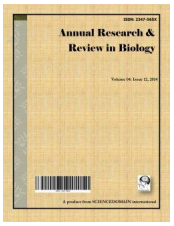

Annual Research & Review in Biology

29(3): 1-11, 2018; Article no.ARRB.44651
ISSN: 2347-565X, NLM ID: 101632869

Advanced Studies on Virulence Genes of *Salmonella* and *Shigella* species Isolated from Milk and Dairy Products

Gamal A. M. Younis[1], Rasha M. Elkenany[1*] and Wesam S. Abd-Elmoati[1]

[1]Department of Bacteriology, Mycology, and Immunology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Author GAMY designed the study, performed the statistical analysis and wrote the protocol. Authors RME and WSA wrote the first draft of the manuscript and managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2018/44651
Editor(s):
(1) Dr. Manikant Tripathi, Department of Microbiology, Dr. Ram Manohar Lohia Avadh University, Faizabad, India.
(2) Dr. George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Vincent Habimana, University of Nairobi, Kenya.
(2) R. Prabha, Karnataka Veterinary, Animal and Fisheries Sciences University, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/27108

Original Research Article

Received 06 August 2018
Accepted 12 October 2018
Published 08 November 2018

ABSTRACT

Salmonella and *Shigella* species are the main health problem in various portions of the world. This study gave rise to detect and enumerate *Salmonella* and *Shigella* species with detection of virulence genes by PCR in randomly collected raw milk and dairy products (ice cream, cheese, yoghurt, rice with milk and cream) from different vendors of village and dairy farms in Mansoura Governorate, Egypt during October 2016. A total of 24 (9.6 %) isolates from 250 samples (raw milk and dairy products) were recognised as *Salmonella* 6.4 % (16/250) and *Shigella* 3.2 % (8/250) species with their high prevalence in raw milk. Amongst serotypes of *Salmonella* species: *S.* Typhimurium 37.5 % (6/16), *S.* Enteritidis, *S.* Tsevie 18.75 % (3/16 each) and other serovars 25 % (4/16). Additionally, the identified *Shigella* species (8/250) were *S. dysenteriae* 50 % (4/8), *S. flexneri* 25 % (2/8) and *S. sonnei* 25 % (2/8). The average of total viable count of samples positive for *Salmonella* and *Shigella* in raw milk and dairy products was 4.47±0.97 $\log^{10}$ CFU/ml or gm and 4.27±1.01 $\log^{10}$ CFU/ml or

**Corresponding author: E-mail: dr_rashavet22@yahoo.com;*

gm, respectively. Furthermore, polymerase chain reaction assay was applied for demonstration of the most common virulence associated genes of *Salmonella* species (*invA*) and *Shigella* species (*invC, ipaH, virA*). The *invA* gene was present in all tested *Salmonella* isolates. The *invC* and *ipaH* genes were present in all *Shigella* isolates, while *virA* gene was absent in all strains. This study recommended that appropriate hygienic measures, as well as continuous monitoring of *Salmonella* and *Shigella* infection, could help to control and prevent the emergence and spread of salmonellosis and shigellosis from milk and dairy products in Egypt.

Keywords: Salmonella; Shigella; milk; dairy products; uniplex PCR; virulence genes.

1. INTRODUCTION

The bacterial contamination of milk and dairy products was largely due to the human factor and unhygienic conditions application. The presence of the pathogenic bacteria in milk considers main public health concerns [1,2]. The environment and food were primarily contaminated with *Salmonella* and *Shigella* by the faecal wastes of the infected animals and humans [3,4] as *Salmonella* and *Shigella* colonised mostly in the gastrointestinal tract [5,6].

Salmonella Enterica cause "salmonellosis" which is one of the most common food-borne disease [7] and associated with a major economic productivity loss in the food and animal industries [8]. Salmonellosis is zoonotic unusually contagious disease, usually self-limiting infection [9]. There are arrays of virulence factors that are responsible for the pathogenic ability of *Salmonella* act in tandem and eventually manifest in the typical symptoms of salmonellosis. In latest years, PCR is commonly applied for disease diagnosis and bacteria identification. The chromosomally located *invA* gene is one of the virulence encoding-gene used for detection of *Salmonella* genus and associated with *Salmonella* pathogenicity islands (SPIs). It triggers the pathogen to invade the host cell and has been considered a universal genetic marker identified from mostly all the *Salmonella* serovars [10,11].

