*Plant Archives* Vol. 21, Supplement 1, 2021 pp. 2501-2512 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
doi link : https://doi.org/10.51470/PLANTARCHIVES.2021.v21.S1.407

# BIOCHEMICAL STUDIES IN FOOD SPOILAGE VIA MOLECULAR DETECTION OF SOME *ENTEROBACTERIACEAE* FOOD BORNE BACTERIA USING MALDI-TOF

**Heba M.Karmy[1], Mahfouz A.Abdelaziz[2], Doaa M. Ghaith[3], Nashwa M.Helmy[1] and Kamla M. El–Saied[4]**

[1.]Department of Biotechnology, Animal Health Research Institute, Giza 12618, Egypt
[2.]Department of chemistry, faculty of science, Cairo University, Giza, A.R. Egypt
[3.]Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt
[4.]Department of Chemistry and Nutritional Deficiency Disorders, Animal Health Research Institute, Giza 12618, Egypt.
*doaa.ghaith@kasralainy.edu.eg, doaaghaith@gmail.com

**ABSTRACT**

Foodborne diseases are caused by consumption of food spoiled by pathogens or their toxins. Sampling beef meat and chicken samples from local markets which is additionally affecting foodborne disease outbreaks, making food safety a universal issue. Biochemical classical microbiological and targeted molecular profiling of *Enterobactericea* using real time PCR, MALDI TOF as well as sequencing confirmation techniques were performed and compromised for speciation of possible foodborne pathogens in meat and tissue samples in randomly collected 300 samples is traced with their health riskimpact on human. Sum of 68 samples out of 300 were speciated; 28 meat (18 *Escherichia coli O157:H7*, 10 *Salmonella*), 18 (10 *Escherichia coli O157:H7*, 8 *Salmonella*) and 22 (10 *E-coil*, 12 *Salmonella*). Six representative positive samples of both *Salmonella* and *Escherichia coli O157:H7* were selected for further confirmation by sequencing as gold standard technique.

***Keywords***: *Enterobacterice*, MALDI -TOF, sequencing, foodborne pathogen, molecular speciation, Bacteriolocal, biochemical methods.

## Introduction

There is a significant increase in the occurrence of foodborne illnesses due to new nutritional trends that support consuming raw, fresh food, the food with low concentration of salt, dry products, and exotic ingredients (Rešetar *et al.,* 2015). Next to these trends, globalization of the food market is additionally affecting foodborne disease outbreaks, making food safety a universal issue *(Akhtar et al., 2012).* Food safety has clearly emerged as a global public health concern. Foodborne pathogen contamination in the food supply is making millions of people sick every year. The World Health Organization estimated in 2015 that 1 in 10 people acquire a foodborne illness per year; which represent an estimated burden of 600 million cases and 420,000 deaths (World Health Organization, 2015). Meat and chicken products are considered as one of the most important products, which attract the consumers for its palatability, affordable price and easily preparation. However, it proved to be available nutrient, but it is liable to harbor different types of microorganisms and constitute the largest potential source of foodborne illness. The common foodborne pathogens which are responsible for most of the foodborne disease out-breaksare *Escherichia coli O157:H7*, *Staphylococcus aureus*, *Salmonella enterica* and *Shigilla* toxin-producing *Escherichia coli* (STEC) (Oliver *et al.*, 2005; Scallan *et al.*, 2011; Zhao *et al.,* 2014).

In the recent years a great attention has been directed towards the evaluation of detection methods for screening meat products for the presence of foodborne pathogens. Several studies have been carried out in the last decade to decrease the time and the amount of manual labor using alternative techniques for accurate identification of foodborne pathogens (Jasson *et al.,* 2010; Wenning *et al.,* 2014).

The conventional methods for detecting the foodborne bacterial pathogens present in food are based on culturing the microorganisms on agar plates followed by standard biochemical identifications (Mandal *et al.,* 2011). They will probably remain the gold standard for the foreseeable future, because they are simple and inexpensive methods. The most common drawbacks are; insufficient suppression of competitive flora, difficulties to distinguish the colony morphology and appearance of target from non-target bacteria, long incubation and time consuming as they depend on the ability of the microorganisms to grow in different culture media such as pre-enrichment media, selective enrichment media and selective plating media. Usually conventional methods require 2 to 3 days for preliminary identification and more than a week for confirmation of the species of the pathogens (Zhao *et al.,* 2014).

The molecular method in particular, has resolved several problems that are experienced with conventional detection methods. (Jeong *et al.,* 2007; Shannon *et al.,* 2007; Moreno *et al.,* 2011). Unfortunately, the PCR sophisticated technique technique is unable to distinguish between viable or dead cells, which could lead to false positive results (Okoh *et al.,* 2007; Moreno *et al.,* 2011). Furthermore, the current gold standard for microbial identification -16SrRNA and 18SrRNA gene sequencing are not favorable because they are expensive, time consuming and requires specialized staff.

(Bizzini *et al.,* 2011). Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been considered as an excellent tool in different research laboratories for detection and discrimination of various types of microorganisms like bacteria and fungi (Liu *et al.,* 2007; Welker *et al.,* 2011; Wieser *et al.,* 2012; Bohme *et al.,* 2016; Elbehiry *et al.,* 2016). MALDI-TOF MS was first introduced for bacterial identification to the genus and species levels depending on their protein profiles (proteomics) by Bright *et al.* (2002). Databases have been developed that include the main pathogenic microorganisms, thus allowing the use of this method in routine bacterial identification from plate culture. Bioinformatics approaches have leveraged the ever increasing amount of publicly available genomic and proteomic data to attain strain-level characterization. Its quickness and reliability makes it fit for counter-bioterrorism, epidemiological tracing of field strains and detection of food contamination (Sandrin*et al.*, 2013). MALDI-TOF MS is approximately two-thirds less expensive than conventional bacteriological methods (Bohme *et al.*, 2012). It is consistent with 16S rRNA gene sequencing, and is expected to substitute for classic biochemical tests (Van Belkum*et al.*, 2012).

Foodborne diseases (FBDs) encompass a wide spectrum of illnesses representing a serious global public health problem (Lai *et al.,* 2016; Ronholm *et al.,* 2016). It became an urgent necessity to develop detection processes to reduce or eliminate pathogenic microorganisms that can be transmitted through foods of animal origin such as *salmonella species* and *Escherichia coli O157:H7* is crucial. For example, non-typhoidal Salmonella accounted for around 47% of the total cases of death from microbial foodborne illnesses (Scallan *et al.,* 2011). Our aim in this article is performing compromise study between molecular profiling techniques in speciation of foodborne randomly collected from local markets and tracing epidemiological contour in some randomly collected human samples

## Material and Methods

### Collection of Samples

A total 300 random samples of foods; 100 samples meat product (luncheon, minced-meat, meat tissue) and 100 chicken tissue (100) were randomly collected from different street vendors, butchers and retail market, In several cities such as Sixth of October City, AL-Munib, Dokki, EL-Sayeda Zeinab, in the governorates of Cairo and Giza. Moreover, 100 stool samples were collected from patients with food poisoning signs (Fever, vomiting, nausea, abdominal pain and diarrhea) admitted in kasralainy hospital.

Samples were collected in sterile polyethylene bags, put in ice box under low temperature and transferred with minimum delay to the laboratory for studying its bacteriological examination.

### Preparation of samples:

Twenty five gram was taken from each collected samples in sterile stomacher bag, mixed with 225 ml buffered peptone water (BPW) and homogenized by using Stomacher 400 circulator

### Bacteriological examination:

### Isolation and identification of *E. coil*:-

According to Swayne *et al.* (1998), Hitchins *et al.* (1998), Dipineto *et al.* (2006) and Kiranmayi *et al*. (2010). These colonies were examined by the standard biochemical tests for identification. *E. coli* isolates were confirmed (Holt *et al.,* 1993) (Swayne *et al.,* 1998).

