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Occurrence and PCR identification of *Salmonella* spp. from milk and dairy products in Mansoura, Egypt

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Abstract

This study was executed to determine the prevalence, serotypes and virulence profiling of *Salmonella* isolated from raw milk and dairy products purchased randomly from small private dairy farms, local groceries and street vendors in Mansoura, Egypt during the period from April through October 2015. A total of 200 samples that comprising 100 raw milk (market and bulk farm, 50 per each) and 100 dairy products (pickled white cheese, fresh soft cheese, Kareish cheese and ice cream, 25 per each) were tested for the presence of *Salmonella* by conventional bacteriological methods followed by serotyping. *Salmonella* strains were molecularly screened by PCR for the presence of virulence associated genes. By culturing, 58 *Salmonella* isolates were recovered with an overall occurrence of 29%, distributed as 52% (26/50), 14% (7/50), 20% (5/25), 8% (2/25), and 72% (18/25) from market milk, bulk farm milk, fresh soft cheese, Kareish cheese and ice cream, respectively. No *Salmonella* isolates were detected from pickled white cheese. Approximately 85% of all *Salmonella* isolates were identified into *S.* Enteritidis (25/58), *S.* Typhimurium (15/58) and *S.* Infantis (9/58). By PCR, all the representative *Salmonella* serovars possessed both *invA* and *stn* genes. Meanwhile, 77.8% of the screened isolates carried the specific amplicon of *avrA* at 422 bp. This study concluded that there is a need for continuous surveillance and monitoring of milk and dairy products especially the locally produced ones to minimize the possibility of human infections with *Salmonella.*

Introduction

Salmonellosis is one of the major foodborne pathogens affects humans causing foodborne illnesses worldwide with serious implications in ill developed countries (Forshell and Wierup, 2006). Milk and dairy products especially those produced from raw or improperly pasteurized milk have been incriminated as potential vehicles for the transmission of different foodborne pathogens to humans. Not only poultry products are the major sources for human non-typhoidal salmonellosis (Capita *et al*., 2003; Halawa *et al*., 2016) but also raw milk and its products are considered other sources.

The contamination of raw milk with pathogens comes from many sources such as feces of infected cattle, contaminated skin, infected udder, milking equipment, air (dust borne infection), feed, animal insects and from milkers (Bergonier *et al*., 2003; Coorevits *et al*., 2008; Callon *et al*., 2008). In Egypt, there is a higher incidence of milk contamination with different pathogenic bacteria especially in rural areas, where small livestock keepers tend to rear dairy animals for the production of milk and native dairy products (Aidaros, 2005).

The manufacturing of different dairy products such as soft cheese, kariesh cheese and ice cream from raw milk improved its microbial quality by increasing the salinity and/or acidity of the manufactured dairy products (Guinee and O'Kennedy, 2007; Al-Ashmawy *et al*., 2016). However, the primitive process of manufacturing under low or uncontrolled hygienic conditions in developing countries is another impediment.

The information obtained from serotyping of *Salmonella* might be helpful during the epidemiological investigation of foodborne outbreaks (Graziani *et al*., 2013). Approximately, more than 2500 *Salmonella* serotypes were identified with a major public health concern given to *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis and *S.* Hadar serovars (Herikstad *et al*., 2002). There are many virulence genes that have been linked to the pathogenicity of *Salmonella* and the severity of infection depends mainly on the presence or absence of these genes. The chromosomally located invasion A *(invA)* gene which

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triggers the pathogen to invade the host cell, has been considered a universal genetic marker identified from mostly all the *Salmonella* serovars (Malorny *et al*., 2003). Also, the enterotoxin *(stn)* gene which is another virulence gene, encodes a protein causing severe diarrhea, regarded as a unique PCR marker for Salmonella identification regardless of their serovars (Moore and Feist, 2007; Zou *et al*., 2012). The *avrA* gene is responsible for the induced cell apoptosis and prevention of the host inflammatory response against infections (Ben-Barak *et al.*, 2006; Jones *et al*., 2008).

