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ERIC-PCR Fingerprinting of Some S. Typhimurium Isolates from Chicken and Humans with Reference to the Microbiological Quality of Retail Chicken Meat in Dakahlia, Egypt

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Abstract: The current study describes the isolation of *Salmonella* species from 100 cloacal swabs and 100 retail chicken meat samples in local pluck-shops at Dakahlia, Egypt. Human workers at the shops were also examined for *Salmonella* species by collecting 50 hand and stool swabs, each. The results showed that *Salmonella* species were identified in 12%, 26%, 4% and 6% of cloacal swabs, meat, hand swabs and stool samples, respectively. Serological and molecular identification showed that *Salmonella typhimurium* (S. Typhimurium) was the only serotype identified from all the examined sources and it accounted for 16 isolates. enterobacterial repetitive intergenic consensus- polymerase chain reactions (ERIC-PCR) fingerprinting of S. Typhimurium isolates produced five clusters indicating the possibility of the serotype transmission between chicken and humans. The discrimination power of ERIC-PCR was high (D=0.966) dividing S. Typhimurium isolates to 12 profiles. The microbiological quality of the examined chicken meat samples was also investigated using aerobic plate, coliform and *Enterobacteriaceae* counts. The results showed the need for improving the hygienic measures during slaughtering, processing and handling of chicken meat.

Key words: S. Typhimurium · ERIC-PCR · Fingerprinting · Chicken · Humans · Egypt

INTRODUCTION

Poultry industry is one of the fastest growing sector of the animal industry in Egypt and worldwide. Consumption of poultry meat has been implicated in food borne outbreaks, therefore, contamination of chicken meat during processing, handling and transportation is of public health importance [1]. The alimentary tract, skin and feather of poultry are considered the most important sources of food borne bacteria, contamination of meat is then possible during slaughter, feather plucking, evisceration and handling [2]. *Salmonella* species has been the leading cause of food borne outbreaks and is considered one of the major causes of human gastroenteritis worldwide [3, 4]. The detection of *Salmonella* species in poultry production is an issue of great concern, since control of this zoonotic disease is in part based on the reduction of the prevalence at the farm level [5]. Amongst more than 2500 serotype, S. Typhimurium has been reported to be the most frequently serovar isolated from humans worldwide accounting for 46% of outbreaks [1, 6].

Serotyping has been shown to have a limited value as an epidemiological tool to trace the source of infection with a low discriminatory power [7, 8]. Molecular typing methods such as repetitive sequence-based polymerase chain reactions (rep-PCR) produce epidemiological markers to trace clonal strains or to relate outbreaks to the source of contamination [8]. The rep-PCR relies on the amplification of highly conserved, short repetitive sequences in the genome of *Enterobacteriaceae*, which include the repetitive extragenic palendronic elements (REP), the enterobacterial repetitive intergenic consensus (ERIC) sequences and the BOX sequences [9]. Based on

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the amplification of the DNA sequences by ERIC or REP primers, it was possible to discriminate between closely related serovars and strains of the same species [9, 10]. ERIC-PCR was chosen over other molecular typing methods during the current study because it has been found to be extremely reliable, reproducible and rapid; furthermore, it offers high discrimination index [10].

The microbiological quality of chicken meat is usually monitored by evaluation of certain parameters such as aerobic plate count (APC), *Enterobacteriaceae* count and most probable number (MPN) of coliforms [2]. These parameters are considered indicators to assess the processing hygiene and storage quality to predict the risk of chicken meat consumption [11].

The aim of the current study was to determine the prevalence of *Salmonella* species in chicken cloacal swabs), freshly slaughtered chicken and in workers in contact with chicken at pluck-shop markets, Dakahlia, Egypt. The possible sources of infection with S. Typhimurium were traced by generating molecular markers using ERIC-PCR to assess the possible relationship among the strains obtained from different sources. Moreover, the microbiological quality of retail chicken meat was investigated.

MATERIALS AND METHODS

Sampling: A total of 100 chicken cloacal swabs and 100 fresh chicken meat samples (breast meat fillet without skin) were collected from local pluck-shop markets at Dakahlia, Egypt. Chicken meat samples were collected directly after the slaughter and evisceration process. Workers at the pluck-shop markets were also examined by collecting 50 hand swabs and stool samples, each. The workers are responsible for slaughtering, evisceration and preparation of chicken meat for marketing.