Shigella is categorised into four serogroups: *S. dysenteriae, S. flexneri, S. sonnei* and *S. boydii* [12]. *Shigella* species have global human health problem by causing: "Shigellosis or bacillary dysentery". Shigellosis, endemic throughout the world, is one of the major causes of morbidity and mortality, particularly amongst children in low and middle income countries [13,14]. The pathogenesis of shigellosis includes inflammation, ulceration, hemorrhage, tissue destruction, and fibrosis of the colonic mucosa, that result in abdominal pain and diarrhea/dysentery; in some cases infertility and endometriosis also have been documented [3,15]. *Shigella* strains have a lot of virulence attributes that are related to their pathogenicity such as *invC, ipaH and virA* genes. The *invC* gene can identify *Shigella* at the genus level [16]. The *ipaH* gene, the invasion plasmid H, is species-specific gene and presents in all *Shigella* strains [17]. The *virA* gene has been implicated in invasion and intercellular spreading [18].

For reduction of milk and dairy products contamination and diseases caused by Salmonella and Shigella infections, the present study was conducted to throw light on the occurrence and enumeration of *Salmonella* and *Shigella* species in randomly collected raw milk and dairy products samples and uses of molecular methods for detection of virulence genes of *Salmonella* and *Shigella* species in Egypt.

2. MATERIALS AND METHODS

2.1 Sampling

A simple random method was adopted to collect a total of 250 raw milk and dairy product samples (150 raw milks, 37 ice creams, 30 kareish cheeses, 20 yogurts, 5 rice with milks, 8 creams) from different vendors of village and dairy farms in Mansoura Governorate, Egypt during October 2016. The samples were maintained on ice box until transported to the laboratory and processed within 1 h of collection.

2.2 Isolation and Identification of *Salmonella* and *Shigella* species

All samples (raw milk and dairy products) were prepared as 25 ml or gm added to 225 ml of sterile buffered peptone water (Oxoid), and then incubated at 37ºC for 6 h. One milliliter of prepared culture was aerobically enriched in

tryptone soya broth (Oxoid) at 37ºC for 24 h for *Shigella* and Rappaport Vassilliadis (RV) broth (Oxoid) at 37ºC and 41ºC for 24 h for *Salmonella*. A single drop of prepared pre-enrichment specimen was inoculated with streaking onto Salmonella-Shigella agar (S-S agar), Xylose Lysine Desoxycholate agar (XLD) and MacConkey`s agar (MAC) (Oxoid) and then incubated at 37ºC for 24 h. Non-lactose fermenting colonies were picked up from culture plates and biochemically tested (triple sugar iron agar, indole, urease and Simmon's citrate agar tests). Serological confirmation of suspected colonies of *Salmonellae* was carried out according to Kauffman White scheme for the determination of somatic (O) and flagellar (H) antigens using monovalent and polyvalent (O&H) *Salmonella* antiserum [19]. Also, *Shigella* serotypes was confirmed by slide agglutination test using *Shigella* antisera (Difco Laboratories) according to the manufacturer's instructions.

2.3 Enumeration of *Salmonella* and *Shigella* Isolates

The bacterial isolates were counted according to [20]. In brief, 10 ml or gm of each sample was aseptically introduced into 90 ml of sterile normal saline solution and homogenised by shaking followed by further decimal dilutions up to 10^{-5} concentrations. A 0.1 ml quantity of appropriately diluted sample was used to inoculate freshly prepared media by spread plate method, and then incubated at 37°C for 24 h. The present colonies were counted and recorded after incubation at 37°C for 24 h, to get the total bacterial count in CFU/ml or gm. The bacterial count was expressed as $\log^{10}$ values of colony-forming units per milliliter (CFU/ml or gm).

2.4 Molecular Determination of Virulence Genes

Uniplex PCR (uPCR) assay was used for the detection of virulence genes of *Salmonella* (*invA* gene) and *Shigella* (*invC, ipaH and virA* genes) isolates. Shortly, extraction of DNA was performed according to QIAamp DNA mini kit instructions. The cycling conditions and specific primers are illustrated in Table (1). The PCR products were electrophoresed on a 1 % agarose gel at 100 V. The agarose gel was stained with 0.5 µg/ml ethidium bromide. The DNA band was visualised by gel documentation system (Biorad, USA) [16,21,22,23].

2.5 Statistical Analysis

The data obtained were analysed using Statistics Package for Social Sciences (SPSS) software and Microsoft Excel 2007. This test combines ANOVA with comparison of differences between means of the treatments at the significance level of $P < 0.05$ [24].