The main objective of this study was to determine the prevalence of *Salmonella* and its serovars in raw milk, pickled white cheese, fresh soft cheese, Kareish cheese and ice cream by conventional bacteriological methods in Mansoura, Egypt. Also, PCR characterization of the virulence associated markers among *Salmonella* serovars was also performed.

Materials and Methods

Sample collection

Two hundred samples that composed of 100 raw milk (market and bulk farm, 50 per each) and 100 dairy products (pickled white cheese, fresh soft cheese, Kareish cheese and ice cream, 25 per each) were included in this study. Milk and dairy products were purchased randomly from small private dairy farms, local groceries and street vendors in Mansoura, Egypt during the period from April through October 2015. All the samples were collected in sterile wide-mouthed jars or kept in their original package, labeled and transported quickly as possible in ice box to the laboratory of Food Hygiene and Control Department, Faculty of Veterinary Medicine, Mansoura University for the conventional identification of *Salmonella*.

Conventional isolation and identification of Salmonella

Twenty five ml of milk sample and also 25 grams of dairy products were homogenized into 225 ml of 0.1% sterile buffered peptone water (BPW; Becton Dickinson, Sparks, MD, USA), and incubated at 37°C for 24 h. One ml of the homogenate was added aseptically to 9 ml of Rappaport Vassilliadis (RV) broth (Oxoid, UK) and kept overnight at 42°C. A loopful from the enriched broth was inoculated onto the surface of xylose lysine desoxycholate (XLD) agar (Oxoid, UK) and then incubated at 37°C for 1 day (Quinn *et al*., 2002). Three to five typical (pink to red colonies with or without dark center on XLD) colonies of *Salmonella* were picked, streaked onto nutrient agar slopes and incubated at 37°C for 18-24 h for the further biochemical identification.

Table 1. List of primers used for PCR identification of *Salmonella* virulence genes.

Target gene	PCR product	Primer sequence	Reference
invA .F	284 bp	5' GTGAAATTATCGCCACGTTCGGGCAA -3'	Oliveira *et al*.,
R		5'- TCATCGCACCGTCAAAGGAACC-3'	2003
stn .F	617 bp	5'- TTGTGTCGCTATCACTGGCAACC -3'	Huehn *et al*.,
R		5'- ATTCGTAACCCGCTCTCGTCC -3'	2010
avrA .F	422 bp	5'- CCTGTATTGTTGAGCGTCTGG -3'	Murugkar *et*
R		5'- AGAAGAGCTTCGTTGAATGTCC -3'	*al*., 2003

The biochemical identification was done according to Macfaddin (2000) by using indole, Vogas-Proskaur, urease, citrate utilization, hydrogen sulphide production, gelatin hydrolysis, oxidation-fermentation, sugar fermentation, detection of ß-galactosidase enzyme (ONPG), ornithine (ODC), lysine (LDC) and argnine (ADH) tests.

Serotyping

The biochemically identified Salmonella isolates were serologically typed according to Kauffmann-White scheme (Popoff *et al*., 2004) at the Faculty of Veterinary Medicine, Department of Food Hygiene and Control, Benha University, Egypt by the slide agglutination technique of both somatic (O) and flagellar (H) antigens.

DNA extraction and molecular identification of Salmonella *virulence genes*

Genomic DNA extraction from *Salmonella* isolates was done by boiling as previously described by Ramadan *et al*. (2016). All DNA samples were stored at -20°C until tested for the presence of *Salmonella* virulence genes. The primer pairs used for the identification of invA, stn and *avrA* genes (target gene, sequence and PCR products) were mentioned in Table 1.

