Molecular characterization of *Salmonella* species isolated from some meat products

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Abstract: The present study was carried out for determining the prevalence of *Salmonella Enterica* as well as screening of virulence genes associated with isolated serotypes from commercial meat product samples by PCR. A total of 200 samples (luncheon, sausage, beef burger, hotdog, kofta meat, chicken meat, basterma and beef) of meat products were collected from different supermarkets and restaurant in Kafr- EL-Sheikh governorate, Egypt. Samples were aseptically processed for the isolation of *Salmonella* by conventional isolation methods. Microbial investigation resulted in the overall isolation percentage of *S. Enterica* was 8.5%. Based on cultural biochemical and serological characteristics, seven different serovars were identified. *S. enteritidis* was the predominant one (47%) followed by *S. typhimurium* (23.52%), *S. infantis, S. haifa, S. virchow, S. muenster* and *S. montevideo* (5.88 % each). All *Salmonella* isolates were screened for the presence of virulence associated genes by PCR. All *Salmonella* isolates were positive for the *inv*A gene. Fimbrial H (*fim*H), hyper-invasive locus (*hil*A), enterotoxin (*stn*) genes were detected in 88.23%, 64.70%, and 41.17% respectively. The present study suggested that *inv*A and *fim*H virulence genes are much conserved in *S. Enterica* isolated from meat products and could use independently as a gene marker for the rapid detection of the virulent strains of *S. Enterica.*

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1. Introduction

Meat and meat products are considered as an excellent source of high quality animal protein, vitamins especially B complex, and certain minerals, especially iron (Gracey, 1986). Also, they are considered as serious sources of food borne diseases and have been frequently linked to major outbreaks of food poisoning all over the world. Possibility of contamination of meat products with food poisoning bacteria especially *Salmonella* organisms has been extensively reported. (Reham, 2004; Erdem *et al.,* 2005 and).

Salmonella species are members of the family *Enterobacteriaceae*, which inhabit the intestinal tract of animals and may be thus recovered from a wide variety of hosts, especially food. Besides, these bacteria may be pathogenic to wild and domestic animals and humans (Holt *et al.,* 1994).

Salmonellosis is a zoonotic bacterial disease of national and international importance. The worldwide distribution of salmonellosis usually parallels the patterns of trade of animal products and food, and the migration patterns of humans and animals (Gilbert *et al.,* 2010). There are more than 2,500 different serovars of *S. Enterica* have been identified and most of them have been described as the cause of human infections. Most reports have mentioned that *S. typhimurium* and *S. enteritidis* are the most common causes of human salmonellosis worldwide (Tavechio *et al.,* 1996). The latest mentioned species were accounted for almost 80% of identified serovars in human in 2006 (Collard *et al.,* 2008).

Salmonella spp. are considered the major cause of foodborne disease in humans, and *S. Enteritidis* is the most frequently isolated serovar in Europe, South America and Asia (Vieira *et al.,* 2009). Phylogenetic analyses show the influence of different factors in the existence and persistence of *Salmonella* spp. in animals, such as cross-contamination among animals, environment and feed (Mello *et al.,* 2011). The ability of *Salmonella* strains to cause disease is attributed to arrays of virulence genes defined in the *Salmonella* pathogenicity islands (SPIs) (Blum *et al.,* 1994). There are at least 60 genes associated with SPIs (Groisman and Ochman, 1997) and the majority of these determinants are located on the chromosome or on large virulence-associated plasmids (Groisman and Ochman, 1996).

Different virulence genes such as *inv*A, *stn*, *fim*H, *hil*A have been identified as major genes responsible for virulence factor in *Salmonella.*

A genetic locus, *inv*, was identified that allows *Salmonella* spp. to enter cultured epithelial cells and responsible for invasion in the gut epithelial tissue of human and animals. *inv*A is a member of this locus and

it is the first gene of an operon consisting of at least two additional invasion genes(Galán, *et al.,* 1992).

