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PUBLIC HEALTH RISK OF *LISTERIA MONOCYTOGENES* IN RAW MILK IN EGYPT: VIRULENCE GENES, ANTIBIOTIC-RESISTANCE AND HIGH-RISK CONSUMPTION PRACTICES

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ABSTRACT

Listeria monocytogenes is an important food-borne pathogen with serious complications in humans. Despite of its public health importance, studies on *L. monocytogenes* are scarce in Egypt. This study aimed to investigate the public health risk of *L. monocytogenes* in raw milk with regard to prevalence, virulence, antibiotic resistance and high-risk consumption practices. A total of 200 milk samples were collected and *L. monocytogenes* was detected in 2% of all samples. On species level, *L. monocytogenes* was isolated from 4% of sheep, 2% of goats, 2% of cows but not from camel milk samples. Molecular analysis showed that *inlA*, *inlB* and *hlyA* virulence genes were detected in 100%, 75% and 75% of the isolates, respectively. All isolates were multi-drug resistant (MDR) to at least 4 antibiotics. Livestock owners' responses with regard to their consumption practices revealed that 62.1% of respondents consume raw milk, 100% of them don't boil milk before dairy processing and 27.1% of them were willing to drink milk from morbid animals. All of these practices are considered of high-risk for transmission of milk-borne pathogens. The high rates of *L. monocytogenes* detection in milk imply unhygienic milk production. The virulence and antibiotic resistance profiles of isolates may pose a potential high risk for the milk consumers in the study region. Continuous monitoring of virulence and antibiotic resistance traits of *L. monocytogenes* pathogens is necessary for accurate risk evaluation and designing of preventive measures in Egypt. Public health education campaigns regarding hygienic milk production and risks of milk-borne pathogens are urgently needed for improving the awareness and reduce the high-risk consumption practices in residents of the study region.

KEYWORDS: Antibiotic Resistance, *L. Monocytogenes*, Public Health, Raw Milk, Virulence Genes

INTRODUCTION

Listeria monocytogenes is a major human bacterial foodborne pathogen that is ubiquitous in nature and has a wide host range (Painter and Slutsker, 2007). Listeriosis is a serious disease in humans with a case fatalities rate of up to 30% (Kasalica et. al. 2011). The disease usually associated with mild gastroenteritis and fever, however serious manifestations may also occur especially in pregnant women causing abortions or premature births, in newborns causing death, sepsis or meningitis and in older or immunocompromised people causing encephalitis or septicemia (Painter and Slutsker, 2007;

Scallan et al. 2011). Listeriosis accounts for 1600 cases, 260 deaths in United States each year (Scallan et al. 2011). The pathogen is resistant to diverse extreme environmental and food processing conditions hence it is a common contaminant in dairy environment. High rates of *L. monocytogenes* isolation in milk usually associated with unhygienic milk production (Osman et al. 2014). Many outbreaks of Listeriosis were associated with consumption of raw milk and dairy products worldwide (Kasalica et. al. 2011). The prevalence rates of *L. monocytogenes* in milk ranged from 2.5 to 6% based on several studies worldwide (Kasalica et. al. 2011). In Egypt, studies on milk-borne *L. monocytogenes* are limited, however few studies detected *L. monocytogenes* in raw milk of different species at rates of 1.5% to 6% (Ahmed et al. 2014; Osman et al. 2014; Abd El-Tawab et al. 2015). This study was conducted in Marsa Matrouh governorate in northwest coast of Egypt as one of the richest governorates in numbers of sheep, goat and camel herds in Egypt. The aim of this study is to investigate the potential public health risk of *L. monocytogenes* in raw milk through; (1) estimation of *L. monocytogenes* prevalence in raw milk collected from sheep, goat, cow and camel, (2) detection of virulence and antibiotic resistance profiles of the isolated pathogens and (3) surveying the high-risk consumption practices of livestock owners.