### Isolation and identification of Salmonella:-

Cultivation and isolation of *Salmonella* was done according to the International Organization for Standardization [ISO] (2002) protocol (ISO 6579: 2002). *Salmonella* isolates were confirmed by biochemical tests (Popoff *et al.,* 2001).

### Confirming the identification of isolated strains using molecular techniques:

### Conventional PCR for *E. coli* and *Salmonella*:

### DNA extraction and PCR amplification:

**Extraction of DNA:** From pure cultures, DNA were extracted by using Thermo -scientific kits (GeneJET Genomic DNA Purification Kit #K0721, #K0722)

**Oligonucleotide primers**: Four sets of primer pairs were synthesized (Midland Certified Reagent Company_ oilgos (USA). Three set of primers was invA1, STM4495 and sefA specific for *Salmonella* spp., S enteritidis and S typhimurium respectively. The fourth primer set was phoA specific for *E coli*. Primer sequences are shown in Table (1).

**DNA amplification**: DNA amplification was done in 25ul reaction volume containing 12.5 ul of Emerald Amp GT PCR mastermix (2x premix, (Takara) Code No. RR310A Kit), 10 PM of each oligonucleotide primers, 5ul of DNA template and fill up to 25 ul with DNAse and RNAse free water. The optimized cycle program for PCR of each primer is shown in Table (2). Samples were considered positive when a specific single band of DNA was shown by fractionation of PCR products on 1.5% agarose gel electrophoresis stained with ethidium bromide, which marked by 100 bp DNA ladder (Genedirex). The electric current was adjusted at 100 V for 30 minutes, and then gel was visualized and photographed using the Bio-Rad gel documentation system.

**Table 1:** Oligonucleotide primers sequences used for detection if *Salmonella* and *E. coli*

| Target M.O. | Gene | | Primer Sequence 5'-3' | Amplified product (bp) | Reference |
|---|---|---|---|---|---|
| ***Salmonella*** | *invA* | F | GTGAAATTATCGCCACGTTCGGGCAA | 284 | **Oliveira *et al*., 2003** |
| | | R | TCATCGCACCGTCAAAGGAACC | | |
| ***E. coli*** | *phoA* | F | CGATTCTGGAAATGGCAAAAG | 720 | **Hu *et al.,* 2011** |
| | | R | CGTGATCAGCGGTGACTATGAC | | |
| ***S. enteritidis*** | *sefA* | F | GCAGCGGTTACTATTGCAGC | 310 | **Akbarmehr *et al*., 2010** |
| | | R | TGTGACAGGGACATTTAGCG | | |
| ***S. typhimurium*** | *STM4495* | F | GGT GGC AAG GGA ATG AA | 915 | **Liu *et al*., 2012** |
| | | R | CGC AGC GTA AAG CAA CT | | |

**Table 2:** Cycling conditions of the different primers during cPCR

| **Target M.O.** | ***Salmonella*** | ***E. coli*** | ***S. enteritidis*** | ***S. typhimurium*** |
|---|---|---|---|---|
| **Gene** | *invA* | *phoA* | *sefA* | *STM4495* |
| **Thermal profile** | 94˚C 5 min. | 94˚C 5 min. | 94˚C 5 min. | 94˚C 5 min. |
| | 35 cycle | 35 cycle | 35 cycle | 35 cycle |
| | 94˚C 30 sec. | 94˚C 30 sec. | 94˚C 30 sec. | 94˚C 30 sec. |
| | 55˚C 30 sec. | 55˚C 40 sec. | 52˚C 30sec. | 50˚C 1 min. |
| | 72˚C 30 sec. | 72˚C 45 sec. | 72˚C 30 sec. | 72˚C 1 min |

**The identification of *Salmonella* and *E. coli* real time PCR:**

**Salmonella invA gene TaqMan® real-time PCR*:***

TaqMan® real-time PCR assays were performed in a final volume of 25 µL, 12.5 µL of 2x QuantiTect Probe RT-PCR Master Mix (Metabion, Germany, Catalogue No. 204443), 10 Pmol of each primer and 5 Pmol of TaqMan® probe, and 5 µl of DNA template and fill up to 25 ul with DNAse and RNAse free water.. Temperature and time conditions of the primers during PCR are shown in Table (3).The reaction was performed in a Strata gene MX3005P real time PCR machine.

***E. coli, Salmonella Enteritidis and Salmonella Typhimorium* SYBR I green real time PCR*:***

DNA amplification were done in a 25- µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Metabion, Germany, Catalogue No. 204141), 10 Pmol of each primer, 5 µl of DNA template fill up to 25 ul with DNAse and RNAse free water. Temperature and time conditions of the primers during PCR are shown in Table (4).The reaction was performed in a Stratagene MX3005P real time PCR machine

**Table 3:** Oligonucleotide sequences of specific primers and probe used for Salmonella spp.: Metabion (Germany) and thermal profile

| **Target gene** | | **Primer sequence (5'-3')** | **Thermal profile** | **Reference** |
|---|---|---|---|---|
| **Salmonella *invA* gene** | F | GCGTTCTGAACCTTTGGTAATAA | 1 cycle 50 $^{o}$C for 2 min. initial denaturation at 95 $^{o}$C for 2 min. then 50 cycles consisting of 95 $^{o}$C for 10 s and 60 $^{o}$C for 1 min. | **Daum *et al*., 2002** |
| | R | CGTTCGGGCAATTCGTTA | | |
| | PROBE | 5′-FAM-TGGCGGTGGGTTTTGTTGTCTTCT-TAMRA-3′ | | |

**Table 4 :** Oligonucleotide primers and Cycling conditions for SYBR green real time PCR:

| **Target gene** | **Primary denaturation 1 cycle** | **Amplification 40 cycles** Secondary denaturation, annealing (Optics on) and extension | **Dissociation curve1 cycle** Secondary denaturation, annealing and final denaturation |
|---|---|---|---|
| | | 94 □C 15 min. | 94 □C 1 min. |
| *E.coli PhoA gene* | 94˚C 5 min | 55 □C 30 sec. | 55 □C 1 min. |
| | | 72 □C 30 sec. | 94 □C 1 min. |
| *S. typhimurium STM4495 gene* | 94˚C 5 min | 94˚C 15 sec<br>50˚C 30 sec<br>72˚C 30 sec | 95˚C 30 sec<br>50˚C 30 sec<br>95˚C 30 sec |
| *S. enteritidissefA*gene | 94˚C 5 min | 94˚C 15 sec<br>52˚C 30 sec<br>72˚C 30 sec | 95˚C 30 sec<br>52˚C 30 sec<br>95˚C 30 sec\ |

**Identification by MALDI-TOF MS analysis:**

**Chemicals and reagents**

Acetonitrile chromosolve grade, was purchased from Riedel –de Haën, Germany. High purity water for HPLC, was supplied from Doprogenic, Kimya, Ankara, Turkey. Ethanol Absolute GR, was supplied from Sigma- Aldrich 99%, Germany. Formic acid GR 99%, was supplied from Oxford Laboratory, India. Trifluoroacetic acid CAS 76-05-1, was supplied from Sigma- Aldrich 99%, Germany. Alpha-cyano-4-hydroxy cinnamic acid (HCCA), was purchased from Bruker Daltonics, Gmbr., Germany. Bacterial Test Standard (BTS) as reference material (peptides) for MALDI-Biotyper, Ref. no. 8256343, LoT no. 0000199130, was obtained from Bruker DaltonicsGmbr., Germany. Matrix reagent solution, was prepared as saturated solution of HCCA (alpha-cyano-4hydroxy cinnamic acid) in organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid).