A uniplex PCR condition was done to detect *invA* gene from *Salmonella* isolates as determined by Oliveira *et al*. (2003) and the cyclic conditions were slightly modified to start with an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 30 sec. A final extension was done at 72ºC for 7 min. To identify stn and *avrA* genes, a duplex PCR was performed with the cyclic conditions similar to that done for *invA* genes with the exception of annealing temperature at 58ºC for 45 sec.

Both uniplex and duplex PCR reactions were conducted in a volume of 25 µl consisting of 0.25 µl of each primer (100 µM each), 12.5 µl of 2X PCR

Table 2. Frequency distribution of *Salmonella* serovars isolated from milk and dairy products.

Product	Total no. of samples	No. of +ve samples	No. of isolates	No. of isolates serotypes				
				S. Enteritidis	S. Typhimrium	S. Infantis	S. Tsevie	S. Malode
Market milk	50	18	26*#	11	8	4	0	3
Bulk farm milk	50	7	7*#	3	1	1	0	2
Pickled white cheese	25	0	0	0	0	0	0	0
Fresh soft cheese	25	4	5*	4	1	0	0	0
Kareish cheese	25	1	2	1	0	0	0	1
Ice cream	25	14	18#	6	5	4	2	1
Total	200	44	58	25	15	9	2	7

*A significant association of the occurrence of *Salmonella* from raw milk and soft cheese (χ^2=9.32).
A significant association of the occurrence of *Salmonella* from raw milk and ice cream (χ^2=12.59).

Master Mix (Promega, Madison, USA), and 5 µl DNA template. Positive amplicons at 284 bp, 617 bp and 422 bp for *invA, stn* and *avrA* genes respectively were electrophresed into 1.5% agarose gel, stained with ethidium bromide and photophraphed under ultraviolet light. DNA extracts from *S.* Typhimurium and *E. coli* O119: H4 were included in each PCR run as positive and negative controls, respectively.

Statistical analysis

The statistical association of *Salmonella* prevalences in raw milk, cheese and ice cream was determined by Chi-square (X^2) test with the usage of statistical packages Microsoft Excel, Win episcope 2.0 and SAS 9.2 (SAS Institute Inc. 2008) software. The probability values were measured at P-values < 0.05.

Results and Discussion

The overall occurrence of biochemically identified *Salmonella* from raw milk and dairy products on XLD was 29% (58/200), distributed as 52% (26/50), 14% (7/50), 0% (0/25), 20% (5/25), 8% (2/25), and 72% (18/25) among market milk, bulk farm milk, pickled white cheese, fresh soft cheese, Kareish cheese and ice cream, respectively. These persumptive *Salmonella* isolates were recovered from a total of 44 samples (Figure 1) with an overall incidence of 22%. Variable incidences of *Salmonella* were reported in many previous literature as determined by Van Kessel *et al.* (2004) (2.65%); Karns *et al.* (2005) (11.8%); Jayarao *et al.* (2006) (6%); Tadesse and Dabassa (2012) (20%); Tesfaw *et al.* (2013) (1.6%) and Gwida and Al-Ashmawy (2014) (12%). At the same time, there are many

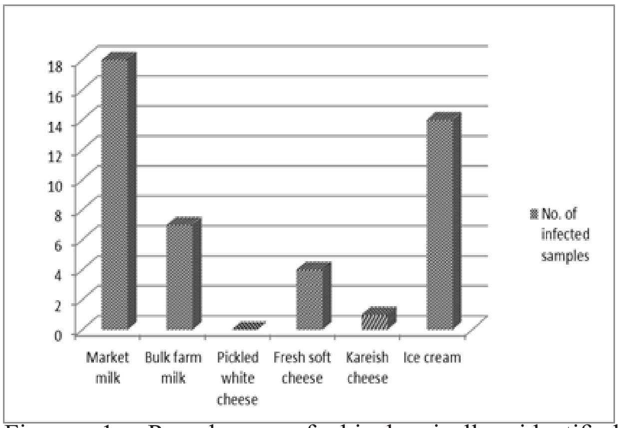

Figure 1. Prevalence of biochemically identified *Salmonella* in raw milk and dairy products.