Sample Preparation

Cloacal, Hand Swabs and Stool Samples: Moistened sterile swabs were used for collection of cloacal swabs, hand swabs and human stool samples. The swabs were then directly inserted into 0.1% peptone water tubes under aseptic conditions and pre-enriched at 37°C for 24 hours.

Chicken Meat Samples: Twenty five grams from chicken meat samples were aseptically transferred to a sterile blender containing 225 ml of 0.1% peptone water for homogenization and pre-enrichment at 37°C for 24 hours. The homogenate of each sample was also used for preparation of serial dilutions for APC, *Enterobacteriaceae* and MPN counts. One ml of the homogenate were transferred into sterile test tube contained 9 ml of 0.1% peptone water, then ten folds serial dilution were prepared up to the required dilution [12].

Bacteriological Examination

Isolation and Identification of Salmonella Species: The isolation and identification of *Salmonella* species were done according to ISO 6579 method [13; 14]. The suspected *Salmonella* colonies were subjected to Gram staining and biochemical identification using oxidase test, hydrolysis of urea, H_2S production and lysine decarboxylation [15]. The biochemically identified *Salmonella* isolates were then subjected to serotyping following Kauffman-White Scheme with commercial antisera (Difco Laboratories Deteroeit, Mitchigeu, USA) for cell wall (O) and flagellar (H) antigen identification [16]. Serological identification was carried out at Animal Health Research Institute, Dokki, Giza.

Molecular Examination

DNA Extraction: DNA extraction from biochemically identified *Salmonella* isolates was performed using bacterial DNA extraction kit (Spin-Column) (BioTeke Corporation, Shanghai, China) according to the manufacturer guidelines.

Salmonella Multiplex PCR: For molecular identification of the most important zoonotic *Salmonella* species (S. Typhimurium and *Salmonella enteritidis*), a multiplex PCR was carried out [17]. Oligonucleotide primers (manufactured by AlphaDNA, Montreal, Quebec, Canada) specific for *Salmonella* species [18], S. Typhimurium [19] and S. Enteritidis [20] producing 204 bp, 401 bp and 304 bp, respectively were used. Positive controls of S. Typhimurium and S. Enteritidis were kindly obtained from Bacteriology Department, Faculty of Veterinary Medicne, Kafr-Elsheikh University.

ERIC-PCR: In order to determine the genetic relationship between the molecularly identified S. Typhimurium isolates from different sources, ERIC-PCR was carried out [21].

Analysis of ERIC-PCR Amplicon Patterns: ERIC-PCR fingerprinting data were transformed into binary code depending on the presence or absence of each band. Similarity between profiles was determined using the Jaccard coefficient [22] and dendrogram was generated by

unweighted pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering routine. The cluster analysis and dendrogram construction were performed with SPSS Inc. version X.

Discriminatory Power of ERIC-PCR: The discriminatory power of ERIC-PCR was measured by the Simpson's index of diversity (*D*) that indicates the average probability that a typing system will assign a different type to two unrelated strains randomly sampled from a population [23]. A *D* value of more than 0.9 indicates good differentiation.

Microbiological Quality of Chicken Meat

Aerobic Plate Count: Spreading of 0.1 ml of each previously prepared serial dilution of the homogenate onto duplicate sterile plates of pre-poured and dried standard plate count agar (Oxoid, CM463, Adelaide, Australia), then the plates were incubated for 48 h at 35°C [24]. The total APC per gram sample was calculated according to the following equation: total APC= number of colonies x dilution factor. The total APC was presented as colony forming units (CFU/g).

Enterobacteriaceae Count: *Enterobacteriaceae* were enumerated by the pour-plating method on violet red bile glucose agar (VRBG; Difco Laboratories Inc., Detroit, Michigan, USA) using the serial dilutions of the sample homogenates [12]. The total *Enterobacteriaceae* count was presented as colony forming units (CFU/g).

Most Probable Number of Coliforms (MPN): The most probable number of coliforms was performed by preparing test tubes with 9 ml of sterile MacConky broth (Oxoid, CM 5a, Adelaide, Australia) containing inverted Durham's tube for gas collection [25]. Positive tubes with acid and gas production were recorded, for each dilution, the results were presented as a fraction as follows: number of positive tubes/number of inoculated tubes. The MPN was then estimated using MPN index [26] and the concentration of coliform bacteria was presented as MPN/g of the sample.

