18].

Detection of *L. monocytogenes* hlyA gene by colony PCR:

L. monocytogenes isolates were screened for the presence of Listeriolysin O (*hlyA*) gene. The sequence of the oligonucleotide primer sets were; Forward *hlyA* 634F: 5'-ACTTCGGCGCAATCAGTGA-3' and Reverse *hlyA* 770R: 5'-TTGCAACTGCTCTTTAGTAACAGCTT-3'. A single colony was picked to grow on Oxford agar plate and incubated at 30°C for 48 h. Subsequently 2-4 colonies from each plate were randomly selected using a sterile toothpick. Colonies were suspended in 50 µl distilled water and incubated at 90°C for 5 min. Following centrifugation at 13 000 rpm/1min, direct colony PCR of the supernatant was performed with the Dreem Tag Green PCR Master Mix (Fermentas). Cycling conditions were initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 20 s., 60°C for 30 s. and 72°C for 50 s., with a

final extension at 72°C for 7 min. PCR products were visualized using ethidium bromide stained 1.2 % agarose gel electrophoresis. The separated PCR products were then visualized under UV light and photographed.

Enrichment PCR Method for Detection of *L. monocytogenes* hlyA Gene: DNA was extracted by thermal cell lysis of suspended bacteria from enrichment broth. After the enrichment of milk (25 ml) or cheese (25 g) in BPW (225 ml) for 24 h at 30 C°, 15 ml of the enrichment was transferred to centrifugal tube and centrifuged at 4000 rpm for 10 min. The supernatant was discarded and the harvested cell pellet was re-suspended in 1 ml sterile distilled water, transferred into 1.5 centrifuge tube and centrifuged for 10 min at 14,000 g. The supernatant was then discarded carefully and the pellet was re-suspended in 100 µl of sterile distilled water by vortexing. The tubes were centrifuged again at 14,000 g for 10min and the supernatant was discarded carefully. The pellet was re-suspended once again in 100 µl of sterile distilled water by vortexing and put in heat block at 95°C for 15 min. After heat treatment, the cell debris was pelleted by centrifugation at 14,000 g for 10 min. The supernatant containing the DNA transferred into a new microcentrifuge tube yielded a volume of 40 to 60 µl, which stored at -20°C until PCR assay was performed. An aliquot of 2 µl of the supernatant was used as the template DNA for PCR analysis to detect *hlyA* gene using the same primer set and PCR cycling protocol that applied for the colony PCR.

Antimicrobial Susceptibility Testing: Antibiotic sensitivity of the isolates was performed according to agar disc diffusion method on Mueller-Hinton agar [19]. The following antimicrobial discs were applied: penicillin G (P/10 IU), tetracycline (TE/30 µg), streptomycin (S/10 µg), cloxacillin (OB/5 µg), rifampicin (RD/5 µg), gentamicin (CN/10 µg), chloramphenicol (C/30 µg), ciprofloxacin (CIP/5µg.), amikacin (AK/30 µg), sulphamethazole/trimethoprim (SXT/25 µg), amoxicillin (AML/10 µg), netilmicin (NET/30 µg), vancomycin (VA/30 µg). Strains were evaluated as susceptible, intermediate or resistant. Multiple antibiotic resistances (MARs) index for each resistant pattern was calculated by the formula given by Singh *et al.* [20].

MAR index= Number of resistance (Isolates classified as intermediate on the basis of inhibition zone were considered as sensitive for MAR index) antibiotics/ Total number of antibiotics tested.