MATERIAL AND METHODS

Study Area and Design

This study was conducted in Marsa Matrouh governorate located in the north west coast of Egypt (31°20′N and 27°13′E). The region contained around 20% of sheep and goat population in Egypt and produces an average of 5000 tons of bovine milk annually (Statistics of livestock, 2011). In addition, Marsa Matrouh governorate harbor the second largest population of camels in Egypt (Statistics of livestock, 2011). There are no formal dairy farms in this region, as all livestock is owned by the private sector (Statistics of livestock, 2011). Majority of the livestock owners in the region belonged to Bedouin tribes who are ruled by old inherited traditions and habits. A cross-sectional study was conducted to investigate the prevalence, virulence and antibiotic resistance of *L. monocytogenes* in apparently healthy raw milk sample collected from different animal species in Marsa Matrouh governorate, Egypt. In addition, survey of high-risk consumption practices by livestock owners was conducted by questionnaires.

Sampling

A total of 200 milk samples were collected from 50 sheep, 50 goats, 50 cows and 50 camels. Apparently healthy raw milk samples (i.e. no blood, flakes or precipitates by strip cub test) were collected under aseptic condition as a composite milk sample per animal. In addition, a total of 59 livestock owners were interviewed using a structured questionnaire for survey of high-risk practices. The questionnaire was designed and piloted by interviewing few livestock owners then revised. After revision, the questionnaire contained all the questions illustrated in Table (4) was used for interviewing all livestock owners.

Isolation and Identification of *L. Monocytogenes*

Isolation of L. monocytogenes was conducted according to the International Organization for Standards (ISO 11290-1, 1996). In brief, milk samples were pre-enriched at rate of 1:9 in Half Fraser broth (Oxoid) and incubated at 30^{o}C for 24 hr. A 0.1 ml inoculum was transferred into 10 ml Fraser broth (Oxoid) and incubated for 48 hr. at 37^{o}C. An inoculum from the broth was spread on PALCAM agar (Oxoid) mixed with selective supplement contained 10mg Polymyxin B, 5mg Acriflavine HCl and 20mg Ceftazidime (Oxoid). The plates were incubated at 37^{o}C for 24 hr. the characteristic colonies were picked and purified by culturing on Tryptone Soya Yeast Extract agar for 18 hr. at 37^{o}C.

Identification of L. monocytogenes was conducted by microscopic characteristics, biochemical examination using API Listeria system (bioMerieux) and species-specific genome amplification by PCR (Lantz et al. 1994).

Molecular Identification and Virulence Genes Detection

DNA extraction was conducted using QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. PCR mixture used for gene amplification was as follow; 12.5µl EmeraldAmp Max PCR Master mix (Takara, Japan), 1µl (20 pmol) of each primer (Metabion, Germany), 6µl of DNA template and water added up to 25µl reaction volume and The reaction was performed in an standard thermocycler (Applied Biosystems). Specific identification of *L. monocytogenes* was conducted according to Lantz et al., (1994) by amplification of a 553 bp of the species-specific region of *16s rRNA* gene using the following primers; forward primer 5' CCTTTGACCACTCTGGAGACAGAGC 3' and reverse primer 5' AAGGAGGTGATCCAACCGCACCTTC 3'. The cycling conditions were as follow; 94˚C (5min); [94˚C (30sec); 60˚C (45sec); 72˚C (45sec)] x 35 cycles; 72˚C (10min). PCR was utilized for amplification of *inlA*, *inlB* and *hlyA* virulence genes. Primers for *inlA* gene (Liu et al., 2007) were forward primer 5' ACGAGTAACGGGACAAATGC 3' and reverse primer 5' CCCGACAGTGGTGCTAGATT 3' amplifying 800bp product and the cycling conditions were 94˚C (5min); [94˚C (30sec); 55˚C (45sec); 72˚C (45sec)] x 35 cycles; 72˚C (10min). For *inlB* gene (Kirkan et al., 2006) the primers were forward primer 5' CTGGAAAGTTTGTATTTGGGAAA 3' and reverse primer 5' TTTCATAATCGCCATCATCACT 3' amplifying 343bp product and the cycling conditions were the same as for *inlA* gene. A 174bp PCR product size was amplified from *hlyA* gene (Deneer and Boychuk, 1991) using the following primers; forward primer 5' GCATCTGCATTCAATAAAGA 3' and reverse primer 5' TGTCACTGCATCTCCGTGGT 3' and the cycling conditions; 94˚C (5min); [94˚C (30sec); 50˚C (30sec); 72˚C (30sec)] x 35 cycles; 72˚C (10min). The PCR products were separated by electrophoresis on 1.5% agarose gel (Applichem) and photographed by a gel documentation station (Alpha Innotech) (Figure 1).