3. RESULTS AND DISCUSSION

3.1 The Prevalence of *Salmonella* and *Shigella* species in Milk and Dairy Products

Although milk and milk products have high nutritive value, they may contain different types of micro-organisms as a result of unhygienic conditions [25]. Bacterial contamination could generally occur from three main sources; within the udder, outside the udder and from the surface of equipment used for the milk handling and storage [26]. The consumption of raw milk and improper processed milk products remains a risk factor for foodborne illness particularly *Salmonella* and *Shigella* infection [7]. *Salmonella* and *Shigella* were shed in the feces of livestock such as cows and buffaloes and could contaminate milk during the milking process. Thus, a total of 24 (9.6 %) isolates from 250 samples (raw milk and dairy products) were identified as *Salmonella* 6.4 % (16/250) and *Shigella* 3.2 % (8/250) species as shown in Table (2 and 3). Among serotypes of *Salmonella* species (16/250): *S.* Typhimurium 37.5 % (6/16), *S.* Enteritidis, *S.* Tsevie 18.75 % (3/16) each, *S.* Infantis 12.5 % (2/16), *S.* Haifa and *S.* Virchow 6.25 % (1/16) each. Furthermore, the identified *Shigella* species (8/250) were *S. dysenteriae* 50 % (4/8), *S. flexneri* 25 % (2/8) and *S. sonnei* 25 % (2/8). A high prevalence of *Salmonella* was detected in kareish cheese (13.33 %), followed by raw milk (7.33 %) and ice cream (2.7 %), while *Shigella* was determined in high percentage in ice cream (8.1 %), and then raw milk (3.33 %) with its absence in kareish cheese and other examined dairy products.

The prevalence of *Salmonella species* in this investigation was consistent with [27, 28, 29] who detected *Salmonella* strains by 8.7 %, 7.7 % and 7.61 % from raw milk and milk products in Nigeria and India, respectively. Also, [30] as well as [31] investigated *Salmonella species* with the prevalence of 15 % (15/100) from milk and cheese and 2 % (16/800) from dairy products in

Egypt, respectively. Additionally, [32] identified *Salmonella species* with a prevalence of 12 % (24/200) from milk and dairy products in Egypt, while [33] detected *Salmonella species* from raw milk in Ethiopia as 20 % (20/100). Other investigators had reported a wide range of prevalence of *Salmonella* (6.7 to 97.6 %) from bulk tank milk and milk filters in United States [34]. Also, this finding detected a high occurrence of *Salmonella* species in Kareish cheese (13.33 %) in similarity to the previous studies in Egypt [35], while several investigators could not recover *Salmonella* species from Kareish cheese [36,37].

Furthermore, the current study revealed a lower prevalence of *Shigella* species. (3.2 %) that was compatible with [31] who isolated *Shigella* species from dairy products in a percentage of 1.4 % in Egypt. Whilst, previous studies [38, 39] showed a prevalence rate of *Shigella* species in raw milk (20 % and 17.5 % respectively), meanwhile, other investigators [40] showed a prevalence rate of *Shigella* species in 11.76 % ice cream. Overall, the results of this study on raw milk and dairy product samples indicated that inadequate hygienic and sanitation practices during milking, processing and manufacture [30,41].

Regarding to serotypes, *S.* Typhimurium was considered the major cause of Salmonella infection among the examined raw milk and dairy products which poses great public health hazards [42]. In this investigation, *S.* Typhimurium was the most dominant serotype amongst *Salmonella* isolates. Similarly, previous studies by [28,32] identified *S.* Typhimurium as the most predominant serotypes recovered from milk and cheese in Egypt and India. On the other hand, [30] detected *S. enteritidis* as the most common serotypes obtained from milk and cheese in Egypt. Moreover, this study showed the dominance of *S. dysenteriae* among *Shigella* species, while [43] found that *S.flexneri* was the most common species isolated from pedha (milk product) samples in India.

3.2 Total Viable Bacterial Count

As illustrated in Table (4), the total viable count of samples positive for *Salmonella* were ranged from 2.92±1.69 to 4.78±1.03 with an average of 4.47±0.97 $\log^{10}$ CFU/ml or gm. Moreover, the total viable count for samples positive for *Shigella* was ranged from 3.99±0.9 to 4.47±1.09 with an average of 4.27±1.01 $\log^{10}$ CFU/ml or gm. Fresh milk drawn from a healthy cow normally contains a low microbial load of less than 10^3 CFU/ml [44]. The presence of pathogenic bacteria such as *Salmonella and Shigella* species in the analysed samples is an indicator of poor hygiene and sanitation during milking and post milking processes [45]. The prescence of *Shigella* in a very small amount (only 10 of them) could cause disease (bacillary dysentery), which was easily transmitted and could cause big outbreaks [5]. Overall, the mean of total viable count of samples positive for *Salmonella* species (4.47±0.97 $\log^{10}$ CFU/ml or gm) and samples positive for *Shigella* species (4.27±1.01 $\log^{10}$ CFU/ml or gm) obtained in this study was similar to that obtained by [46] who detected *Salmonella* and *Shigella* count in raw cow's milk with a range of 4.456 ± 0.443 log CFU/ml in India, respectively. Also, other investigators [38] noticed the *Salmonella* and *Shigella* count in raw milk with a mean 4.7 log CFU/ml and 6.04 log CFU/ml in Sudan, respectively. However, [20] found that the total *Salmonella-Shigella* count was ranged between 5.69-6.04 log CFU/ml in raw milk samples collected from dairy farms in Nigeria. Other researchers [40] detected the total *Salmonella* and *Shigella* count with a mean of 3.95 log CFU/ml and 3.7 log CFU/ml in ice cream, respectively. Previous study [47] reported that the mean of *Salmonella* count in local cheese was 6.3 log CFU/ml. This study showed that the quality of milk and dairy products resulted in the study areas were relatively poor. This was clear from the high values of total bacterial count (TBC) and there was the need for adequate sanitary measures and good personal hygiene at handling and different stages of production and consumption to reduce the public health risk.