RESULTS AND DISCUSSION

Prevalence of *L. Monocytogenes* in Milk and Soft Cheese Samples: In this study, the conventional biochemical tests for identification of *L. monocytogenes* indicated that 36% of all the examined samples (58% of market raw milk, 36% of Bulk tank milk, 28% of Damietta cheese and 24% of Kareish cheese) were positive for *L. monocytogenes*. A total of 109 colonies that were suggested biochemically to be *L. monocytogenes* were further genetically confirmed by colony PCR. Colony PCR method confirmed the presence of *L. monocytogenes* in 14% (28/200) among all milk and soft cheese samples tested with a prevalence of 24% (12/50), 8% (4/50) and 24% (12/50) in market raw milk, Bulk tank milk and Damietta cheese, respectively, while it couldn't be detected in any of Kareish cheese tested. With the use of enrichment PCR method, however, *L. monocytogenes* was detected in 23% (46/200) among all milk and soft cheese samples tested with prevalence of 24% (12/50), 8% (4/50), 28% (14/50) and 32% (16/50) in market raw milk, Bulk tank milk, Damietta cheese and Kareish cheese samples, respectively (Fig. 1). Interestingly, all positive samples of Kareish cheese by enrichment PCR method were negative by colony PCR method.

Several studies were conducted in different countries to screen milk and soft cheeses for the presence of *L. monocytogenes*. A study in Uganda has identified *L. monocytogenes* at very high prevalence (60%), in raw milk [5]. Another study in Ethiopia revealed that 13% of raw milk and 1% of cottage cheese were positive for *L. monocytogenes* [21]. In USA, 12.6% of milk filters were positive for *L. monocytogenes* [22]. Other studies revealed different prevalence of *L. monocytogenes* in raw milk. For instances, it was 21.7% in Iran [23], 6.3% in Ireland [24], 5.5% in Finland [25] and 1.9% in Portugal [3]. Nonetheless *L. monocytogenes* could not be detected in any of raw milk samples tested in Austria [26], Turkey [27] and Brazil [28].

Although *L. monocytogenes* could not be detected by colony PCR method in Kareish cheese tested in the present study, Damietta cheese samples exhibited a high prevalence rate of 24% for *L. monocytogenes*. Lower prevalence rates, however, were reported in soft cheese samples in Brazil (9.6-13.3%) [29], Mexico (6-12%) [30], Jordan (11.1%) [31], while it could not be detected in any of the cheese samples in Austria [26], Turkey [27] and Portugal [3].

Prevalence of *L. monocytogenes* in Human Samples:

Regarding to the examined human samples, only two out of 40 (5%) stool specimens from apparently healthy dairy handlers was positive for *L. monocytogenes*, while it could not be detected in both stool specimens from diarrheic patients and hand swabs from dairy handlers. Most cases of Listeriosis arise from the ingestion of contaminated foods and food products [32]. In spite of the wide exposure of this organism, little is known about the provenance of *L. monocytogenes* in human stool and it is not known whether human fecal dispersal contributes to human foodborne transmission. The obtained results clearly explain that *Listeria* are not enteropathogenic organism, nevertheless, they belong to the intestinal flora as transient or resident flora [33]. Presence of *L. monocytogenes* in stool specimens of apparently healthy dairy workers is a very important and complex issue for microbiological risk assessment and may be in drastic healthy problems and economic losses for the industry [34]. Therefore, we cannot ignore the importance of the asymptomatic carrier workers who can contaminate milk and dairy products during unhygienic handling.

Detection of *L. monocytogenes* by Conventional Method:

Milk and soft cheeses are highly perishable food that may incriminated in *L. monocytogenes* infections, therefore a rapid and accurate method for detection should be applied. Subsequently, conventional method alone for detection and isolation of *L. monocytogenes* is not adequate method. Taponen *et al.* [35] reported that all common mastitis bacteria can occur in large quantities in clinical mastitis samples that exhibit no growth in conventional culture and can be detected using real-time PCR assay. Makino *et al.* [36] reported that *L. monocytogenes* could be directly detected from food by PCR when its amount $\geq 10^3$ cfu/0.5 g of sample. In our study, using of the enrichment PCR method overcome the negative results may occur due to failure of *L. monocytogenes* to grow on selective medium especially with the presence of other competitors as shown by Colony PCR method, as well as the high detection limit needed in direct sample PCR.