**Sample preparation**

Smear biological material (single colony) as a thin film directly onto a spot on a MALDI target plate. Before that step sample prepared by addition 1 µL of 70% formic acid to the bacterial pellet and allow to dry at room temperature. After 50 µl of acetonitrile was added, the components were mixed thoroughly again and allowed to dry at room temperature. Subsequently, the sample was overlaid with 1 µl of matrix (Standard solvent (acetonitrile 50%, water

47.5% and trifluoroacetic acid 2.5%) from Sigma-Aldrich (# 19182) and dried again.

**Instrument conditions**

The instrument used was MALDI Microflex LT, Bruker Daltonics, Bremen, Germany. Peptide mass fingerprint product ion spectra were acquired in a linear positive mode at laser frequency of 60 Hz within a mass range of 2,000 to 20,000 daltons. Instrument parameter settings were as follows. Ion source I at 20 kV, ion source II at 18 kV, lens at 6 kV, extraction delay time of 120 ns, initial laser power of 50%, maximal laser power of 60%, and laser attenuation offset of 25% (range of 19%). For each spectrum, 240 laser shots in 40 shot steps from different positions of the target spot (random walk movement) were automatically acquired with AutoXecute acquisition control software (Flex control version 3.0; Bruker Daltonics, Leipzig, Germany).

Main spectra projection (MSP) creation was performed with a total of 68 spectra acquired for each isolate.

**Cross identification against the created MSP and the Bruker database**

Before assigning the MSPs to their respective nodes on the taxonomy tree, all spectra were loaded into the Biotyper software, and identification was carried out against the MSPs available in the created library. Following the creation of 68 MSPs of strains, each MSP was subjected for identification, and crossmatching was also performed. For comparison of two spectra (Karger *et al.*, 2012), MALDI Biotyper calculates MSP-based similarity scores ranging from 0 (no similarity) to 3 (complete identity). Efficiency check of the database search was performed using BTS. The database (main spectra) for the newly investigated bacteria was constructed using the MaldiBioTyper device (Bruker Daltonik, GmbH). The software requires log (score) values ≥2.0 for species-level identification and values <2 and ≥1.7 for genus-level identification. The results based on the log (score) values <1.7 were rated as being unidentifiable by the software

**Partial 16S rDNA gene sequencing:**

Primers and excess nucleotides were removed from the amplified DNA using a PCR clean-up kit (Qiagen Inc. Valencia CA).A purified RT-PCR product was sequenced in the forward and/ or reverse directions on an Applied Biosystems 3130 automated DNA Sequencer *(*ABI, 3130, USA*)*. Using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit. The master mix reaction was done according to the instruction of the manufacture. (Perkin-Elmer/Applied Biosystems, Foster City, CA), with Cat. No. 4336817. A BLAST® analysis (Basic Local Alignment Search Tool) (Altschul *et al.,* 1990) was initially performed to establish sequence identity to Gen Bank accessions.

Finally DNA sequencing was done for 6 sample, 3 for *E.coli*, 3 for salmonella as a golden test for conformance as in Table 5.

**Table 5:** Sequence analysis of functional 16S rDNA genes in *Escherichia coli* and *Salmonella*:

| Bacteria | Gene | | | Sample No. | Amplified segment |
|---|---|---|---|---|---|
| *E.coli* | phoA | Food Samples | Meat products | 5 | 720 bp |
| | | | Chickens | 7 | |
| | | Human | From stool | 3 | |
| *Salmonellae* | invA | Food Samples | Meat products | 3 | 284 bp |
| | | | Chickens | 6 | |
| | | Human | From stool | 2 | |

## Results and Discussion

**Results:**

**Prevalence of pathogenic bacteria isolated by bacteriological and biochemical classical techniques:**

Bacterial isolationfrom different foods and human samples by bacteriological and biochemical classical techniques as shown in figure1

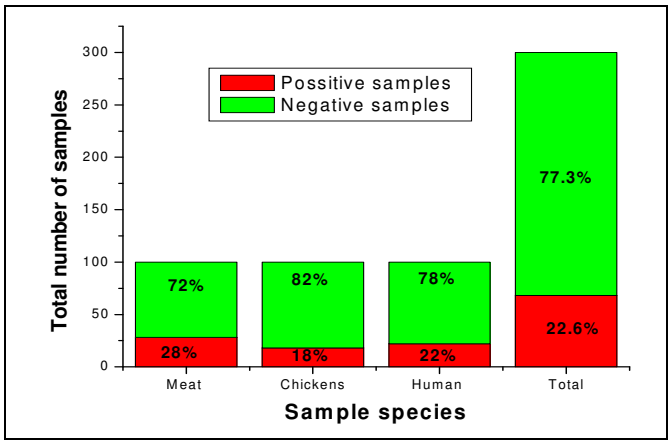


**Fig. 1 :** Prevalence of foodborne pathogens isolated from examined samples*:*

**Incidence of positive *Bacteria* from different foods and human samples:**

The different foods and human contact samples was examined by bacteriological tests as shown in Table (12). *E.coli* was isolated from(18) meat products, (10)chickens and from (10) stool positive strains while the other samples were negative**.** On the other hand, *Salmonellae* **was** isolated(10) meat samples, (8) chickens and (12) human] **as shown in figure 2**

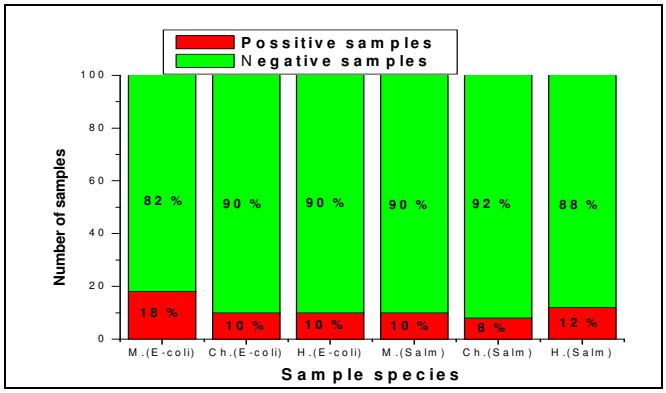


**Fig. 2 :** Incidence of *E.coli* and salmonella in different foods and human samples

**PCR and RT-PCR for Detection of Virulence Gene of *E. coli* and *Salmonella* isolates:**

PCR using primers fragments listed in materials and methods for amplification of (*phoA*) gene from the isolates of *E.coli* and (*inv*A) gene from the isolate *Salmonella* are represented in figure 3

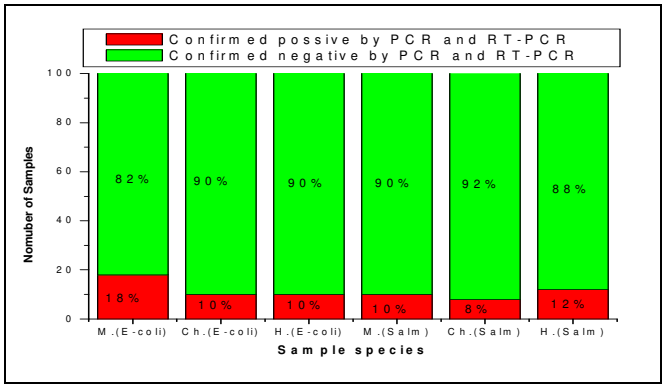


**Fig. 3:** PCR for the detection of virulence Gene *of E.coli and Salmonella* in foods and human samples:

Agarose gel electrophoresis showing PCR results for detection of *phoA* gene specific for *E.coli* in meat, chicken, human DNA respectively. L is M DNA marker (100 bp ladder as molecular size DNA marker) according to following details; A-L1 to L18 are E.*coli* positive meat samples (the product DNA fragment is 720bp). From L1 to L10 are *E.coli* positive chicken samples (the product DNA fragment is 720bp). And finally from L1 to L10 are *E.coli* positive human samples (the product DNA fragment is 720bp).