Antibiotic Resistance Testing

All *L. monocytogenes* isolates were tested using standard disc diffusion method (CLSI, 2013). Nine antibiotic discs (Oxoid) were used as illustrated in Table (2). The breakpoint of inhibition zone diameter for Ampicillin, Erythromycin and Sulfamethoxazole-Trimethoprim were according to the recommendation of EUCAST (2016) for L. monocytogenes. For the rest of antibiotics, the breakpoint for *Staphylococcal aureus* was used according to CLSI, (2013).

RESULTS AND DISCUSSIONS

The overall prevalence of *L. monocytogenes* in collected raw milk samples was 2% (Table 1). Lower prevalences were reported in Egypt (1.57%) by Ahmed et al. 2014 and in Portugal (1.1%) by Almeida et al. (2013), however higher rate was recorded in Moroco (5.9%) by El-Marnissi et al. (2013). The prevalences of *L. monocytogenes* in sheep and goat were estimated as 4% and 2% of milk samples collected in this study, respectively. These prevalences were higher than those reported by Osman et al. (2014) in both sheep's milk (1.4%) and goat's milk (1.9%). Moreover, *L. monocytogenes* was found in 2% of cow's milk, which was lower than Abd El-Tawab et al., (2015) who reported *L. monocytogenes* in 6% of cow's milk samples. None of camel's milk samples showed the existence of *L. monocytogenes*, which agreed with report of Ombarak and Elgabory, (2014). However, *L. monocytogenes* was reported in 1% of camel's milk samples in another study (Alall et al., 2012). Variation in the prevalences of *L. monocytogenes* in this study and other studies in Egypt or elsewhere may be attributed to differences in milk sampling, detection methodologies, animals' husbandry and hygienic

milking conditions. *L. monocytogenes* pathogens are ubiquitously distributed in animals' environment as well as intestinal content. Hence, the relatively high prevalence of *L. monocytogenes* detected in milk samples in this study highlighted the unhygienic milking conditions that usually associated with either poor hygiene of animals' udder or milkers' practices. It also implies a relatively high zoonotic risk for milk consumers in the study area, especially when the majority of the residents owned livestock and consume their animals' milk.

Virulence of *L. monocytogenes* is controlled by many factors, yet *internalins* A and B encoded *inlA* and *inlB* genes, respectively and *Listeriolysin* O encoded by *hlyA* gene remain the major determinants of pathogenicity (Nishibori et al., 1995; Bergmann et al. 2002). These virulence genes are responsible for intestinal adhesion, invasion and systemic spread of the pathogen within host (Nishibori et al., 1995; Bergmann et al. 2002). Molecular detection of *inlA*, *inlB* and *hlyA* virulence genes in the isolated L. monocytogenes revealed that all isolate (100%) contained at least 2 of the 3 examined genes by PCR (Table 3 and Figure 1). The *inlA*, *inlB* and *hlyA* genes were reported in 100%, 75% and 75% of examined isolates, respectively. All the three virulence genes were previously reported in virulent *L. monocytogenes* isolated from milk, dairy products and human patients (Jaradat et al. 2002; Osman et al. 2014; Abd El-Tawab et al. 2015). In agreement with this study, L. monocytogenes isolates that lacked *hlyA* gene was previously reported by Nishibori et al., (1995). This finding indicates that the common use of *hlyA* gene for detection of L. monocytogenes is not suitable for accurate epidemiological investigation and it should be replaced by detection of the conserved species-specific *16s rRNA* gene for detection of the pathogen. Bergmann et al. (2002) reported that *L. monocytogenes* isolates defective in *inlB* gene showed reduced activity of *inlA*-dependent invasion of human tissues, while lacking *hlyA* gene resulted in loss of virulence in *L. monocytogenes* isolates in another study (Nishibori et al., 1995).