previous studies that did not isolate *Salmonella* from raw milk samples (Ekici *et al.*, 2004; Mhone *et al.*, 2012; Zeinhom and Abdel-Latef, 2014). The variable prevalences of *Salmonella* from milk and its products could be linked to the sampling techniques, source of samples, geographical differences, seasonal variation, farm husbandry practices, bacteriological techniques used for pathogen isolation and process of product manufacturing (Oliver *et al.*, 2005).

The distribution of *Salmonella* serovars among raw milk samples and different dairy products is shown in Table 2. It was found that *S.* Enteritidis, *S.* Typhimurium and *S.* Infantis represented approximately 85% (49/58) of the *Salmonella* serovars isolated from raw milk samples and dairy products. The over representing of the above serovars especially *S.* Enteritidis and *S.* Typhimurium that were commonly associated with public health concern worldwide (CDC, 2008; Foley *et al.*, 2008) verified the significant role of milk and its products in transmission of salmonellosis to humans.

The presence of *Salmonell*a was significantly

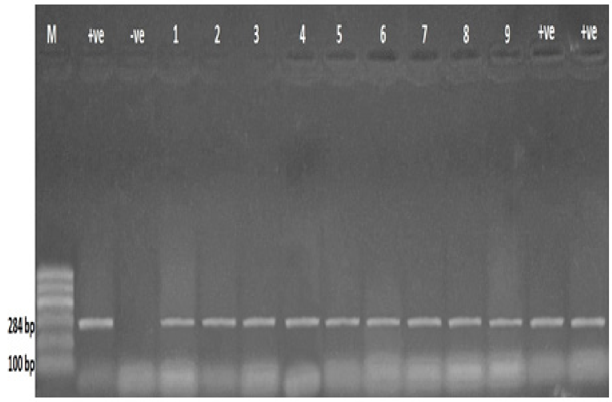

Figure 2. 1.5% agarose gel electrophoresis of the uniplex PCR amplification of *invA* gene in *Salmonella* isolates from milk and dairy products. Lane M: 100 bp DNA ladder. Lane +ve: positive control. Lane -ve: negative control. Lane 1-9: positive *Salmonella* serovars with *invA* gene (284 bp).

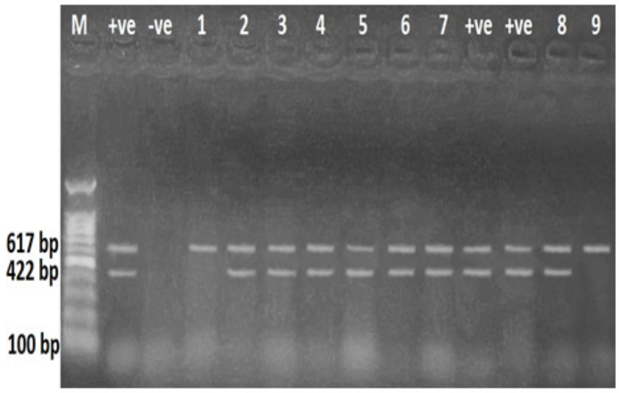

Figure 3. 1.5% agarose gel electrophoresis of the duplex PCR amplification of *stn* and *avrA* genes in *Salmonella* isolates from milk and dairy products. Lane M: 100 bp DNA ladder. Lane +ve: positive control. Lane -ve: negative control. Lane 2-8: positive *Salmonella* serovars with both stn and *avrA* genes. Lane 1 and 9: positive *Salmonella* serovars with *stn* gene only (617 bp).

higher (χ^2=9.3221, P <0.05) among raw milk (33%, 33/100) than soft cheese (pickled and fresh) samples (10%, 5/50). This could be attributed to the manufacturing process of cheese that related to ripening and storage in brine solution for a period not less than 3 months which creates undesirable condition for the survival and growth of *Salmonella* (Sung and Collins, 2000; Kousta *et al.*, 2010). Another possible explanation could be related to the fecal contamination of raw milk with foodborne pathogen during its collection or the shedding of pathogens into milk from infected udder.