3.3 Molecular Determination of Virulence Genes

A PCR assay targeting *invA* gene (target size: 284-bp) of *Salmonella* species as well as *invC* (target size: 875-bp), *ipaH* (target size: 600-bp) and *virA* (target size: 215-bp) genes of *Shigella* species was applied for identification of virulent strains of *Salmonella* and *Shigella* isolates. The *invA* gene was found in all *Salmonella* strains (16/16, 100 %) (Fig. 1). Furthermore, the *ipaH* and *invC* genes were detected in all *Shigella* strains (8/8, 100 %), while *virA* gene was absent in all *Shigella* strains (Fig. 2). A PCR targeting invasion (*invA)* gene conferred rapid identification of *Salmonella* isolates as all *Salmonella* serovars have the *invA* gene as a unique character [48]. The detection of *invA* gene

by PCR was rapid, sensitive and specific method for the identification of *Salmonella* genus in many samples [10,11,49]. The *invA* is the first gene of an operon containing three or possibly more genes arranged in the same transcriptional unit [50]. This gene has been shown to be present and functional in most (if not all) *Salmonella* serotypes [51]. This study showed the presence of *invA* gene in 100 % of the *Salmonella* isolates tested. This result was consistent with other investigators [52] who detected *invA* gene in all isolated serovars of *Salmonella* from food samples. It was predictable since the *invA* is an invasion gene conserved among *Salmonella* serotypes, so all the *Salmonella* isolates were found highly invasive.

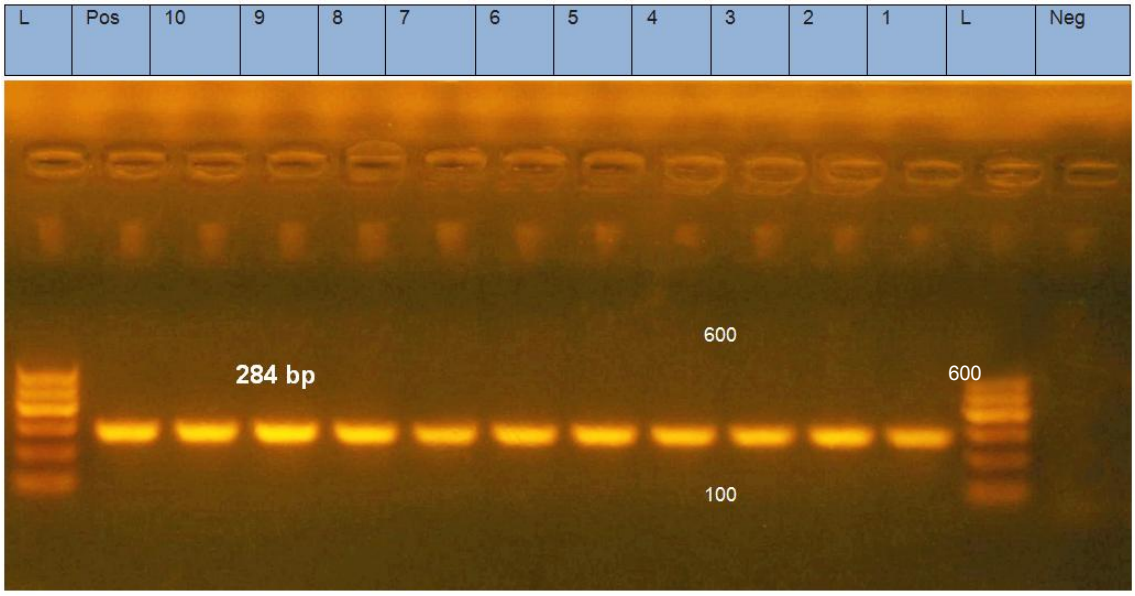

Fig. 1. Representative agarose gel electrophoresis of *Salmonella* serovars showing PCR amplification for *invA* gene (284 bp). (L) ladder 100 bp; lane (1-10) positive samples, Neg (negative control), Pos (positive control).