RESULTS

Prevalence of *Salmonella* Species: The prevalence rates of *Salmonella* species in different samples are summarized in Table 1. Overall, 43 *Salmonella* isolates (14.3%) were identified from 300 samples collected from different sources. The isolation rate of *Salmonella* species from chicken meat samples was 26% followed by 12% from cloacal swabs. Out of 50 hand swabs from human workers at the chicken pluck-shop markets, 4% were positive for *Salmonella* species, while 6% of stool samples from the same workers were positive (Table 1).

Serotyping and PCR Confirmation: Different serotypes were identified (data not shown), S. Typhimurium was the most frequently identified serotype from the examined samples comprising 16 isolates (Table 1), while no S. Enteritidis was detected. For confirmation of the serotyping results, PCR for identification of S. Typhimurium was performed and the results showed the confirmation of the 16 isolates as S. Typhimurium serotype (Figure 1 and Table 1).

ERIC-PCR Fingerprinting: *Salmonella typhimurium* was the only serotype identified from all the examined sources (Table 1), therefore it was subjected to ERIC-PCR fingerprinting to evaluate the genetic relationship between the isolates from chicken and humans. ERIC-PCR profiles were discriminated by the number and position of amplified fragments. Visual comparison of the banding patterns of S. Typhimurium DNA revealed multiple DNA fragments ranged in sizes between 100 and 1200 bp (Figure 2). Two small size common bands of about 170 bp and 220 bp were observed in all 16 isolates. ERIC-PCR primer sets produced 12 profiles (referred to as E1 to E12). Table 2 shows the number and source of isolates in each profile. The discriminatory power of ERIC-PCR was calculated to be 0.966 by the Simpson's index of diversity (*D*).

The dendrogram analysis of the examined isolates showed five clusters and two separate isolates (Figure 3 and Table 2). Two isolates from chicken meat and two human isolates (from hand swab and stool, one isolate, each) fall in the same cluster (cluster I) showing genetic relationship. The similarity index between profile E1 and E2 was 86%, E1 and E3 was 83%, while the similarity between E2 and E3 was 71%. Profiles E4 (one isolate from cloacal swabs) and E5 (one isolate from chicken meat) formed one cluster with 89% similarity. Cluster III contained profiles E6 (one isolate from meat and stool, each) and E7 (one isolate from cloacal swabs), with a similarity index of 90%. Profiles E8 (one isolate from cloacal swabs and human stool each) and E9 (one isolate from hand swabs) formed one cluster with a similarity index of 78%. The results showed that one human stool

Table 1: Prevalence of *Salmonella* species in the examined samples

Type of samples	Number examined	Number positive [%(CI)]	S. Typhimurium (number)
Cloacal swabs	100	12 [12% (6.4-20)]	5
Chicken meat	100	26 [26% (17.7-35.7)]	6
Human hand swabs	50	2 [4% (5-13.7)]	2
Human stool	50	3 [6% (1.3-16.5)]	3
Total	300	43 [14.3% (10.6-13.8)]	16