It has been proposed that *Salmonella* enterotoxin (*Stn)* is a putative virulence factor and causes an enterotoxic effect on epithelial cells, leading to enteric disorder (Chopra *et al*., 1994; Asten and Dijk, 2005). Interestingly, it has been shown that the *stn* gene is specifically distributed in *Salmonella* spp. irrespective of their serotypes (Lee *et al*., 2009). This second finding indicates that the *stn* gene might be useful for the identification or detection of *Salmonella* and that *Stn* might be involved in functions unique to *Salmonella*. *Fim*H is lectin-like adhesion, located on the tip of the fimbrial shaft, is directly responsible for bacteria binding to oligomannosidic structures carried by many eukaryotic membrane-bound and secreted glycoprotein (Krogfelt *et al.,* 1990). The *hil*A gene encodes the central regulator *hil*A, which is necessary for the expression of the Type three secretion system (TTSS) components. *hil*A is also required to invade epithelial cells and induce apoptosis of macrophages (Bajaj *et al.,*1996). Conventional methods used to detect and identify *Salmonella* including selective enrichment and plating followed by biochemical tests are time consuming (Burtscher *et al.,* 1999; Bennasar, *et al.,* 2000;. On the other hand, polymerase chain Reaction (PCR) is a rapid and reliable method for detection and identification of food borne pathogens such as Salmonella as a complementary to conventional (Chiu and Ou, 1996; Rodulfo *et al.,* 2012). The purpose of this study was to evaluate the potential virulence of *Salmonella* isolates from some meat products by detecting the presence of the *inv*A, *stn*, *fim*H and *hil*A virulence genes using the polymerase chain reaction (PCR).

2. Material and Methods

Sampling

A total of 200 samples (50 meat luncheon, 40 beef burger, 21 sausage, 22 hotdog, 38 kofta meat, 20 chicken meat, 6 basterma and 3 beef) of meat products were collected from different supermarkets and restaurants in Kafr- EL-Sheikh governorate, Egypt. The collected samples were labeled and placed in icebox to keep the samples cool and transferred quickly to the laboratory as soon as possible under complete aseptic condition to prevent cross contamination. This included the use of sterile sampling materials and wearing disposable gloves. The frozen samples were allowed to thaw in the refrigerator (2-7 °C). The samples were immediately examined bacteriologically for the detection of *Salmonellae.*

Isolation and identification of *Salmonella* species

The standard protocol described by ISO 6579 (2002) was adopted for the isolation of *Salmonella* spp. from meat product samples. Briefly, 25 grams of each meat product sample were taken, cut into pieces, using sterile forceps and scissors and blended for two minutes in sterile blender jar containing 225 ml of buffered peptone water (BPW) (0.1%) as a pre-enrichment broth and incubated at 37 °C for 24 hrs. Subsequently, 0.1 ml mixture was transferred to a tube containing 10 ml of the Rappaport Vassiliadis broth (Oxoid) and then incubated at 41.5 ± 1ºC for 24 ± 3hrs. Loopful of enrichment culture was streaked onto Xylose lysine desoxycholate (XLD) (Oxoid) agar plates then incubated at 37 °C for 24 hrs. Typical colonies of *Salmonella* were pink colonies with or without black centers. According to (Edwards and Ewing, 1972) *Salmonella* confirmed by biochemical tests as Triple Sugar Iron (TSI), Lysine decarboxylase (LIA), Urease, Indole, Methyl red, Voges-Proskauer and Simmons citrate utilization. The colonies identified as *Salmonella* were preserved in 20 percent glycerol broth at -20 C for further characterization. According to Kauffman – White scheme (Kauffman, 1974). Typical *Salmonella* isolates were serotyped by a standard slide and tube agglutination test using commercial polyvalent and monovalent O and H antisera (Denka-Seiken, Tokyo, Japan) to identify *Salmonella* serovars.

Molecular examination of *Salmonella* serotypes

Bacterial DNA extraction was done by using QIA amp DNA mini and Blood mini kit according to the manufacturer's instructions. The primer pairs used in PCR protocols for detection of *inv*A, *stn*, *fim*H and *hil*A were selected from published papers based on specificity, compatibility and ability to target the potential virulence genes of interest. The nucleotide sequences and anticipated molecular sizes of PCR amplified fragments for these gene-specific oligonucleotide primer sets are outlined in (Table 1). The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany) using 25 µl of PCR mixture. The reaction mix invariably consisted of 5 µl of the bacterial lysate, 5 µl of 10x assay buffer for Taq polymerase containing 1.5 mM $MgCl_2$, 2 µl of 10mM dNTP mix 1 µl each of forward and reverse primer (10 pmol) and 1.25 U of Taq DNA polymerase made upto 50 µl using sterile distilled water. The PCR cycling protocol was applied as follow: An initial denaturation at 94°C for 60 sec, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 64°C for 30 sec and extension at 72°C for 30 sec, followed by a final extension at 72°C for 7 min. Finally, 5 µl of each amplicon was electrophoresed in 1.5 % agrose gel (Sigma–USA, stained with ethidium bromide and visualized and captured on UV transilluminator.