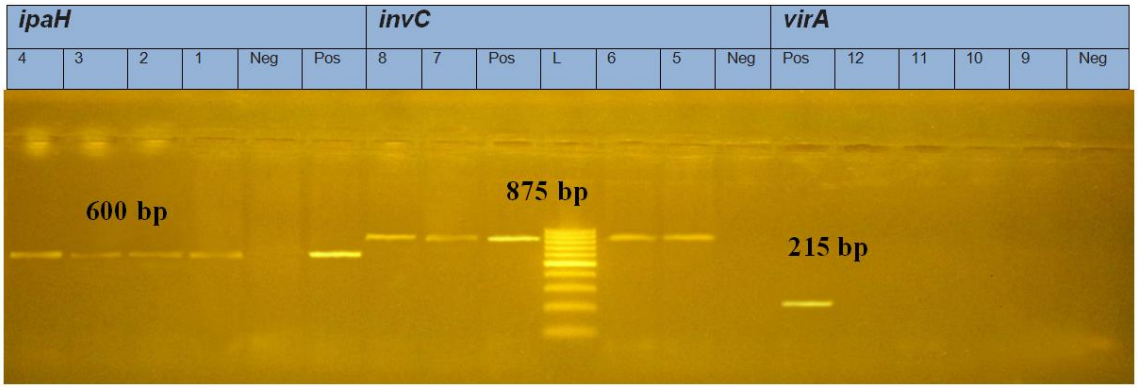

Fig. 2. Representative agarose gel electrophoresis of *Shigella* serovars showing amplification for *ipaH, invC* and *virA* genes. (L) ladder 100 bp; lane (1-4): positive *ipaH* gene (600bp), Lanes (5-8): positive *invC* gene (875bp) and lane (9-12): negative *virA* gene (215bp). Neg (negative control), Pos (positive control).

Table 1. Cycling of specific primers during uniplex PCR for virulence genes of *Salmonella* and *Shigella* species

Target M.O.	Gene	Primer Sequence 5'-3'	Amplified product (bp)	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension	Reference
Salmonella	*invA*	5'-GTGAAATTATCGCCACGTTCGGGCAA-3' 3'-TCATCGCACCGTCAAAGGAACC-5'	284	94˚C/5 min.	94˚C/30 sec.	55˚C/30 sec.	72˚C/30 sec.	35	72˚C/7 min.	Oliveira et al., 2003
Shigella	*invC*	5'-TGC CCA GTT TCT TCA TAC GC-3' 3'-GAA AGT AGC TCC CGA AAT GC-5'	875			60˚C/45 sec.	72˚C/45 sec.		72˚C/10min.	Ojha et al., 2013
	ipaH	5'-GCCGGTCAGCCACCCT CTGAGACTAC-3' 3'-GTTCCTTGACCGCCTTTCCGTACCGT-5'	600			55˚C/45 sec.				Jiménez et al., 2010
	virA	5'-CTG CAT TCT GGC AAT CTC TTC ACA TC-3' 3'-TGA TGA GCT AAC TTC GTA AGC CCT CC-5'	215			60˚C/30 sec.	72˚C/30 sec.			Villalobo and Torres, 1998

Table 2. The prevalence of *Salmonella* species in milk and dairy products

Types of samples	No. of samples	*Salmonella* serovars						
		S. Typhimurium	*S. Enteritidis*	*S. Infantis*	*S. Tsevie*	*S. Virchow*	*S. Haifa*	Total
Raw milk	150	5	1	1	2	1	1	11(7.33%)
Ice cream	37	0	1	0	0	0	0	1(2.7%)
Kareish cheese	30	1	1	1	1	0	0	4(13.33%)
Yoghurt	20	0	0	0	0	0	0	0(0%)
Rice with milk	5	0	0	0	0	0	0	0(0%)
Cream	8	0	0	0	0	0	0	0(0%)
Total	250	6	3	2	3	1	1	16(6.4%)

Table 3. The prevalence of *Shigella* species in milk and dairy products

Types Of Samples	No. of samples	*Shigella* serovars			
		S. dysenteriae	***S. flexneri***	***S. sonnei***	**Total**
Raw milk	150	3	1	1	5(3.33%)
Ice cream	37	1	1	1	3(8.1%)
Kareish cheese	30	0	0	0	0(0%)
Yoghurt	20	0	0	0	0(0%)
Rice with milk	5	0	0	0	0(0%)
Cream	8	0	0	0	0(0%)
Total	250	4	2	2	8(3.2%)

Table 4. *Salmonella* and *Shigella* densities in raw milk and dairy products

Positive samples	*Salmonella* serovars	log CFU/ml*	Positive samples	*Shigella* serovars	log CFU/ml*
1	*S.* Typhimurium	4.66±1.06	1	*S. dysenteriae*	4.37±1.05
2		4.02±0.8	2		4.22±1.01
3		2.92±1.69	3		4.43±1.06
4		4.7±0.96	4		3.99±0.9
5		4.46±0.92	5	*S. flexneri*	4.1±0.98
6		4.44±1	6		4.47±1.09
7	*S.* Enteritidis	4.67±1.07	7		4.29±1.04
8		4.12±0.83	8	*S. sonnei*	4.26±0.95
9		4.78±1.03			
10	*S.* Infantis	4.54±1.18			
11		4.53±0.9			
12	*S.* Tsevie	4.64±1.06			
13		4.48±1.03			
14		4.39±0.98			
15	*S.* Haifa	4.51±0.99			
16	*S.* Virchow	4.65±0.99			
Over all mean		4.47±0.97	Over all mean		4.27±1.01