Cheeses are made from both unpasteurized and pasteurized milk. Referring to results of our study, *L. monocytogenes* in cheese showed lower prevalence rates in comparison with that of milk. The survival and growth of *L. monocytogenes* in cheese depends on the intrinsic factors as pH and extrinsic factors as competing micro flora, as well as whether the milk is pasteurized or not. Donnelly [37] and Genigeogis *et al.* [38] reported that

cheese which supports the growth of *L. monocytogenes*, are typically have pH values < 5.6 as cream cheese. Damietta soft cheeses are the most popular variety of soft cheeses consumed by Egyptian people. It was made by clotting the milk with coagulating enzyme in rennet. Once the desired coagulum has been reached, it is cut into small cubes or blocks. The curd may be salted first or formed first then salted by placing cheese in brine and then the unripened cheese is ready for maturation. Damietta soft cheese are consumed as fresh product (a few days to 4 weeks) or as ripened cheese. Therefore, Damietta cheese considers one of the dairy products which support the growth of *L. monocytogenes*. Pasteurization is a key listeriocidal step in controlling *L. monocytogenes* in RTE soft cheese.

On the other hand, unpasteurized cheese with $\text{pH} \leq 4.2$ may present particular challenges with respect to control the growth of *L. monocytogenes*. Many research results support that the inhibition of *L. monocytogenes* may be due to lactic acid content in food [39-42]. Studies dealing with the inhibition of *L. monocytogenes* in cheeses with complex microbial community have shown that there is a significant decrease in the detectability of *L. monocytogenes* [40, 41, 43-45].

Kareish cheese showed the lower prevalence rate among all samples examined in this study, Kareish cheese (acid- curd skim milk cheese) is a kind of soft cheese manufactured in Egyptian villages and it can be considered the main protein supplement consumed by most farmers. Its manufacture is still primitive and unhygienic, a fact that may expose the product to serious contamination. Failure to detect *L. monocytogenes* from Kareish cheese samples by colony PCR method may be attributed to the low pH and other antimicrobial compounds produced by lactic acid bacteria incorporated in Kareish, as well as the complex heavy microbial community. These factors are therefore having a marked effect on the ability of *L. monocytogenes* to survive and grow. Even though, an outbreak in Germany occurs in 2006 due to consumption of a commercial acid curd cheese [46].

PCR Assay for Detection of *L. monocytogenes*: One of the best ways to detect and confirm the pathogen is through the detection of one of the virulence factors, the most virulence factor associated with *L. monocytogenes* is listeriolysin O (LLO) produced by the microorganism [47] encoded by *hlyA* gene [48]. The LLO-encoding gene (*hlyA*) is present only in virulent strains of the species and is required for virulence.

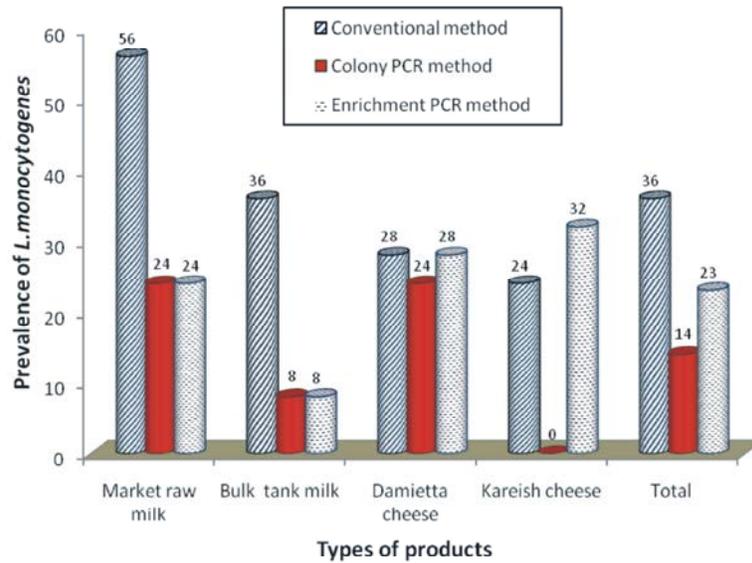


Fig. 1: Prevalence rate of *L. monocytogenes* in milk and soft cheese samples.