In the last decades, *L. monocytogenes* showed an emerging increase in resistance to multiple drug types (Pesavento et al. 2010). The results of antibiotic resistance (Table 2 and 3) showed that all *L. monocytogenes* isolates were multi-drug resistant for at least 4 antibiotics (100%). All isolates were resistant to Ampicillin, Cephalothin and Sulfamethoxazole-Trimethoprim and 75% of them showed resistance for Erythromycin and Tetracycline. Resistance for Cephalothin was reported by Osman et al. (2014), while resistance for other antibiotics at varying degrees was demonstrated by Pesavento et al. (2010). However sensitivity for most of these antibiotics was previously reported (Altuntas et al. 2012; Osman et al. 2014). Ampicillin, Gentamicin and Sulfamethoxazole-Trimethoprim are the drugs of choice for treatment of Listeriosis in human cases (Pesavento et al. 2010; Altuntas et al. 2012). Despite the retained sensitivity for Gentamicin by all isolates (100%), resistance to two (Ampicillin and Sulfamethoxazole-Trimethoprim) of three drugs of choice is considered an alarming public health risk.

Lack of correct knowledge about disease transmission may result in spread of high-risk practices (Jayarao et al., 2006; Hegazy et al., 2016). Livestock owners' responses revealed that 40% of them denied the possible transmission of food-borne pathogens in milk and dairy products and further 20% did not know the correct answer. This means that around 60% of participants in this study lacked correct information regarding milk-borne pathogens. The low awareness rate reported by participant in this study (40%) was much lower than other reports that recorded awareness of milk-borne diseases by over 90% of the participants in Nile-Delta region of Egypt (Holt et al., 2011) and over 60% of participants in USA (Jayarao et al., 2006). As an expected result of high rate of lack of awareness, majority of the participant consume raw milk (62.1%), none of them boil milk before dairy processing (100%) and around one third (27.1%) of them were willing to drink milk from morbid animals even without boiling (62.5%). All of these practices are considered of high-risk

for transmission of milk-borne pathogens (Jayarao et al., 2006; Holt et al., 2011) especially when milk harbor dangerous pathogens as *L. monocytogenes*. Despite sheep milk showed the highest prevalence of *L. monocytogenes*, none of the participant consumes sheep milk for the claim that ewe's milk is only sufficient for her offspring. Many of sheep flocks are raised with goats or cows in study region and elsewhere in Egypt (Hegazy et al., 2016) so Listeriosis within the sheep flock may be a source of infection for other in contact animals that used for milk production and consumption. Interestingly, 25% of cow's milk, 72.4% of goat's milk and 100% camel's milk consumers do not boil milk for the belief of the safety of these kinds of milk. Our findings demonstrated L. monocytogenes in milk from cows and goats, while camel's milk was free. Yet, other studies demonstrated the possible contamination of camel milk by *L. monocytogenes* and other food-borne pathogens as well (Alall et al., 2012; Ombarak and Elgabory, 2014). These findings highlight the incorrect awareness of milk safety and the dangerous tradition and habits of consumption by residents in this study region. The low rate of awareness may correlate with inadequate health educational campaign, low educational level, inheritance of bad traditions and habits of consumption from closed old ancestor Bedouin communities and tribes widely distributed in this region of the country.

In conclusion this study aimed to assess the potential public health risk of *L. monocytogenes* in raw milk collected from different species in western of Egypt with regard to prevalence, virulence, antibiotic resistance and high-risk consumption practices survey. *L. monocytogenes* were detected in relatively high rates in milk samples from sheep, goat and cow but not camel, which may imply unhygienic milking conditions. All *L. monocytogenes* isolates contained a minimum 2 out of 3 virulence genes and showed multi-drug resistance for at least 4 antibiotics, which may pose a public health threats to consumers of milk contaminated by these pathogens. Majority of the livestock owners showed low rates of disease awareness and high tendency for high-risk consumption practices as consumption and processing of raw milk. Variation in virulence capabilities of *L. monocytogenes* and the emerging increase in resistance for antibiotics recorded in this study necessitate regular surveillance for *L. monocytogenes* isolates for correct risk assessment of these pathogens in food and human clinical cases. Also, urgent and comprehensive educational campaigns regarding hygienic milk production and risks of food-borne pathogens are required for mitigation or prevention of high-risk consumption practices in communities of the study region.