On the other hand Agarose gel electrophoresis showing PCR results for detection of invA gene specific for *Salmonella* in meat, chicken, human DNA respectively. L is M DNA marker (100 bp ladder as molecular size DNA marker). From L1 to L10 are Salmonell positive meat samples (the product DNA fragment is 284bp). From L1 to L8 are Salmonella positive chicken samples (the product DNA fragment is 284bp). Finally from L1 to L12 are Salmonella positive chicken samples (the product DNA fragment is 284bp).

RT-PCR result using primer of *E. coli* phoA gene in positive samples of meat, chicken and human will be discussed in details. Amplification plots of Real Time PCR of genomic DNA isolated from meat, chicken, human samples respectively. The primers represent the phoA gene of *E. coli* where the threshold line, of the control positive DNA and 18 positive meat samples (all above the threshold line), and the control negative DNA below the threshold line. Again, 10 positive chicken samples (all above the threshold line), and the control negative DNA below the threshold line. Finally, 10 positive human samples (all above the threshold line), and the Control Negative DNA below the threshold line.

RT-PCR result using primer of *Salmonella*invA gene in positive samples of meat, chicken and human:

Amplification plots of Real Time PCR of genomic DNA isolated from meat, chicken, human samples respectively using the primers represent the invA gene of *Salmonella*.

10 DNA samples expressed the invA gene, indicating the presence of Salmonella organisms in the meat samples, 8 DNA samples expressed the invA gene, indicating the presence of Salmonella organisms in the chicken sample and finally 12 DNA samples expressed the invA gene, indicating the presence of *Salmonella* organisms in the human samples.

**Direct bacteriological isolation, conventional PCR and RT- PCR for the detection**

PCR result using primer of *Salmonella Typhimurium* STM4495 gene in positive samples of meat, chicken and human: Agarose gel electrophoresis showing PCR results for detection of serotyping salmonella typhimurium in meat, chicken, human samples respectively. L is M DNA marker (100 bp ladder as molecular size DNA marker). From L1 to L8 are salmonella typhimurium positive meat samples (the product DNA fragment is 915 bp). From L6 to L8 are salmonella typhimurium positive chicken samples (the product DNA fragment is 915 bp). And finally, from L1 to L10 are *salmonella* typhimurium positive Meat samples (the product DNA fragment is 915 bp).

PCR result using primer of *Salmonella enteritidis*sefA gene in positive samples of meat, chicken and human: Agarose gel electrophoresis showing PCR results for detection of serotyping salmonella Enteritidis in meat, chicken, human samples respectively. L is M DNA marker (100 bp ladder as molecular size DNA marker). From L1 and L2 are *salmonella Enteritidis* positive Meat samples (the product DNA fragment is 284 bp). From L1 to L5 are *salmonella Enteritidis* positive Chicken samples (the product DNA fragment is 310 bp). From L1 and L2 are *salmonella Enteritidis* positive Human samples (the product DNA fragment is 284 bp).

RT-PCR result using the primer of *Salmonella Typhimurium* STM4495 gene in positive samples of meat, chicken and human are explained. Amplification plots of Real Time PCR of genomic DNA isolated from meat, chicken, human samples are shown respectively. The primers represent the Serotyping *Typhimurium* gene of *Salmonella*. 8 positive samples (all above the threshold line) and the Control Negative DNA below the Threshold line, 3 positive samples (all above the threshold line), and the Control Negative DNA below the Threshold line and finally, 10 positive samples (all above the threshold line), and the Control Negative DNA below the Threshold line

RT-PCR results using primer of *Salmonella Enteritidis sefA* gene in positive samples of meat, chicken and human. Amplification plots of Real Time PCR of genomic DNA isolated from meat, chicken, human samples respectively. The primers represent the Serotyping Enteritidis gene of Salmonella. 2 positive samples (all above the threshold line), and the Control Negative DNA below the Threshold line, 5 positive samples (all above the threshold line), and the Control Negative DNA below the Threshold line and 2 positive samples (all above the threshold line), and the Control Negative DNA below the Threshold line. Figure 4 is shown that.

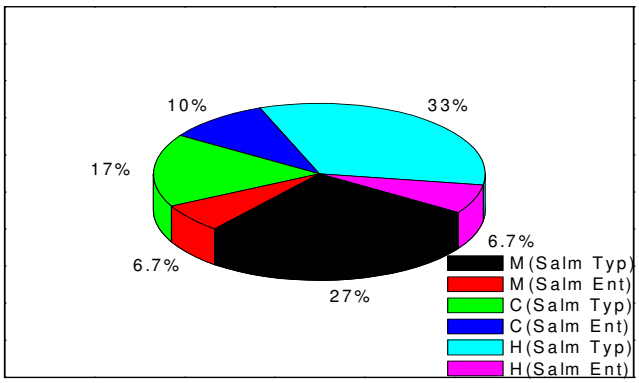


**Fig. 4 :** Serotyping of Salmonella spp. isolated from food and human samples:

**Proteomic identification of foodborne pathogen using MALDI-TOF:**

In recent study**,** all bacteria isolated from various food samples or human contact (handles food) were identified by MALDI-TOF-MS fingerprinting and the spectra obtained were compared with the spectra stored in the broker database, As shown in figure 5 and table 6 ; 38 of *E.coli* isolates were properly identified (92%)while *Salmonella* isolates various in identification were meat product, chickens and Human contact properly identified (80%), (87.5%), (83.33%) we notice that 18 of *E.coli* samples identified with a score value ranging from (2.00 to 3.00) while 12 of *Salmonella* samples identified at the same value. Also in Figure 6, we can see Salmonella serovar was100% correctly identified by PCR and RT-PCR assay while MALDI-TOF MS was limited in identifying Salmonella at serotype levels. Figure 7, 8 also represents an overview of the matrix-assisted laser desorption-ionization-time-of-flight mass spectra of 3 *Escherichia coli* and 3 *Salmonella* which are selected for further sequencing.

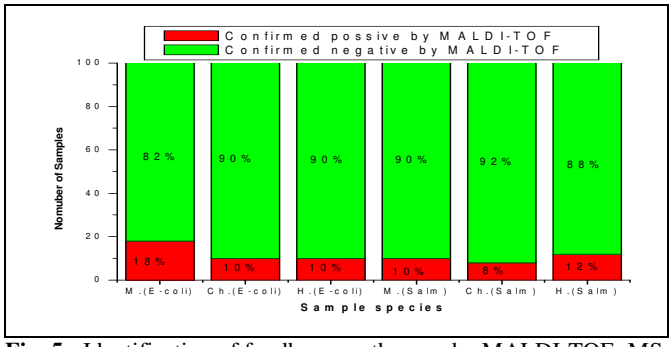


**Fig. 5 :** Identification of foodborne pathogens by MALDI-TOF- MS Fingerprinting Systems for *E.coli* and *Salmonellae*