**Mean of log ±Standard deviation.*

Moreover, three highly specific genes *(invC, ipaH, and virA* genes*)* to *Shigella* were detected by PCR. The *invC* gene was present among all of the *Shigella* species [16]. The *ipaH* gene is one of the most important virulence gene that is differentiated *Shigella* from *Entero-invasive E. coli* (EIEC)*,* since *Shigella* and EIEC have similar physio-biochemical characteristics [53]. The *virA* gene located upstream and transcribed divergently from *icsA* (*virG*), is involved in invasion and spreading, it is the only gene outside the main virulence gene operons to be regulated by the *virB* protein [54]. In this investigation, the invC and *ipaH* genes were detected in 100 % of *Shigella* strains tested, whereas *virA* gene was absent in all *Shigella* strains. This result was compatible to [55] who determined *ipaH* gene in all isolates of *Shigella* from food samples. [16] found *invC* gene in 96.7 % of *Shigella* strains isolated from human. In contrast, [21] determined *virA* gene in all *Shigella* strains isolated from mayonnaise.

4. CONCLUSION

Raw milk and dairy products could be a source of virulent strains of *Salmonella* species based on *invA* gene and *Shigella* species based on *invC, ipaH, virA genes* that has a public health hazard. Therefore, it should be overcome by proper hygienic measures during milking, handling of milk and manufacture of dairy products as well as effective training and education of the farmers to improve consciousness of milk borne zoonosis. This study improved the understanding of epidemiologic feature of salmonellosis and shigellosis and delivered a scientific basis for control and prevention of such diseases in Egypt.

ETHICAL APPROVAL

This article does not contain any studies with animals performed by any of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Alvarez AM. Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial diseases. Ann. Rev. Phytopath. 2004;42:339-366.
2. Claeys WL, Cardoen S, Daube G, Block JD, Dewettinck K, Dierick K, Zutter LD, Huyghebaert A, Imberechts H, Thiange P, Vandenplas Y, Herman L. Raw or heated cow milk consumption: Review of risks and benefits. Food Control. 2013;31:251–262.
3. Ashkenazi S. Shigella infections in children: New insights. Seminars in Pediatric Infectious Diseases. 2004;15(4): 246–252.
4. Ponce E, Khan AA, Cheng CM, Summage WC, Cernglia CE. Prevalence and characterization of Salmonella enteric serovar Weltevreden from imported sea food. Food Microbiology. 2008;25(1):29-35.
5. Warren BR, Parish ME and Schneider KR. Shigella as a foodborne pathogen and current methods for detection in food. Crit. Rev. Food Sci. Nutr. 2006;46(7):551-67.
6. Abulreesh HH. Salmonellae in the environment. In: Annous B, Gurtler JB, editors. Salmonella distribution, adaptation, control measures and molecular technologies. In. Tech. 2012;19-50.
7. Hald T. Advances in microbial food safety: 2. Pathogen update: Salmonella. Elsevier Inc. 2013;Chapters. p. 2.2.
8. Malkawi HI, Gharaibeh R. Rapid and simultaneous Identification of two Salmonella enterica serotypes Enteritidis and Typhimurium from chicken and meat product by multiplex PCR. Biotechnol. 2004;3(1):44-48.
9. Chompook P. Shigellosis. Encyclopedia of Environmental Health. 2011;26–32.
10. Malorny B, Hoorfar J, Bunge C, Helmuth R. Multicenter validation of the analytical accuracy of Salmonella PCR: towards an international standard. Applied and Environmental Microbiology. 2003;69(1): 290-296.
11. Amini K, Zahari TS, Gholamreza N, Reza R, Javid A, Banou SA. Molecular detection of invA and spv virulence genes in Salmonella enteritidis isolated from human and animals in Iran. African Journal of Microbiology Research. 2010;4(21):2202-2210.
12. Hale TL. Genetic basis of virulence in Shigella species. Microbiol. Rev. 1991;55: 206–224.
13. Bardhan P, Faruque ASG, Naheed A and Sack DA. Decreasing shigellosis-related deaths without *Shigella* spp.-specific

interventions. Asia. Emerg. Infect. Dis. 2010;16(11):1718-1723.