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APPENDICES

Table 1: Frequency Distribution of *L. Monocytogenes* Detection in Milk Samples Collected in this Study

Species	*L. monocytogenes*		
	No.	+ve	%
Sheep	50	2	4
Goat	50	1	2
Cow	50	1	2
Camel	50	0	0
Total	**200**	**4**	**2**

Table 2: Antibiotic Resistance of *L. Monocytogenes* Pathogens Isolated from Milk in this Study

Antibiotic	Conc. (µg)	*L. monocytogenes* (n= 4)		
		*Bp	**R	%
Ampicillin (AMP)	10	16	4	100
Cephalothin (KF)	30	14	4	100
Gentamicin (CN)	10	12	0	0
Kanamycin (K)	30	13	0	0
Erythromycin (E)	15	25	3	75
Chloramphenicol (C)	30	12	0	0
Rifampin (RD)	5	16	1	25
Tetracycline (TE)	30	14	3	75
Sulfamethoxazole-Trimethoprim (SXT)	25	29	4	100

*Bp: Breakpoint. **R; Resistance

Table 3: Resistance and Virulence Profiles of *L. Monocytogenes* Pathogens Isolated from Milk in this Study

Isolate ID	Antibiotic Resistance		Virulence genes		
	Phenotype	**MDRI	*hlyA*	*inlA*	*inlB*
*Lm (4)	AMP, KF, E, RD, TE, SXT	0.7	+	+	+
Lm (2)	AMP, KF, E, TE, SXT	0.6	+	+	+
Lm (3)	AMP, KF, E, SXT	0.4	-	+	+
Lm (1)	AMP, KF, TE, SXT		+	+	-

*LM; L. monocytogenes isolate (no.). **MDRI; Multi drug resistance index, calculated by dividing number of resistant antibiotics by the total number of tested ones.

Table 4: Responses of Livestock Owners Regarding Their Milk Consumption Practices

Question	Answer	(%)
Can milk or dairy products consumption transmit disease to man?	Yes	40
	No	40
	Don't Know	20
Do you consume milk from your owned animals?	Yes	98.3
	No	1.7
What kind of milk do you regularly consume?	Sheep	0
	Goat	82.9
	Cow	22.8
	Camel	8.6
Do you boil the milk before consumption?	Yes	37.9
	No	62.1
Do you process dairy products from your animals' milk?	Yes	93.2
	No	6.8
Do you boil the milk before processing?	Yes	0
	No	100
Do you consume milk from morbid animals?	Yes	27.1
	No	72.9
Do you boil milk from morbid animals before consumption?	Yes	37.5
	No	62.5

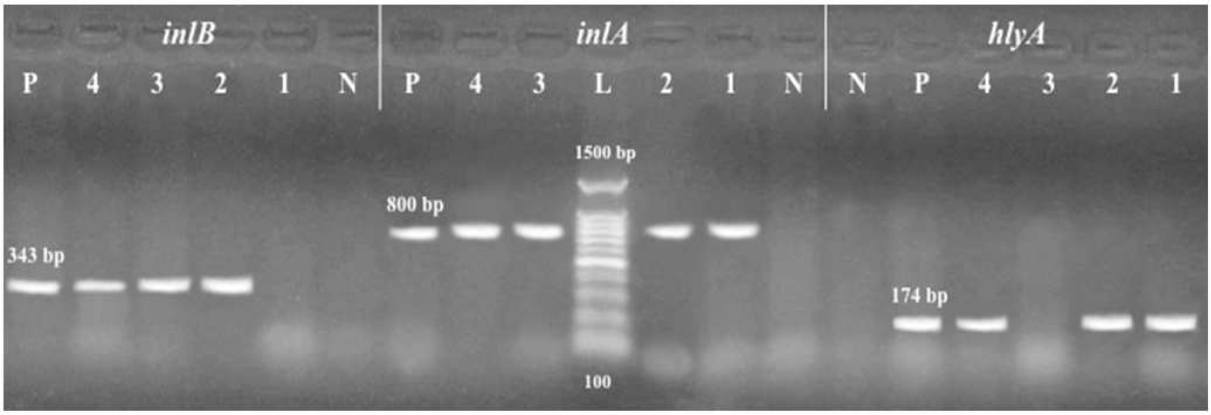

Figure 1: PCR Detection of *Inla, Inlb* and *Hlya* Virulence Genes in the *L. Monocytogenes* Isolates in this Study

Lanes1-4; L. monocytogenes isolates. P; positive control. N; negative control. L; ladder.