**Table 6 :** Score value of foodborne pathogens identified by MALDI-TOF- MS Fingerprinting Systems for *E.coli* and *Salmonellae*

| **Bacteria** | **Origin** | | **Total Number** | **MALDI-TOF MS Fingerprinting** | | | | **Microorganisms not match with either bacteria *E.coli* or Salmonella** |
|---|---|---|---|---|---|---|---|---|
| | | | | **0.000-1.699 ( - )** | **1.700-1.999 ( + )** | **2.000-2.299 ( ++ )** | **2.300-3000 ( +++ )** | |
| ***E.coli*** | **Food Samples** | **Meat products** | 18 | **1 not reliable identification sample no 3 (Analyte ID 2a)** | 0 | 12 | 2 | **3** |
| | | **Chickens** | 10 | 0 | 0 | 3 | 7 | **-** |
| | **Human** | **From stool** | 10 | 1 *not reliable identification* sample no 6 (Analyte ID 6) | 0 | 8 | 1 | **-** |
| | **Total** | | 38 | 2 | 0 | 23 | 10 | **3** |
| **Salmonellae** | **Food Samples** | **Meat products** | 10 | 0 | 0 | 2 | 6 | 2 **proteus mirabilis samples no 9,10 (Analyte ID 9,10)** |
| | | **Chickens** | 8 | 0 | 0 | 1 | 6 **samples no 8** | 1 **proteus mirabilis sample no 1** |
| | **Human** | **From stool** | 12 | 1 **not reliable identification sample no 1 (Analyte ID s1)** | 1 | 2 | 6 | 2 **proteus mirabilis samples no 3,4 (Analyte ID S3, S5)** |
| | **Total** | | 30 | 1 | 1 | 5 | 18 | 5 |

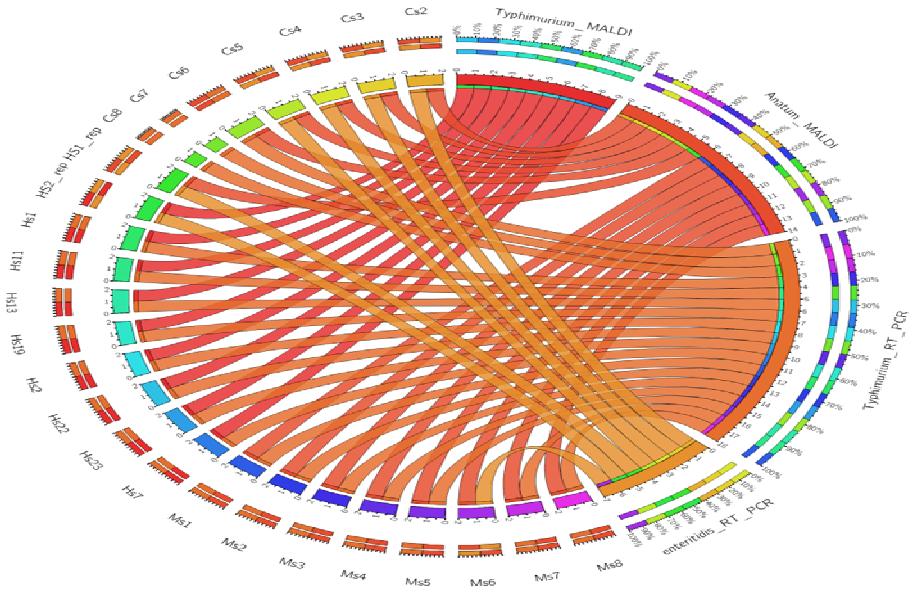


**Fig. 6:** Compromise of representative positive samples throughout genomic molecular techniques and proteomics using mass spectroscopic techniques.