3. Result

1. Prevalence of *Salmonella* in some meat product samples

A total of 200 meat product samples were collected and processed for isolation and identification of *Salmonella* spp. Based on cultural, morphological, biochemical and serological characteristics. It was observed that 17 (8.5%) samples were found to be positive for various species of *Salmonella*. A total of 17 isolates of *Salmonella* were identified (8.5%) including seven different serotypes. *S. Enteritidis* was the predominant one (8 isolates / 47 %), followed by *S. typhimurium* (4 isolates / 23.52 %), *S. Infantis*, *S. Haifa*, *S. Virchow*, *S. Muenster* and *S. Montevideo* (one isolate / 5.88 % each). Out of 21 susage samples, 2 were found to be positive for *Salmonella*, among them one was *S. Enteritidis* and one was *S. Typhimurium*. Among 40 beef burger samples, 5 *Salmonella* isolates were identified (2 *S. Enteritidis*, 1 *S. Montevideo*, 1 *S. Haifa* and 1 *S. Virchow*). Two *S.* Enteritidis isolates were identified from 22 hot dog samples. Out of 38 Kofta meat samples, 6 *Salmonella* isolates were recovered (3 *S.* Enteritidis, 1 *S.* Typhimurium, 1 *S.* Muenster and 1 *S.* Infantis), while in chicken meat samples, two isolates of *S.* Typhimurium were identified. No *Salmonella* were recovered from luncheon meat, basterma and beef meat samples (Table 2).

Table 1. Oligonucleotide primer sequences used for genotyping of *Salmonella* serovars

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
*inv*A (F)	5′ GTGAAATTATCGCCACGTTCGGGCA ′3	284	Shanmugasamy *et al.* (2011)
*inv*A (R)	5′ TCATCGCACCGTCAAAGGAACC ′3		
Stn (F)	5′ CTTTGGTCGTAAAATAAGGCG ′3	260	Makino *et al.* (1999)
stn (R)	5′ TGCCCAAAGCAGAGAGATTC ′3		
*hil*A (F)	5′ CTGCCGCAGTGTTAAGGATA ′3	497	Guo *et al.* (2000)
*hil*A (R)	5′ CTGTCGCCTTAATCGCATGT ′3		
*fim*H (F)	5′ GGA TCC ATG AAA ATA TAC TC ′3	1008	Menghistu (2010)
*fim*H (R)	5′ AAG CTT TTA ATC ATA ATC GAC TC ′3		

invasion A (invA), Enterotoxin (stn), hyper-invasive locus (hilA) and fimbrial (fimH) genes

Table 2. Prevalence of salmonella spp in various meat product samples

Type of samples	Total sample processed	Total positive	*Salmonella* isolates					
			Serotypes	Positive cases	Percentage	Group	Antigenic structure	
							O	H
Luncheon	50	-	-	-	-	-	-	-
Sausage	21	2	*S. enteritidis* *S. typhimurium*	1 1	4.8 4.8	D1 B	1,9,12 1,4,5,12	g,m: - i: 1,2
Beef burger	40	5	*S. enteritidis* *S. montevideo* *S. virchow* *S. Haifa*	2 1 1 1	5 2.5 2.5 2.5	D1 C1 C1 B	1,9,12 6,7,14 6,7,14 1,4,5,12	g,m: - g,m,s: 1,2,7 r: 1,2 Z10: 1,2
Hot dog	22	2	*S. enteritidis*	2	9.1	D1	1,9,12	g,m: -
Kofta meat	38	6	*S. enteritidis* *S. typhimurium* *S. infantis* *S. muenster*	3 1 1 1	7.9 2.6 2.6 2.6	D1 B C1 E1	1,9,12 1,4,5,12 6,7 3,10,15,34	g,m: - i: 1,2 r: 1,5 e,h: 1,5
Chicken meat	20	2	*S. typhimurium*	2	10	B	1,4,5,12	i: 1,2
Basterma meat	6		-	-	-	-	-	
Beef meat	3		-	-	-	-	-	