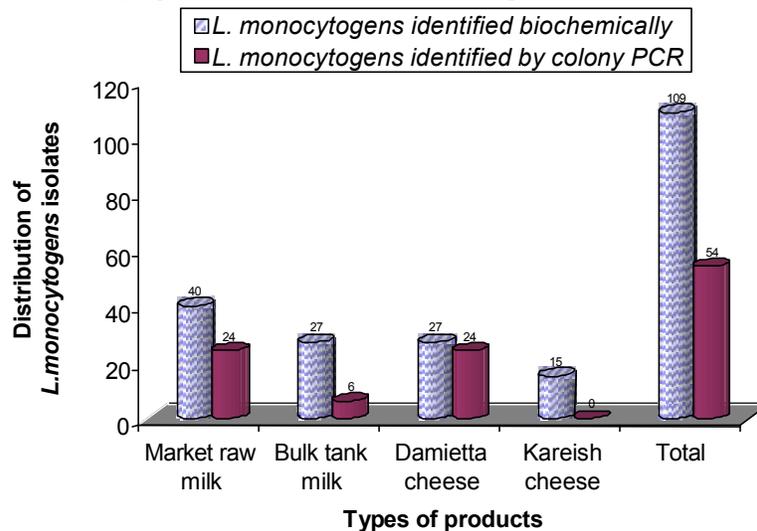


Fig. 2: Distribution of isolated *L. monocytogenes* in milk and soft cheeses

Colony PCR for detection of *hlyA* gene indicated that 24, 8, 24 and 0% of market raw milk, bulk tank milk, Damietta cheese and Kareish cheese samples were positive for such gene (Figure 1) with an overall mean of 14% among the 200 samples tested. Fifty four *L. monocytogenes* isolates (24 isolates derived from market raw milk, 6 from Bulk tank milk and 24 from Damietta cheese) have been identified by colony PCR (Figure 2). The amplified gene was detected at the expected molecular size of 136 bp (Figure 3). Enrichment PCR, however, verified the existence of *hlyA* gene in 24, 8, 28 and 32 % of examined market raw milk, bulk tank milk, Damietta cheese and Kareish cheese samples, respectively (Figure 1, 4) with an overall mean of 23%

among all tested samples. Surprisingly, Kareish cheese samples, which were assumed by colony PCR method to be negative for *L. monocytogenes* exhibited 16 (32%) positive samples for *L. monocytogenes* by enrichment PCR method.

In accordance with the European economic Communities standards [49] which reported that milk and dairy products should be free from *L. monocytogenes* and the U.S Department of Agriculture (USDA) Food Safety Inspection Service (FSIS), which has a zero-tolerance policy, under which RTE products contaminated with *L. monocytogenes* in contact with contaminated surfaces are consider adulterated and may not be distributed in commerce [50], Egyptian Standards [51, 52] designated

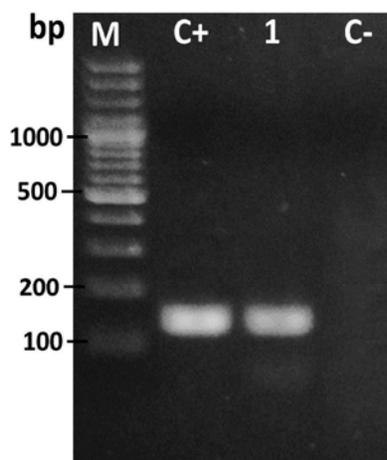


Fig. 3: Representative agarose gel electrophoresis show single PCR of positive strain isolated from examined samples tested with hylA gene primer specific for a suspected *L. monocytogenes* isolates. C-: Control negative, C+: Control Positive, 1 express a sample of isolated *L. monocytogenes* strains.