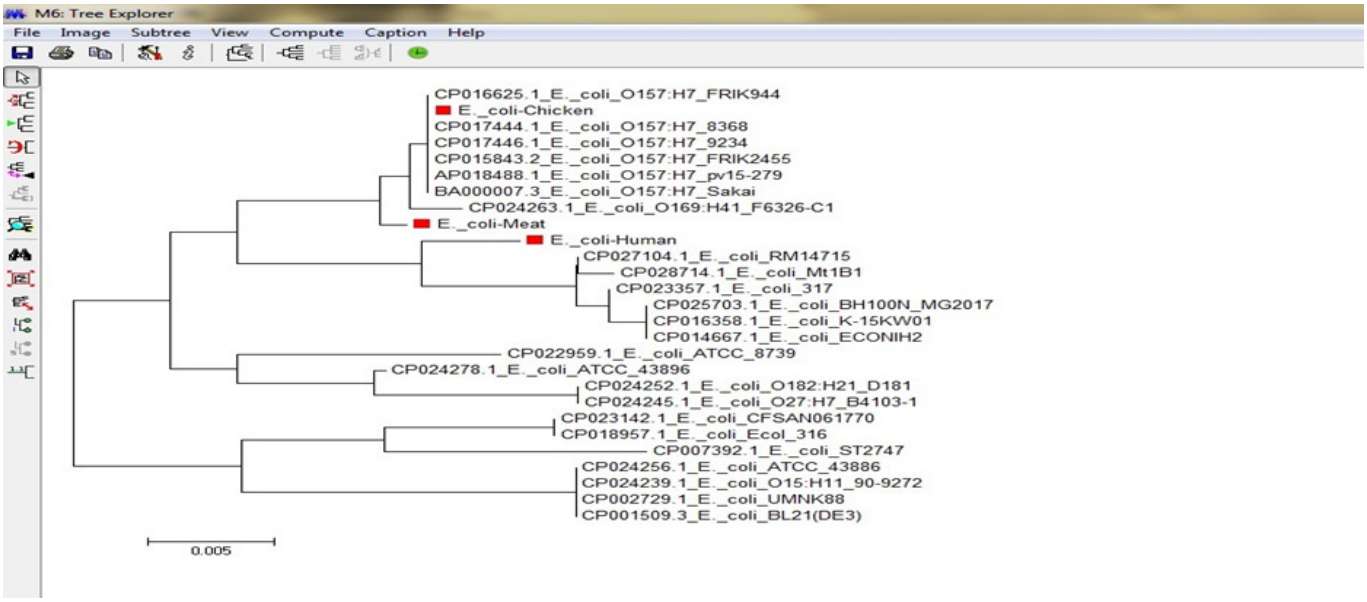


**Fig. 7 :** The homology percentage of *E-coli* nucleotide sequence results

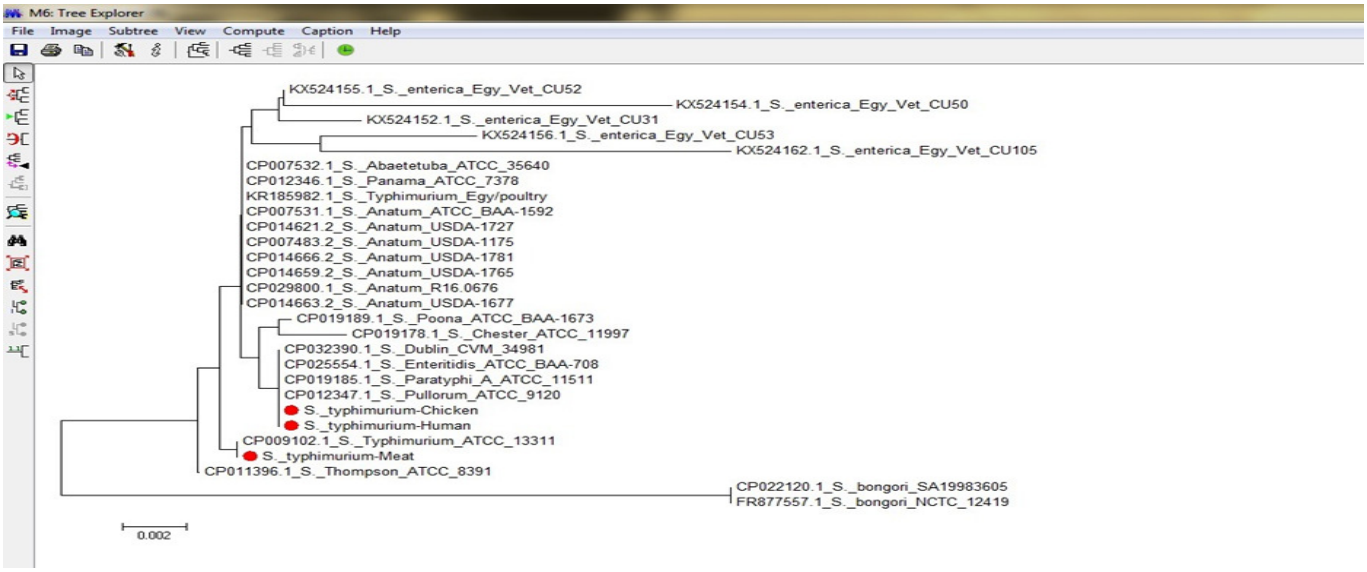


**Fig. 8 :** The homology percentage of *Salmonela* nucleotide sequence results

## Discussion

Overview of the matrix-assisted laser desorption-ionization-time-of-flight mass spectra of 38 *Escherichia coli* sample and 30 Salmonella which are selected in food and Human sample. Accurate and rapid identification of pathogenic foodborne is of critical importance in disease treatment and public health. So these foodborne pathogen must be identified at the species, subspecies and serovar level. MALDI-TOF MS has proven to be a useful method Identify the broad bacterial species (Rettinger *et al.,* 2012; Bader *et al.,* 2013; Egli *et al.*, 2015). The application of MALDI-TOF MS fingerprinting for the identification of bacterial strains isolated from meat products, chickens and from patient has shown to be a competent tool for the rapid and accurate differentiation of bacterial species, due to the resulting highly specific spectral profiles, named fingerprints (Giebel *et al.,* 2010). In order to have a solid ground and a fair judgment, one must have a gold standard to refer to regarding the typing of field isolates to the biovar level. The pros of nucleic acid methods are the cons of MALDI-TOF MS technology, where the technique is usually applied on bacterial culture extracts and in rare cases on cultures directly. When it comes to rapidity in binomial identification of a bacterial culture, MALDI-TOF MS is the fastest even when compared with PCR and RT-PCR. The collected strains were analyzed by PCR to detect phoA gene from the isolate *E.coli* and *inv*A gene from the isolate *Salmonella*. PCR eliminates the need for isolation and further biochemical identification, especially with fastidious microorganisms that need unique medium requirement (Bayatzadeh *et al.,* 2011). Further, PCR can detect target genetic sequences regardless the growth stage of the target cells (Gong *et al.,* 2002) in contrast to the bacterial culture where cells in samples may exist in a variety of different growth stages or in small number, some of them may not be fit to grow in culture (Lawsoni *et al.,* 1998). However, culture is still always recommended to determine the susceptibility of isolated strains.

Our results indicate that the results of PCR and RT-PCR for detection of the phoA gene from 38 isolates of *E. coli* were properly identified (100%). Moreover; PCR assay was carried out for the detection of the *inv*A gene from isolated strains has revealed that the gene was present in all of the isolates (100%) which agrees with a study performed and recorded the same results by (Dione *et al.,* 2011; Gole *et al.,* 2013).

Surveillance of *Salmonella* serovars and phagetypes from human and animal sources is relevant for detecting national and global outbreaks, for identifying the source of an infection and for implementing prevention and control measures since the distribution of Salmonella serovars may differ between countries. Molecular methods used again to discriminate among closely related serovars. We observed that *salmonella Typhimurium* and *salmonella Enteritidis* are the most common serovars in our research and isolated from humans, livestock and chickens. *Salmonella Typhimurium* was the most prevalent serovar in isolates of human origin. A similar finding was reported from Europe, Australia and America (Wray and Wray *et al.,* 2000; van Duijkeren *et al.,* 2002).

In the present study, identification by MALDI-TOF MS yielded a valid score for 60 (88.24%) of 68 at the species level and 8 discordant results were identified. The discordant results shown in fig. 6 were mainly due to inaccurate taxonomic assignment of a given spectra in the MALDI-TOF MS database. MALDI-TOF MS gave a valid score for genus and species identification of 92% when used in identification of previously identified *E. coli* culture using conventional methods. Molecular techniques and sequencing for some sample this agrees with Ge *et al.* (2016), Jesumirhewe *et al.* (2016) and Naiara *et al.* (2017). Which achieved species identification of *E. coli* isolates from meat products, chickens and from stool using MALDI-TOF MS of 83.33%, 100%, and 100%, respectively, when compared with traditional methods of identification. Although, in our study recognized the inability of the MALDI-TOF spectra to discriminate closely related species in *E. coli*. It consisted of three isolate *Enterobacter hormaechei, Enterobacter asburiae, Kosakoniacowanii* which were identified as *E. coli* by MALDI-TOF MS. All this studies not identified *E. coli* to sub species level due to the inability of the MALDI-TOF spectra to discriminate closely related species. Most of our samples are closely related to Shigella and not definitely distinguishable Because *Shigella spp. and E. coli* exhibit great similarity at the genomic level (Johnson *et al*., 2000). it was expected that their similar proteomes prevented their differentiation by MALDI-TOF MS.

Furthermore, pure colonies previously identified as *Salmonella* isolates using traditional methods gave valid score of 83.33% using MALDI-TOF MS assay. This result agrees with (Ulrich *et al.,* 2011). However, MALDI-TOF MS can still be used in primary identification and screened *Salmonella* can be further serotyped using other method one of them the White-Kauffmann-LeMinor scheme. as we know Determination of the serotype of Salmonella could provide more information to clinical therapy. In our dataSalmonella serovar was 100% correctly identified by PCR and RT-PCR assay. The isolated serovar was identified as follows: *Salmonella typhi* isolated from meat and poultry products and from faeces 27%, 10%, and 33%, respectively. While *salmonella Enteritidis* from meat and poultry products and from human stool was 6.7%, 17%, and 6.7%, respectively. However, MALDI-TOF MS was limited in identifying *Salmonella* at serotype levels. The accuracy of serotype determination using MALDI-TOF MS was poor, as *Salmonella typhi* was detected in humans at 27% while *salmonella Enteritidis* was interlaced in this work with another biovar, especially *Salmonella anatum*. We demonstrated that MALDI-TOF MS method presented in this study was not useful for serovar assignment currently, and we also found this method may not be suitable to subtype a specific serovar as well, since the discriminatory ability of this method is relatively low when the distance level was raised to ensure good reproducibility. In addition, whether the dendrogram of protein spectra can reflect a clonal outbreak is not confirmed. By examination of *E. coli* and Salmonella isolates and strains revealed by MALDI-TOF MS, 10-20 prominent ion peaks were identified in the mass spectra. Range of these prominent ion peaks were from the 3000 and 10,500 m/z, with the highest-intensity peaks being in the range of 4367-9723 m/z with *E.coli* isolates while in the case of Salmonella isolates, range of these spectra peaks were from the 3000 and 11,000 m/z, with the highest-intensity spectra peaks being in the range of 4369-9532 m/z. Examination of mass spectra reveals specific peaks of the strain that fall into a range At 4367, 5384, 6259,6319

,7163,7190, 9561 and 9723 m / z for all *Escherichia coli* Isolates consistent with Christner *et al.* (2014) and also reveals strain-specific peaks at 4369, 5385, 5618, 5775, 6099, 6260, 6319, 7100, 7669, 8300, and 9532 m/z for all *Salmonella* isolates which agree to large extent with Dieckmann *et al.* (2011) fig(pattern). Previous study presumed that a pattern recognition approach was limited in serovar level identification, because of the complexity of the peak patterns. Dieckmann *et al.* (2011) established weighted pattern matching approaches, which could achieve robust results in serovar level identification of *E. coli and Salmonella*. We tried to find serovar-specific biomarkers using Dieckmann's approach and failed. Some consensus peaks were indeed found. However, these peaks could not be considered as biomarkers for serovar identification because these peaks are not serovar-specific and not exclusive to other serovars. We found that mass lists of strains of different serovars are so similar that we cannot find any serovar-specific peaks exclusively present in one serovar. Therefore, these peaks could not be considered as biomarkers for serovar identification. In addition, consensus peaks found in the present study were largely different from previous studies, (Leuschner *et al.,* 2004). In this sense, in order to determine specific peaks and achieve a better identification of an unknown strain, a wide number of strains and species considered as reference would improve the accuracy of the method. Mass spectra of *E. coli and salmonella* strains show that protein profile similarity does not correlate always with the traditional genus/ species/ biovar ranking system. Protein patterns for some species and biovars may be closer to other species than to other biovars of the same species of *E. coli* and *Salmonella* isolates.