14. Wen X, Wu Y, Bian F, Sun Y, Zheng X, Zhang Y, Jiang C, Yuan G. High prevalence of atypical class 1 integrons and class 2 integrons in multi-drug resistance Shigella flexneri isolated from China. Afr. J. Microbiol. Res. 2012;6:6987-6993.
15. Kodati VL, Govindan S, Movva S, Ponnala S, Hasan Q. Role of Shigella infection in endometriosis: A novel hypothesis. Medical Hypotheses. 2008;70(2):239–243.
16. Ojha SC, Yean CY, Ismail A, Singh KB. A Pentaplex PCR assay for the detection and differentiation of Shigella species. BioMed Research International. 2013:9.
17. Zhang L, Wei Q, Han Q, Chen Q, Tai W, Zhang J, Song Y, Xia X. Detection of Shigella in Milk and Clinical Samples by Magnetic Immunocaptured-Loop-Mediated Isothermal Amplification Assay. Front. Microbiol. 2018;9:94.
18. Uchiya K, Tobe T, Komatsu K, Suzuki T, Watarai M, Fukuda I, Yoshikawa M, Sasakawa C. Identification of a novel virulence gene, virA, on the large plasmid of Shigella, involved in invasion and intercellular spreading. Mol. Microbiol. 1995;17(2):241–250.
19. Grimont PAD, Weill FX. Antigenic Formulae of the Salmonella Serovars, 9th Edn. Paris: WHO Collaborating Centre for Reference and Research on Salmonella, Institute Pasteur Broilers in Egypt. Microbial Immunol. 2007;56(4):254-261.
20. Oladipo IC, Tona GO, Akinlabi EE, Bosede OE. Bacteriological quality of raw cow's milk from different dairy farms in Ogbomoso, Nigeria. Int. J. Adv. Res. Biol. Sci. 2016;3(8):1-6.
21. Villalobo E, Torres A. PCR for detection of *Shigella* spp. in mayonnaise. Appl. Environ. Microbiol. 1998;64(4):1242-5.
22. Oliveira SD, Rodenbusch CR, Ce MC, Rocha SLS, Canal CW. Evaluation of selective and non-selective enrichment PCR procedures for Salmonella de-tection. Lett. Appl. Microbiol. 2003;36:217-221.
23. Jiménez KB, McCoy CB, Achí R. Detection of shigella in lettuce by the use of a rapid molecular assay with increased sensitivity. Braz. J. Microbiol. 2010;41(4): 993–1000.
24. Anderson M, Hinds P, Hurditt S, Miller P, McGrowder D, Alexander-Lindo R. The microbial content of unexpired pasteurized milk from selected supermarkets in a developing country. Asian Pac. J. Trop. Biomed. 2011;1(3):205-211.
25. Dwarkanath CT, Srikanta S. Study on the microbiological quality of traditional Indian sweetmeat products. Arogya- J. Health Sci. 1978;4:123.
26. Oliver SP, Jayarao BM, Almeida RA. Foodborne pathogens in milk and the dairy farm environment: Food safety and public health implications. Foodborne Pathogens and Disease. 2005;2(2):115-129.
27. Karshima NS, Pam VA, Bata SI, Dung PA, Paman ND. Isolation of Salmonella Species from milk and locally processed milk products traded for human consumption and associated risk factors in Kanam, Plateau State, Nigeria. J. Anim. Prod. Adv. 2013;3(3):69-74.
28. Kaushik P, Anjay, Kumari S, Bharti SK, Dayal S. Isolation and prevalence of Salmonella from chicken meat and cattle milk collected from local markets of Patna, India. Veterinary World. 2014;7(2):62-65.
29. Singh P, Singh RV, Gupta B, Tripathi SS, Tomar KS, Jain S, Sahni YP. Prevalence study of Salmonella spp. in milk and milk products. Madhya Pradesh, India. Asian Journal of Dairy and Food Research. 2018;37(1):7-12.
30. El-Baz AH, El-Sherbini M, Abdelkhalek A, Al-Ashmawy MA. Prevalence and molecular characterization of Salmonella serovars in milk and cheese in Mansoura city, Egypt. Journal of Advanced Veterinary and Animal Research. 2017;4(1):45-51.
31. Ahmed AM, Shimamoto T. Isolation and molecular characterization of *Salmonella enterica, Escherichia coli* O157:H7 and *Shigella* spp. from meat and dairy products in Egypt. International Journal of Food Microbiology. 2014;168-169:57-62.
32. Gwida MM, AL-Ashmawy MAM. Culture versus PCR for Salmonella species identification in some dairy products and dairy handlers with special concern to its Zoonotic importance. Veterinary Medicine International. 2014:5.
33. Tadesse T, Dabassa A. Prevalence and antimicrobial resistance of Salmonella isolated from raw milk samples collected

from Kersa District, Jimma Zone, Southwest Ethiopia. Journal of Medical Sciences. 2012;12(7):224-228.