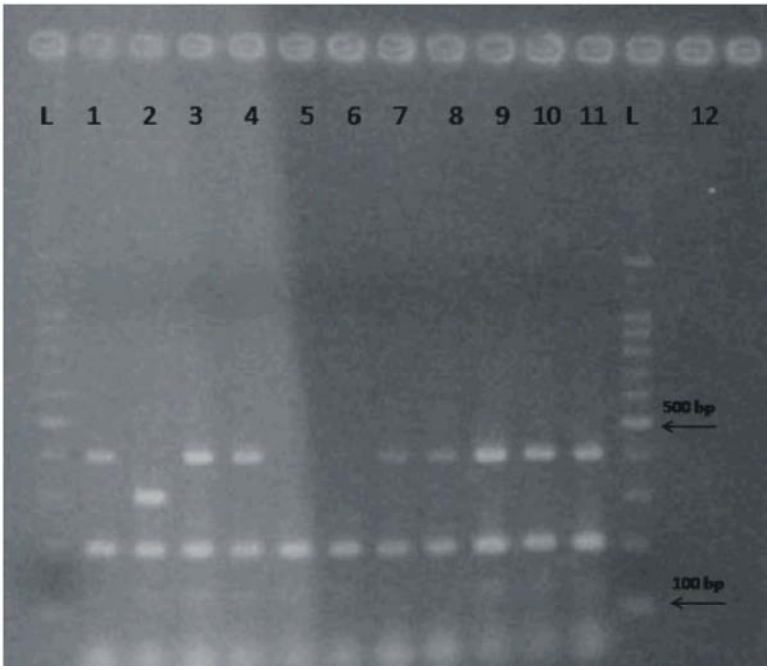

Fig. 1: PCR results of *Salmonella* multiplex PCR in 1.2% agarose gel (L: 100 bp ladder, 1: S. Typhimurium positive control, 2: S. Enteritidis positive control, [3-4 and 7-11]: S. Typhimurium positive samples, [5-6]: *Salmonella* species positive samples, 12: Negative control)

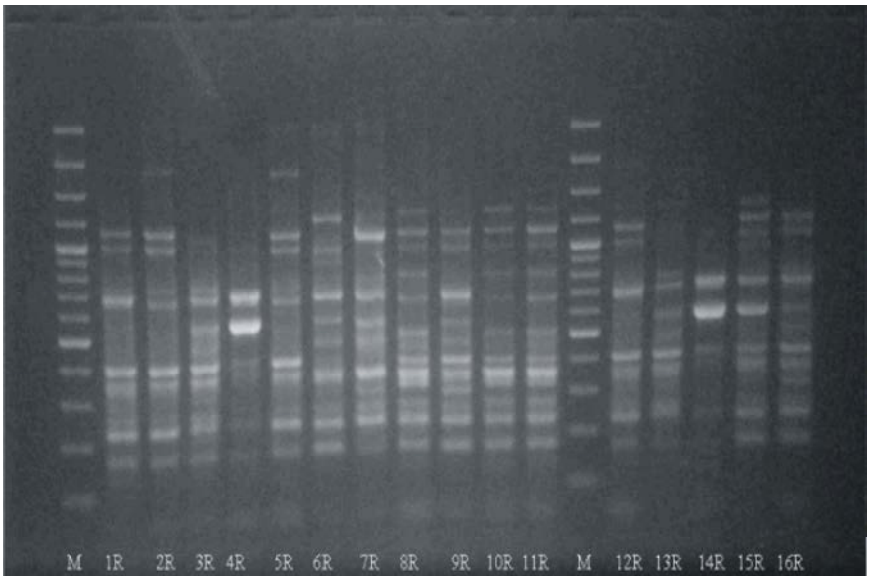

Fig. 2: PCR results of S. Typhimurium ERIC-PCR profiles in 1.2% agarose gel (M: 100 bp ladder, 1R-16R: S. Typhimurium isolates)

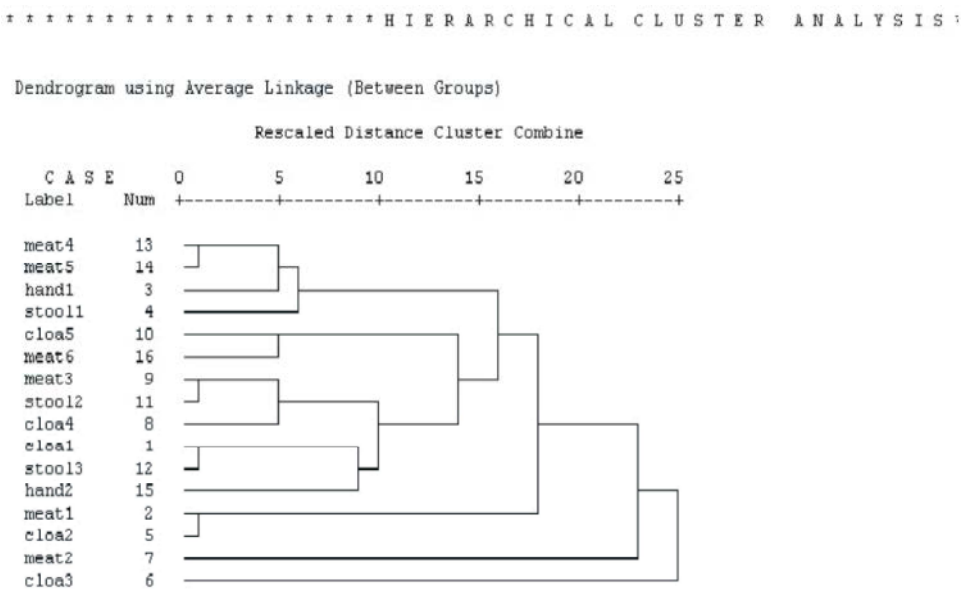

Fig. 3: Dendrogram showing the relatedness of S. Typhimurium strains isolated from human (hand, stool), chicken meat (meat) and chicken cloacal swabs (cloa) as determined by the DNA fingerprint analysis performed by ERIC-PCR using the SPSS computer software program