Further confirmation of selected 6 samples were judged for *E-coli* and *Salmonella* speciation. A copy of DNA pattern with sequence distance and phylogenic tree for both *E.coli* and *Salmonella* are represented in fig. 7 and fig. 8; respectively. Phyloproteomic analysis of protein spectra and phylogenetic studies of 16S rDNA genes was also conducted for 6 samples (*3E. coli* and *3Salmonella*) and the correlation of the clusters with the species and serotype was observed. Dendrograms in figures; 7 and 8. help in grouping of *E. coli O157:H7* originated from various food samples and feces of human with renal failure symptoms. Figure 3 show most of isolates have similarity between (95 to 100%). This result indicated most of isolates can be grouped in the same species according to the Committee on Reconciliation of Approaches to Bacterial Systematic. The committee wrote that generally would include strains with approximately 70% or greater DNA-DNA relatedness (Rosello-Mora and Amann, 2001; Doolittle and Zhaxybayeva, 2009). The highest of similarity coefficients among isolates are showed by *E. coli*-Chicken with CP015843.2 *E. coli* O157:H7 FRIK2455, CP017446.1 *E. coli* O157:H7 9234, CP017444.1 *E. coli* O157:H7 8368, CP016625.1, *E. coli* O157:H7 FRIK944 and AP018488.1 *E. coli* O157:H7 pv15-279 isolates with similarity coefficient "1 "or 100 % similarly and there is (99.7%) similarity between *E. coli-Chicken and E. coli-Meat.* Higher similarities are showed by E. coli-Human with the other isolates also. The presence of *E. coli* 0157:H7 strains would pose greater threat to consumers due to the fact that these strains are responsible for a range of disease ranging from mild diarrhea to complicated conditions.

The nucleotide sequence of bp PCR fragment representing the invA gene of *Salmonella typhimurium* isolate was compared with other 25 *S. typhimurium* strains published sequences on GenBank. The homology percentage of nucleotide sequence results showed high homology (100.0%) between *Salmonella typhimurium*-Chicken, *Salmonella typhimurium*-Human, *Salmonella typhimurium*-Meat strains and CP032390.1 *SalmonellaDublin* CVM 34981, CP025554.1 *Salmonella Enteritidis* ATCC BAA-708, CP019185.1 S. *Paratayphi*A ATCC 11511, CP012347.1 S. *Pullorum* ATCC 9120. Ironically, there is (100.0%) homogeneity between *Salmonella typhimurium*- Chicken and *Salmonella typhimurium*-Human. These results agree with Shi *et al.* (2012) who found the high invA gene homology between *Salmonella* strains (72.9-97.2%). Both Phylogenetic trees (Fig.7, 8) demonstrated that the Egyptian isolate is closely related to the other isolate in GenBank but in a separate cluster due to some amino acid substitutions when comparing with others strains. No clear correlation could be established between the DNA profiles and locations (towns) where the food samples were purchased.

MALDI-TOF MS can be used as an application in assisting in both detection of salmonella and determination at species level and subspecies level. MALDI-TOF MS is suitable for identification of *E.coli and salmonella* at species level with high accuracy. In the case biovar level was more difficult by visual comparison to reference spectra. The serotype of *E. coli and salmonella* can provide more information for clinical detection and treatment. However, MALDI-TOF MS was limited in identification of Samples at serotype levels. In summary, MALDI-TOF mass fingerprinting proved to be an accurate and cost-effective technique, with potential for routine identification of bacteria in the food sector as well as in clinical microbiology (Seng *et al.,* 2009). In this sense, some authors consider that, taking into account the cost of materials and staff, the cost of bacterial identification by MALDI-TOF MS fingerprinting is around two-thirds less than conventional methods (Hsieh *et al.,* 2008; Nassif *et al.,* 2009). On the other hand, it has several advantages over other fast methods relying on genomics, such as PCR, RT-PCR, DNA sequencing, DNA microarrays, because fewer steps are necessary to achieve bacterial identification and thus, fewer errors are introduced along the analyzing process. Another advantage of MALDI-TOF mass fingerprinting is the effortless analysis of results, since no extensive data processing and statistical analysis is required, as it is the case in other rapid methods for bacterial identification, such as PCR, RT-PCR, DNA sequencing and DNA microarrays. It is envisaged that molecular identification through MALDI-TOF MS will rapidly establish a robust position for surveillance of microbial pathogens in the complex interwoven network of origin, producers, traders and consumers. Our results are consistent with previous work by Shell *et al.* (2017) where it was stated that MALDI-TOF MS showed significant promise in *E. coli* and *Salmonella* identification on genus and species levels and can be also used as a tool for sub species and serovar typing, but it will require additional studies and modifications to existing protocols and commercial and the extended database. The identification using MALDI-TOF MS method could analyze pure positive culture rapidly (may be within minutes especially when direct cultural identification methods used rather than ethanol: Formic acid extraction

method) and also reliable manner. However, identification by traditional methods needs more facilities, media, chemicals, experiences, and time and this in contrast with the non-requirement of high technical expertise, the simple extraction procedure and low running cost identification using MALDI-TOF MS which provide more advantages over other methods for identification. However, the applications have to be carried out with cautions because the accuracy decreases using of too much of chemicals and materials and the samples have to be spotted with the matrix solution with care to avoid the presence of the liquid smear between spots, which increase possibility of cross-contamination. The sample size used for this study is low as it is a preliminary study to use this technique in diagnostic laboratories in Egypt, but anyhow, more samples are needed in future studies to detect sensitivity, reliability, and performance of this type of bacterial identification.

All bacterial isolates used in our study were identified by MALDI-TOF MS fingerprinting (protein fingerprinting) in less than 2 h. By contrast, identification by biochemical colorimetric or molecular methods can take several hours or days (Böhme *et al.,* 2016; Elbehiry *et al.,* 2016).

## Conclusion

This study demonstrated that Bruker MALDI-TOF MS Biotyper is a reliable fast and economic tool for the identification of Gram-negative bacteria, especially *E. coli* and *Salmonella* which could be used as alternative regular diagnostic tool for routine identification and differentiation of microorganism especially in the field of food-borne pathogens. MALDI-TOF MS method presented in this study was not useful for biovar assignment of *E. coli* and *Salmonella* currently, and was evident in *Salmonella* subtypes as the MALDI-TOF MS was not able to distinguish subspecies *S. typhimurium, S. enteritidis* from other subspecies. Because of robust differentiation of subspecies or subtypes demands more selectivity than provided by a fingerprint of m/z values of intact proteins. MALDI-TOF MS need more validation and verification and more study on the performance of direct colony and extraction methods to detect the most sensitive one and also need using more samples to detect sensitivity, reliability, and performance of this type of bacterial identification. Future research may be directed to applying suitable algorithms for discrimination of isolates at different taxonomic levels. Still, MALDI-TOF MS is rapid, robust, and promising identification tool, which can be used in primary identification, screening and diagnostics of foodborne pathogen. For MS-based proteome analysis of two species, pan proteome for protein identification might be inconvenient due to the difference in the entry IDs for each species. Therefore, further future works for the sake of effective protein identification is needed for better species, subspecies and strain by collecting and enhancing data in libraries of MS.