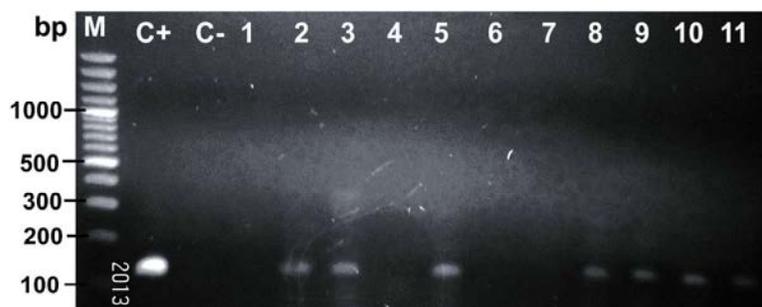


Fig. 4: Agarose gel electrophoresis of hylA gene amplicons specific for *L. monocytogenes* by enrichment PCR method. C+: Control Positive, C-: Control negative, Lanes from 4 to 14 are representative run for PCR results of 11 extracted DNA expressed some examined samples showing positive (lanes 2,3,5,8-11) and negative (lanes 1,4,6,7) amplicon hylA gene.

that raw milk and soft cheeses must be free from *L. monocytogenes*. Our PCR results verified that 24% and 8% of market raw milk, bulk tank milk samples; and 28% and 32% of Damietta cheese and Kareish cheese samples were contaminated with *L. monocytogenes* and hence not fulfill both national and international standards.

National governments should provide more attention to ready-to-eat food as soft cheeses from production time and through their expected shelf life and rapid, specific and accurate protocols for *L. monocytogenes* detection should be provided and applied.

Antimicrobial Susceptibility of Isolated *L. monocytogenes*: The antimicrobial drug susceptibility pattern for the 54 isolated microorganisms from milk and soft cheeses are shown in Table 1. In this study 100% of *L. monocytogenes* isolates show resistance against penicillin G, tetracycline, streptomycin, cloxacillin and

rifampicin, followed by chloramphenicol (81.5%), amoxicillin (81.5%) and vancomycin (81.5%), sulphamethazole/ trimethoprim (70.4%), ciprofloxacin (51.9%), gentamicin (48.2%), amikacin and netilmicin (40.7%). On the contrary to our results, Osaili *et al.* [31] reported that all *L. monocytogenes* isolated from brined white cheese in Jordan were susceptible to gentamicin, vancomycin, sulphamethazole/trimethoprim, erythromycin, tetracycline, rifampicin, but resistant to oxacillin. Likewise, Jamali *et al.* [23] revealed that 49.4% of *L. monocytogenes* isolates from raw cow's milk were resistant to tetracycline and 43.4% were resistant to penicillin G, while all the isolates were susceptible to gentamicin, vancomycin and rifampicin.

The increased antimicrobial administration to animals could be the cause of antimicrobial resistance in *L. monocytogenes* [53]. Multidrug resistant *L. monocytogenes* strains isolated from foods and human

Table 1: Antimicrobial sensitivity pattern of *L. monocytogenes* isolated from milk and soft cheeses