Moreover, *Salmonella* were also isolated from the native type of soft cheese (Kareish cheese) which is mostly manufactured by smallholders and purchased from local markets in Egypt. The primitive methods used for manufacturing Kariesh cheese from unpasteurized raw milk with the absence of standard hygienic measures afford the pathway for its contamination with different foodborne pathogens (Brooks *et al.*, 2012) and subsequently hazard to consumers. According to the Egyptian standards of Kariesh cheese No.1008/2005 there is an obligation for the pasteurization of raw milk and the absence of *Salmonella* and other pathogens in 25 g.

The higher occurrence of *Salmonella* from ice cream samples in this study was unexpected. It was noticeable that heat treatment of raw milk before its manufacturing and the lower storage temperature of ice cream provide unfavorable conditions for the growth of different pathogens (Lejeune and Rajala-Schultz, 2009). However, the repeated freezing and thawing cycles of ice cream due to the repeated loss of electricity especially in developing countries beside the post processing contamination especially in small scale manufacturer could attribute to the higher incidences of pathogenic bacteria in ice cream.

A total of 9 representative *Salmonella* serovars isolates identified from milk and dairy products were examined for the presence of *invA, stn* and *avrA* genes by PCR. The 9 representative isolates included 3 isolates (*S.* Enteritidis, 1; *S.* Infantis, 1 and *S.* Malode, 1) from market milk, 2 isolates (*S.* Enteritidis, 1; *S.* Typhimurium, 1) from fresh soft cheese and 4 isolates (*S.* Typhimurium, 1; *S.* Infantis, 2 and *S.* Malode, 1) from ice cream. It was found that all the representative *Salmonella* serovars isolates carried the specific amplicons of both *invA* and *stn* genes (Figure 2 and 3) at 284 bp and 617 bp, respectively. However, the specific amplified product of avrA at 422 bp was identified in 77.8% (7/9) of the examined isolates (Figure 3).

The existence of *invA* gene mostly in all Salmonella serovars and its absence from the other bacteria rather than *Salmonella* proved it as a genetic marker for the identification of *Salmonella* (Daum *et al.*, 2002; Gallegos-Robles *et al.*, 2009; Osman *et al.*, 2013; Rowlands *et al.*, 2014 and Sallam *et al.*, 2014). Likewise, the presence of *stn* gene, a virulence gene with an enterotoxic activity, in all examined *Salmonella* isolates was in agreement with that determined by Murugkar *et al*. (2003) and Sallam *et al.* (2014). Regarding *avrA* gene, its presence in approximately 80% of the examined *Salmonella* isolates was similar to that reported by Streckel *et al.* (2004). Controversy to *invA* and *stn* genes, not all *Salmonella* serotypes have *avrA* gene (Collier-Hyams *et al*., 2002; Prager *et al*., 2003). Meanwhile, previous studies by Ben-Barak *et al.* (2006) and Borges *et al.* (2013) noticed that *avrA* gene was predominately detected among the *Salmonella* serovars causing severe outbreaks in humans as *S*. Typhimurium and

S. Enteritidis.

The above findings highlighted the presence of *Salmonella* in a higher proportion in raw milk and its products such as soft cheese, kareish cheese and ice cream in Mansoura, Egypt and considerably public health concern. It could be concluded that the implementation of hygienic measures during milking and manufacturing of dairy products are required to minimize the risk of human infections with *Salmonella*. Moreover, the application of PCR targeting i*nvA* and *stn* genes provides a valuable tool for the rapid identification of *Salmonella* from food samples especially milk and dairy products.

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