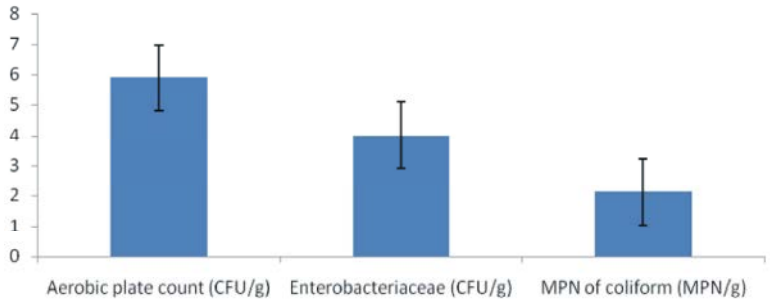

Fig. 4: Mean log APC, Mean log *Enterobacteriaceae* and Mean log MPN per gram of examined chicken meat samples, error bars show standard deviation

Table 2: ERIC-PCR fingerprinting profiles and associated clusters

Profile	Number of isolates	Source	Cluster
E1	2	Chicken meat	Cluster I
E2	1	Hand swab	
E3	1	Human stool	
E4	1	Cloacal swab	Cluster II
E5	1	Chicken meat	
E6	2	Chicken meat, Human stool	Cluster III
E7	1	Cloacal swab	
E8	2	Cloacal swab, Human stool	Cluster IV
E9	1	Hand swab	
E10	2	Chicken meat, Cloacal swab	Cluster V
E11	1	Chicken meat	Single isolate
E12	1	Cloacal swab	Single isolate

isolate shared identical banding profile with chicken meat isolate (profile E6). Also, another human stool isolate shared the same profile with cloacal swab isolate (profile E8).

Microbiological Quality: The results illustrated in Figure 4 shows that APC of the examined chicken meat samples ranged from 4.6 to 6.9 with a mean ±SD value of 5.89 ± 0.72 $\log_{10}$ CFU/g. The count of *Enterobacteriaceae* in the examined samples ranged from 3.3 to 4.6 with an average of 4 ± 0.45 $\log_{10}$ CFU/g, while MPN of coliforms ranged from 0.95 to 2.95 with a mean of 2.13±0.75 MPN/ g.

DISCUSSION

Salmonella is an important zoonotic pathogen acquired mainly due to consumption of contaminated food especially poultry meat [3]. The worldwide incidence of nontyphoidal salmonellosis is estimated to be 1.3 billion cases and 3 million deaths annually [27, 28]. The obtained results showed that *Salmonella* species were isolated from 12% of chicken cloacal swabs (Table 1).

This finding was consistent with 12.4% prevalence rate of *Salmonella* species recovered from chicken cloacal swabs in poultry farms at Beni-Suef, Egypt [29]. Also, nearly similar results (11.4%) were reported in Alexandria from living layer flocks in poultry farms [30].

Contamination of chicken meat at retail markets could be originated from the intestinal contents of the carcass during evisceration, defeathering process, handling and cross-contamination during storage [31]. The current study revealed that 26% of retail chicken meat samples examined were contaminated with *Salmonella* species. These results were comparable with a prevalence rate of 26.4% in Ireland [32], 27% in Russia [33], 27% in Colombia [34], 29% in UK [35] and 29.3% in India [36]. Lower isolation rate of 4.4% was reported in retail chicken meat samples in Egypt [6]. Moreover, the obtained isolation rate of *Salmonella* contamination of chicken meat is lower than 35.8% in Spain [37], 36.5% in Belgium [38] and 60% in Portugal [39]. The obtained results revealed a significantly higher prevalence rate of *Salmonella* species in chicken meat compared to cloacal swabs ($p=0.1$) that highlights the importance of chicken meat as a vehicle for *Salmonella* transmission to humans [32]. The relatively high isolation rate of *Salmonella* from chicken carcasses during the current study could be attributed to the fact that in Egypt, most of chicken are sold in pluck-shop markets that devoid hygienic measures leading to increased chances for contamination of slaughtered chicken carcasses with *Salmonella* species.