34. Kessel V, Sonnier J, Zhao S and Kams JS. Antimicrobial resistance of Salmonella enterica isolates from bulk tank milk and milk filters in United States. J. Food Prot. 2013;76:18-25.
35. El-Kosi OHR. Occurrence of some enteric pathogens and their indicators in some Egyptian raw milk products. Assiut Veterinary Medical Journal. 2001;45(89): 48–60.
36. Bahout AA and Moustafa AH. Occurrence of microorganisms in relation to public health in Kariesh cheese. Assiut Veterinary Medical Journal. 2006;52(11):85–92.
37. Fadel HM and Ismail J. Prevalence and significance of *Staphylococcus aureus* and enterobacteriaceae species in selected dairy products and handlers International Journal of Dairy Science. 2009;4(3):100–108.
38. Yagoub SO, Awadalla NE and El-Zubeir IEM. Incidence of some potential pathogens in raw milk in Khartoum north (Sudan) and their susceptibility to antimicrobial agents. Khartoum, Sudan. Journal of Animal and Veterinary Advances. 2005;4(3):341-344.
39. Reta MA, Bereda TW and Alemu AN. Bacterial contaminations of raw cow's milk consumed at Jigjiga City of Somali Regional State, Eastern Ethiopia. International Journal of Food Contamination. 2016;3:4.
40. Edward KC, Owuamalam PO, Onyekachi OV, Nnochiri OA, Akumah CN. Microbial Quality Assessment of Ice Cream Sold in Umuahia, South-Eastern Nigeria: A Comparative Study. Journal of Biology, Agriculture and Healthcare. 2017;7(16):40-48.
41. Ojokoh AO. Microbiological examination of ice cream sold in Akure, Pakistan. J. Nutrition. 2006;5(6):536 538.
42. Hendriksen RS, Vieira AR, Karlsmose S, Lo Fo Wong DM, Jensen AB, Wegener HC and Aarestrup FM. Global monitoring of Salmonella serovar distribution from the world health organization global food borne infections network country data bank: results of quality assured laboratories from 2001 to 2007. Foodborne Pathogens and Disease. 2011;8(8):887-900.
43. Tambekar DH and Bhutda SA. Prevalence of Bacterial Pathogens in Pedha (A Milk Product) Sold in Amravati (India). International Journal of Dairy Science. 2006;1(1):32-35.
44. Wallace RL. Bacteria counts in raw milk. Dairy cattle Management. 2009;1-4:67-72
45. Chye FY, Abdullah A and Ayob MK. Bacteriological quality and safety of raw milk in Malaysia. Food microbiology. 2004;21(5):535-541.
46. Minj AK and Behera N. A comparative microbiological quality assessment of rural and urban milk samples. African Journal of Food Science. 2012;6(21):519-523.
47. Falegan CR and Akere GA. Isolation of Salmonella spp. in 'WARA' (local cheese) from three different locations in Ado-Ekiti, Ekiti State, Nigeria. 2014;23(4):1628-1634.
48. Herrera-Leon S, McQuiston JR, Usera MA, Fields PI, Garaizar J and Echeita MA. Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of Salmonella spp. Journal of Clinical Microbiology. 2004;42(6):2581-2586.
49. Lampel KA, Orlandi PA and Kornegay L. Improved template preparation for PCR-based assay for detection of food-borne bacterial pathogens. Applied and Environmental Microbiology. 2000;66(10): 539-4542.
50. Galin JE and Curtiss RIII. Cloning and molecular characterization of genes whose products allow Salmonella typhimunum to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA. 1989;86(16):6383-6387.
51. Galin JE and Curtiss RIII. Distribution of the invA, -B, -C, and -D genes of Salmonella typhimurium among other Salmonella serovars: invA mutants of Salmonella typhi are deficient for entry into mammalian cells. Infect. Immun. 1991;59:2901-2908.
52. Ahmed OB, Asghar AH, Abd El-Rahim IH and Hegazy AI. Detection of *Salmonella* in Food Samples by Culture and Polymerase Chain Reaction Methods. J. Bacteriol. Parasitol. 2014;5(3):187.
53. Abu Ud-Din and Wahid S. Relationship among Shigella spp. and enteroinvasive *Escherichia coli* (EIEC) and their differentiation. Braz. Journal of Microbiology. 2014;45(4):1131–1138.
54. Adler B, Sasakawa C, Tobe T, Makino S, Komatsu K and Yoshikawa M. A. Dual

transcriptional activation system for the 230 kb plasmid genes coding for virulence-associated antigens of Shigella flexneri. Mol. Microbiol. 1989;3(5):627–635.

55. Mokhtari W, Nsaibia S, Majouri D, Hassen AB, Gharbi A and Aouni M. Detection and characterization of Shigella species isolated from food and human stool samples in Nabeul, Tunisia, by molecular methods and culture techniques. Journal of Applied Microbiology. 2012;113(1):209–222.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/27108