2. Molecular detection of *Salmonella* virulence associated genes*:*

A total of 17 *Salmonella* isolates were screened by multiplex PCR to detect four virulence genes *inv*A, *stn*, *hil*A and *fim*H (284 bp, 260 bp, 497 bp, 1008 bp) respectively. The results showed that *inv*A gene was the most prevalent one (100%) in all serovars, followed by *fim*H (88.23%) present in 15 serovars (8 *S. enteritidis*, 3 *S. typhimurium*, 1 *S. Infantis,* 1 *S. Virchow,* 1 *S. Muenster* and 1 *S. Montevideo*), *hil*A (64.70%) present in 11 serovars (5 *S. Enteritidis*, 3 *S. Typhimurium*, 1 *S. Virchow,* 1 *S. Infantis* and 1 *S. Muenster*) and *stn* in 7 serovars (41.17%) present in (2 *S. Enteritidis,* 3 *S. Typhimurium*, 1 *S. Virchow* and 1*S. Muenster*) as shown in figure 1, table 3.

Table 3 virulence genes present in different serovars of *Salmonella*

Salmonella Serotypes	No	Virulence genes			
		*inv*A	*fim*H	*hil*A	*Stn*
S. Enteritidis	8	8	8	5	2
S. Typhimurium	4	4	3	3	3
S. Infantis	1	1	1	1	0
S. Virchow	1	1	1	1	1
S. Haifa	1	1	0	0	0
S. Muenster	1	1	1	1	1
S. Montevideo	1	1	1	0	0

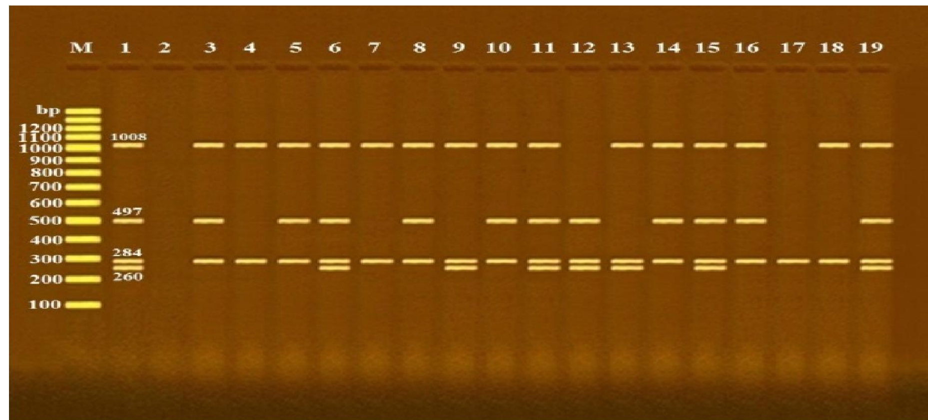

Figure (1): Agarose gel electrophoresis of multiplex PCR of stn (260 bp), invA (284 bp), hilA (497 bp) and fimH (1008 bp) virulence genes for characterization of *Salmonella* species. Lane M: 100 bp ladder as molecular size DNA marker, Lane 1: Control positive strain for *stn*, *inv*A, *hil*A and *fim*H genes, Lane 2: Control negative, Lanes (3 – 10): *S. Enteritidis* & lanes (11-14)*: S. Typhimurium* & lane (15): *S. Virchow* & lane (16): *S. Infantis* & lane (17): *S. Haifa* & lane (18): *S. Montevideo* & lane (19): *S. Muenster*.

4. Discussion

Salmonellosis is considered one of the anthropozoonotic diseases of a serious medical problem and raises great concern in the food industry (Ashton, 1990). Meat products such as minced meat, sausage and beef burger have popularity because they represent quick and easy prepared meat meals and solve the problem of the shortage in fresh meat of high price, which is not within the reach of large number of societies with limited income. Despite of the traditional food hygiene efforts for eliminating of agents responsible for food borne illness, *Salmonella* remains as one of the major food borne health hazards, and meat and meat products plays an important role, as a reservoir, in disseminating *Salmonellae.* (Mohammed, 2000).

In the current study, the overall incidence of *Salmonella* was 8.5 % of the all examined meat product samples.

Our results are in agreement with the earlier findings reported from different geographical regions (Iraqui, 2002; Siriken *et al.,* 2006; Cortez *et al.*, 2008) with an incidence of 8%, 7% and 7.5% respectively. However, other investigators (Eleiwa, 2003; Mrema *et al.,* 2004; Ejeta *et al.* 2004) have reported 12%, 20% and 14.4% prevalence of *Salmonella* respectively. On the other hand, there are also other studies that have found lower frequencies of salmonella isolated from meat products (Zaki, 2003; Bosilevac, 2009; Abd El-Tawab *et al.,* 2015) with an incidence of 5%, 4.2% and 1.33% respectively.