Type of Antimicrobials	No. of <i>L. monocytogenes</i> isolates (n = 54)		
	S (%)	I (%)	R (%)
Penicillin G (10 IU)	-	-	54(100)
Tetracycline (30 µg)	-	-	54(100)
Streptomycin (10 µg)	-	-	54(100)
Cloxacillin (5 µg)	-	-	54(100)
Rifampicin (5 µg)	-	-	54(100)
Gentamicin (10 µg)	12(22.2)	16(29.6)	26(48.2)
Chloramphenicol (30 µg)	10(18.5)	-	44(81.5)
Ciprofloxacin (5 µg)	4(7.4)	22(40.7)	28(51.9)
Amikacin (30 µg)	26(48.2)	6(11.1)	22(40.7)
Sulphamethazole/trimethoprim (25 µg)	12(22.2)	4(7.4)	38(70.4)
Amoxicillin (10 µg)	4(7.4)	6(11.1)	44(81.5)
Netilmicin (30 µg)	28(51.9)	4(7.4)	22(40.7)
Vancomycin (30 µg)	4(7.4)	6(11.1)	44(81.5)

Table 2: Antimicrobial sensitivity profile of *L. monocytogenes* isolated from milk and soft cheeses

No of antimicrobials	No. of isolates	Types of antimicrobials	MAR* index
12	6	2 P10, TE30, S10, OB5, RD5, C30, AML30, VA30, SXT25, CIP5, CN10, AK30 4 P10, TE30, S10, OB5, RD5, C30, AML30, VA30, SXT25, CIP5, CN10, NET30	0.923
11	6	4 P10, TE30, S10, OB5, RD5, C30, AML30, VA30, SXT25, CN10, NET30 2 P10, TE30, S10, OB5, RD5, C30, AML30, VA30, SXT25, CIP5, CN10	0.846
10	22	6 P10, TE30, S10, OB5, RD5, C30, AML30, VA30, SXT25, CIP5 4 P10, TE30, S10, OB5, RD5, SXT25, CIP5, CN10, AK30, NET30 4 P10, TE30, S10, OB5, RD5, C30, AML30, VA30, SXT25, CN10 4 P10, TE30, S10, OB5, RD5, C30, VA30, CN10, AK30, NET30 2 P10, TE30, S10, OB5, RD5, AML30, VA30, SXT25, AK30, NET30 2 P10, TE30, S10, OB5, RD5, C30, SXT25, CN10, AK30, NET30	0.769
9	10	6 P10, TE30, S10, OB5, RD5, C30, AML30, VA30, SXT25 4 P10, TE30, S10, OB5, RD5, C30, AML30, VA30, AK30,	0.692
8	10	6 P10, TE30, S10, OB5, RD5, C30, AML30, VA30 4 P10, TE30, S10, OB5, RD5, AML30, AK30, NET30	0.615
≥ 7	0	0 -	≤ 0.538
Total	54	54 -	1.00

*MAR: Multiple Antimicrobial Resistance index

Listeriosis has been previously reported by Safdar and Amstrong [54] and Marian *et al.* [55]. The overall frequencies and patterns of resistance can vary remarkably from one country to another.

Interestingly, 100% of *L. monocytogenes* strains isolated in this study from milk and soft cheeses were multidrug resistant (Table 2), which indicated that *L. monocytogenes* isolates resist at least 8 of the 13 antimicrobials used. This result indicated that there is a seriously misused of antimicrobials and there is a real threat for public health, which suggested the need of continuous surveillance and more prudent use of antibiotics.

CONCLUSION

This study indicate that raw milk and soft cheeses sold in Mansoura markets may be considered as a

threat to consumers since they are contaminated with *L. monocytogenes* organisms which show high multiple antimicrobial resistance, so, they should be treated as RTE food in which the growth of *L. monocytogenes* can occur. Pasteurized milk and soft cheeses made from pasteurized milk should be only permitted to be sold in markets. The study also suggested the need of continuous surveillance and more prudent use of antibiotics. Risk assessment should be applied at various levels along food production chain which reflects the current state of knowledge about the contamination of foods with *L. monocytogenes* and rates of Listeriosis. These need an accurate and reliable method for detection of this pathogen. Molecular diagnostic technique used in this study is proven to be highly sensitive, precise and fast. Additional educational approaches for dairy farmers, farm visitors and stock handlers are necessary.

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