Human workers at the pluck-shop markets were apparently healthy; however, examination of stool samples revealed that 6% were carriers for *Salmonella* species (Table 1). The obtained proportion from human healthy carriers was lower than 12% [40] and 35% [41] isolation rates from apparently healthy personnel in Thailand. Moreover, higher isolation rate of 46.4% was reported from human stool samples in contact with poultry in Pakistan [42], while, in Mexico, 13.3% of human stool samples were found positive for *Salmonella* species, with S. Typhimurium predominating [43]. In Nigeria, stool samples from workers in poultry farms showed 0.5% prevalence of *Salmonella* species, of which, two were identified as S. Typhimurium [44]. Differences in the obtained isolation rate of *Salmonella* species from humans in the current study compared to the aforementioned results could be attributed to several factors such as sampling methods, isolation and identification protocols and the degree of human contact with poultry and other animals [41].

Hand swabs from workers at the pluck-shop markets were also examined for *Salmonella* contamination in order to investigate the role of workers in cross contamination of chicken meat with *Salmonella* species during slaughtering and evisceration process. The results showed that 4% of hand swabs were positive for *Salmonella* species and only S. Typhimurium serotype was identified. Nearly similar isolation rate of 3.1% from hand swabs of humans in contact with poultry was previously reported in Sharkia, Egypt [45]. Also, other studies in Egypt documented the isolation of *Salmonella* species from 8.9% [29] and 8.3% [46] of hand swabs from human in contact with poultry. Lower isolation rate of 1.8% was also reported in USA from hand swabs of humans in contact with poultry and poultry products [47].

The isolation of S. Typhimurium from different sources provide evidence that contact with poultry and consumption of poultry meat could pose health hazards for humans [44]. In order to investigate the genetic relationship between *Salmonella* isolates from different sources to trace back the source of infection and to investigate the possibility of transmission of certain genotype to humans from chicken, S. Typhimurium was chosen and subjected to ERIC-PCR genotyping. The choice of S. Typhimurium strain was for the reason that this was the only serotypes isolated from all the sources under investigation.

ERIC-PCR fingerprinting has been shown to have a greater discriminatory power, together with the advantages of being simple and fast method for relating bacterial strains associated with food-borne epidemics [8]. The obtained results showed high discriminatory power of ERIC-PCR (D=0.966) dividing S. Typhimurium in to 12 profiles and five clusters (Table 2). In accordance, several studies have reported the potential of rep-PCR with high discriminatory power in the epidemiological studies of bacterial strains [8; 48]. For instance, great heterogeneity in 31 S. Enteritidis was reported indicating high discriminatory power (0.985) of ERIC-PCR and usefulness of the method for epidemiological evaluation of S. Enteritidis isolates in Poland [10]. Moreover, a high discriminatory power of ERIC-PCR was found in 13 S. Enteritidis isolates dividing these isolates in to six clusters [3]. Also, the obtained results in the current study is consistent with a study reported that the obtained ERIC-PCR fingerprints from 89 *Salmonella* isolates of 22 serotypes did not correlate with serotypes, indicating that ERIC-PCR was able to discriminate among *Salmonella* isolates sharing similar serotypes [49].

In contrast, a study was performed on 65 *Salmonella* isolates of 49 serotypes and the authors concluded that the isolates within one serotype produced the same profile [50]. Furthermore, Millemann *et al.* [51] evaluated ERIC-PCR on 56 S. Typhimurium and S. Enteritidis isolates. They reported that ERIC-PCR was not able to discriminate serotypes, since all S. Enteritidis isolates and some S. Typhimurium isolates shared the same fingerprint [3, 51]. A possible explanation for the discrepancy of the aforementioned studies could be the PCR conditions, some studies reported that elevated annealing temperatures of primers improved the reproducibility and resolving power of rep-PCR [3].

The presence of two common bands at 170 bp and 220 bp in all 16 isolates indicate that these two band profiles are strain specific for S. Typhimurium (Figure 2). This observation was also reported for S. Typhimurium isolates from human and food sources in Mauritius [8].