The high prevalence of *Salmonella* in meat products may indicate contamination of meat during cutting or processing as well as contamination from grinders, air, packaging materials and hands of the workers. Temperature rise (2 – 4°C) during grinding could also increase the incidence of *Salmonella* organisms, (Ismail, 2006)**.**

In the present investigation, *Salmonellae* serotypes isolated from meat product samples were identified serologically. *S. Enteritidis* was the most predominant pathogenic serotypes detected with an incidence of 47% of the all *S. Enterica* isolates. This result differs from other previous studies (Iraqui, 2002; Molla *et al.,* 2003; Charles Hernades *et al.,* 2007) with an incidence of 2%, 12.1% and 20% respectively. *S. Typhimiurium* was the second predominant serotypes (23.52%) of the all *Salmonella* isolates. In contrast to our result, lower prevalence of *S. Typhimurium* was reported by Essam (2010), Edris *et al.* (2011) and (Ahmed and Shimamoto, 2013) with an incidence of 8%, 8% and 2.5% respectively.

With regard to other recovered *Salmonella* serotypes, one isolate for each *S. Infantis*, *S.* Haifa, *S. Virchow S. Montevideo* and *S. Muenster* was identified with an incidence of 5.88%. other previous studies higher prevalence rate for *S. Infantis* (Ejeta *et al.,* 2004), *S. Haifa* (Karmi, 2014; Hassanin *et al.,* 2014), *S.* Virchow (Olsen *et al.,* 2001), while similar prevalence rate for *S. Montevideo* and *S. Muenster* was reported by Faten, *et al.* (2015). No *Salmonella* were recovered from luncheon, beef meat or Basterma meat samples. These results confirm the finding reported by Reham (2004), Ismail (2008) and Mohamed (2009). Absence of *Salmonella* in these meat products may be due to the addition of food additives such as spices and preservatives, which have an antimicrobial activity and inhibit survival and multiplication of micro-organisms (Libby, 1975).

In the present study, a total of 17 *Salmonella* serotypes were screened for the presence of four virulence genes, including *inv*A, *fim*H, *hil*A and *stn* by using multiplex PCR. The virulence profile of *Salmonella* isolates is shown in Table 3.

Our results revealed that *inv*A was the most prevalent in all *S. Enterica* isolates (100%), thus, all the *Salmonella* isolates were found highly invasive. This finding was consistent with many previous reports (Abouzeed *et al.,* 2000; Chuanchuen 2010; Dione *et al.,* 2011) that established the presence of *inv*A gene in nearly all *Salmonella* irrespective of serovar or source. However, Kumarss *et al.* (2010) reported a lower percentage of *inv*A in *Salmonella* isolates recovered from meat products (11.4%).

Based on the results obtained in our study, *hil*A gene was detected with an incidence 64.70% of all *Salmonella* serotypes, this in contrast to Ammar et al. (2016) who detected *hil*A in 88.24%. However, other studies (Amini *et al.*, 2010; Campioni *et al.,* 2012; Craciunas *et al.,* 2012) concluded that *inv*A and *hil*A genes would be detected in all of the isolates due to their importance for cell invasion.

With regard to *stn* gene, it was detected in 41.17% of all examined *Salmonella* serotypes. This in contrast to the data described by Murugkar *et al.* (2003) and Ammar *et al.* (2016), who reported higher prevalence of *stn* gene (100% and 58.82%) respectively. In the present study, *fim*H was detected with an incidence of 88.23%. Similar higher incidence rate was also reported by Borowsky *et al.* (2009).

Conclusion

The obtained results in the present study revealed that meat products contaminated with pathogenic *Salmonellae* and the more prevalent serotypes were *S.* Enteritidis *and S.* Typhimurium and the lesser prevalent isolates were *S.* Heifa*, S.* Infantis*, S.* Virchow*, S.* Montevideo and *S.* Muenster. Results showed that *Salmonella* possesses genes that are responsible for virulence and establishment of infection in host cells. One of such genes is *inv*A gene, which is the most prevalent in our study followed by *fim*H, *hil*A and *stn*. PCR is a useful tool for the rapid detection of *Salmonella* spp., and *inv*A and *hilA* genes can be considered target genes for the detection of this genus. The frequency of these genes may predict the ability of virulence of different strains. On the other hand, careful handling, preparing, washing, cleaning, and all personal hygiene awareness would help to minimize such contamination and subsequently prevent consumers from food borne illness.

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