Raw poultry meat is believed to be vehicles for *Salmonella* transmission and when food safety precautions are inadequate, there may be a serious risk of salmonellosis outbreaks [8, 52]. The presence of some S. Typhimurium isolates from chicken meat, cloacal swabs and humans in the same clusters (Figure 3 and Table 2) sharing a degree of similarity indicates the possibility of the transmission of such strain to humans due to consumption of contaminated chicken meat from the gut of birds during slaughter [53]. These findings were consistent with the reported similarity between some *Salmonella* isolates originated from humans and food sources [8]. Moreover, another study reported a high degree of similarity between *Salmonella* isolates from humans and one isolate from a turkey [54].

The microbiological quality of retail chicken meat was assessed in the current study by determination of aerobic plate count, *Enterobacteriaceae* count and most probable number of coliforms. The results of aerobic plate count ranged from 4.6 to 6.9 with a mean value of 5.89 ± 0.72 $\log_{10}$ CFU/g of examined chicken muscle (Figure 4). Our results coincide with 5.13 $\log_{10}$ CFU/g reported in Egypt [55] and 5.19 $\log_{10}$ CFU/g in Spain [11]. However, higher APC levels of 6.6 $\log_{10}$ CFU/g in Morocco [56] and 6.5 $\log_{10}$ CFU/g in Kuwait [57] were observed. A possible explanation of the aforementioned higher levels of APC than the current study could be the sampling of breast meat with neck skin [56, 57], due to the fact that breast skin seems to be presenting a high degree of bacterial contamination [58]. However, during the current study, only breast meat samples without skin were examined. Moreover, the studies in Kuwait and Morocco evaluated the hygienic quality of chicken meat after refrigeration, while, in the present study, the examined fresh chicken meat samples were collected directly after the slaughtering and evisceration process. The higher APC levels in chicken meat during refrigeration storage are expected because psychrotrophs (bacteria capable of multiplying during refrigeration) are included in APC [11, 59]. Although there are no established microbiological norms for raw chicken meat in Egypt, the obtained levels of APC are within the marginally acceptable level compared to the maximum contamination levels (6 $\log_{10}$ CFU/g) reported in Spain and USA [60; 61].

The average number of *Enterobacteriaceae* in the examined samples was 4.0 ± 0.45 log10 CFU/g (Figure 4). These results are higher than 2.92 ± 0.70 $\log_{10}$ CFU/g in Turkish chicken meat [62] and 2.58-3.53 $\log_{10}$ CFU/g in retail cut chicken meat in Spain [11]. However, higher levels of 4.6 $\log_{10}$ CFU/g were reported in Kuwait [57]. The mean counts of *Enterobacteriaceae* in the present study exceeded the acceptable limits of 2 $\log_{10}$ CFU/g when compared to Spanish regulations [60].

In order to explain the discrepancy between obtaining marginally accepted level of APC and unacceptable level of *Enterobacteriaceae* count in the present study, low percentages of *Enterobacteriaceae* count (≤ 1%) within APC has been previously reported indicating low correlation between APC and *Enterobacteriaceae* count [11; 57].

The presence of coliforms in meat may be responsible for their substandard quality resulting in economic losses and their presence in great number reflects a public health hazard [12]. In the current study, the MPN of coliforms ranged from 0.95 to 2.95 with average of 2.13 ± 0.75 MPN/g (Figure 4). The obtained results are comparable with 2 $\log_{10}$ CFU/g detected in Turkish chicken meat [11], while higher MPN of 3.6 $\log_{10}$ CFU/g [57] and 3 $\log_{10}$ CFU/g [61] were also reported.

In conclusion, the current study reported the isolation of *Salmonella* species from retail chicken breast fillet at Dakahlia, Egypt. Fingerprinting of S. Typhimurium isolates identified from chicken and human sources indicates genetic relationship between the isolates, which in turn suggest the possibility of transmission of the organism between chicken and humans. ERIC-PCR is of high discriminatory power for molecular and epidemiological investigation of genetic relatedness among S. Typhimurium isolates, however, investigation of greater and more diverse *Salmonella* isolates is recommended. The microbiological quality of the examined chicken meat samples was also investigated and indicated the need for improving the hygienic parameters during processing and handling.

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