## References

Akbarmehr, J.; Salehi, T.Z. and Brujeni, G.H.N. (2010). Identification of Salmonella isolated from poultry by MPCR technique and evaluation of their hspgroEL gene diversity based on the PCR-RFLP analysis. African Journal of Microbiology Research 4(15): 1594-1598.

Akhtar, S.; Sarker, M.R.; Hossain, A. (2012). Microbiological food safety: a dilemma of developing societies. Crit. Rev. Microbiol. 40: 348–359.

Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W. David and Lipman D.J. (1990). Basic Local Alignment Search Tool. Journal of Molecular Biology 215: 403–410.

Bader, O. (2013). MALDI-TOF-MS-based species identification and typing approaches in medical mycology. Proteomics. 13: 788–799.

Bayatzadeh, M.A.; Pourbakhsh, S.A.; Homayounimehr, A.R.; Ashtari, A.; Abtin, A.R. (2011). Application of culture and polymerase chain reaction (PCR) methods for isolation and identification of Mycoplasma synoviae on broiler chicken farms. Archives of Razi Institute, 66(2): 87-94.

Bizzini, A.; Jaton, K.; Romo, D.; Bille, J.; Prod'hom, G. and Greub, G. (2011). Matrix-assisted laser desorption ionization–time of flight mass spectrometry as an alternative to 16S rRNA gene sequencing for identification of difficult-To-identify bacterial strains. J. Clin. Microbiol. 49: 693–696.

Böhme, K.; Fernández-No, I.C.; Barros-Velázquez, J.; Gallardo, J.M.; Cañas, B. and Calo-Mata, P. (2012). SpectraBank: an open access tool for rapid microbial identification by MALDI-TOF MS fingerprinting. Electrophoresis, 33(14): 2138-2142

Böhme, K.; Antelo, C.S.; Fernández-No, I.C.; Quintela-Baluja, M.; Barros-Velázquez, J.; Cañas, B. and Calo-Mata, P. (2016). Detection of Foodborne Pathogens Using MALDI-TOF Mass Spectrometry. Antimicrobial Food Packaging 15: 203-214.

Bright, J.J.; Claydon, M.A.; Soufian, M.; Gordon, D.B. (2002). Rapid typing of bacteria using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry and pattern recognition software. J. Microbiol. Methods 48 (2-3): 127-138.

Cheng, K.; Chui, H.; Domish, L.; Hernandez, D. and Wang G. (2016) Recent development of mass spectrometry and proteomics applications in identification and typing of bacteria Proteomics Clin Appl. 10(4): 346–357.

Christner, M.; Trusch, M.; Rohde, H.; Kwiatkowski, M.; Schluter, H.; Wolters, M.; Aepfelbacher, M. and Hentschke, M. (2014) Rapid MALDI-TOF mass spectrometry strain typing during a large outbreak of Shiga-toxigenic *Escherichia coli.PLoS One*, 9(7): e101924.

Daum, L.T.; Barnes, W.J.; McAvin, J.C.; Neidert, M.S.; Cooper, L.A.; Huff, W.B.; Gaul, L.; Riggins, W.S.; Morris, S.; Salmen, A. and Lohman, K.L. (2002) Real-Time PCR Detection of Salmonella in Suspect Foods from a Gastroenteritis Outbreak in Kerr County, Texas. J Clin Microbiol. 40(8): 3050–3052.

Dieckmann, R. and Malorny, B. (2011). Rapid screening of epidemiologically important *Salmonella enterica* subsp. *Enterica* serovars by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry.*Appl. Environ. Microbiol.*77(12): 4136-4146.

Dione, M.M.; Saha, D.; Mohammed, N.I.; Adegbola, R.A.; Ieven, M.; Antonio, M. (2011). Antimicrobial resistance and virulence genes of nontyphoidal Salmonella isolates in The Gambia and Senegal. J. Infect. Dev. Ctries, 5: 765-775.

Dipineto, L.; Santaniello, A.; Fontanella, M.; Lagos, K.; Fioretti, A. and Menna, L.F. (2006):Presence of Shiga toxin-producing *Escherichia coli* O157:H7 in living layer hens. Letters in Appl. Microbiol., 43(3): 293–295.

Doolittle, W.F. and Zhaxybayeva, O. (2009). On the origin of prokaryotic species. Genome Res., 19: 744-756.

Egli, A.; Tschudin-Sutter, S.; Oberle, M. *et al*. (2015). Matrixassisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) based typing of extended-spectrum beta-lactamase producing E. coli–a novel tool for real-time outbreak investigation. PLoS One. 10: e0120624.

Elbehiry, A.; Al-Dubaib, M.; Marzouk, E.; Osman, S.; Edrees, H. (2016). Performance of MALDI biotyper compared with Vitek™ 2 compact system for fast identification and discrimination of Staphylococcus species isolated from bovine mastitis. Microbiology Open 5: 1061-1070.

Ge, M.; Kuo, A.; Liu, K.; Wen, Y.; Chia, J.; Chang, P.; Lee, M.; Wu, T.; Chang, S. and Lu, J. (2016) Routine identification of microorganisms by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: Success rate, economic analysis, and clinical outcome. *J. Microbiol. Immunol. Infect*.; XX, 1-7.

Giebel, R.; Worden, C.; Rust, S.M.; Kleinheinz, G.T.; Robbins, M.; Sandrin, T.R. (2010). Microbial fingerprinting usingmatrix-assisted laser desorption ionization time-of-flight massspectrometry (MALDI-TOF MS): Applications and challenges, Advances in Applied Microbiology, pp 149–184. New York: Academic.

Gole, V.C.; Chousalkar, K.K. and Roberts, J.R. (2013). Survey of Enterobacteriaceae contamination of table eggs collected from layer flocks in Australia. Int. J. Food Microbiol.; 164: 161-16.

Gong, J.; Forster, R J.; Yu, H.; Chambers, J R.; Wheatcroft, R.; Sabour, P M.; Chen, S. (2002). Molecular analysis of bacterial populations in the ileum of broiler chickens and comparison with bacteria in the cecum. FEMS Microbiology Ecology Journal, (41): 171-179.

Hitchins, A.D.; Feng, P.; Watkins, W.D.; Rippey, S.R. and Chandler, L. A. (1998). Bacteriological Analytical Manual, Edition 8, Revision A, Chapter 4. U.S. Food & Drug Administration Center for Food Safety & Applied Nutrition.

Holt, J.G.; Krieg, N.R.; Sneath, P.H.A.; Staley, J.T. and Williams, S.T. (1993). Bergey's Manual of Determinative Bacteriology, 9th ed. Williams and Wilkins, Hagerstown, MD.

Hsieh, S.-Y.; Tseng, C.-L.; Lee, Y.-S.; Kuo, A.-J.; Sun, C.-F.; Lin, Y.-H.; *et al*. (2008). Highly efficient classification and identification of human pathogenic bacteria by MALDI-TOF MS. Molecular &Cellular Proteomics, 7(2): 448–456.

Hu, Q.; Tu, J.; Han, X.; Zhu, Y.; Ding, C. and Yu, S. (2011): Development of multiplex PCR assay for rapid detection of Riemerella anatipestifer, Escherichia coli, and Salmonella enterica simultaneously from ducks. Journal of Microbiological Methods 87: 64–69.

International organization for standardization (ISO 6579 part 1, 2002): Microbiology of food and animal feeding stuffs – Horizontal method for the detection of Salmonella. International Standards Organization, Geneva. 4th Edition. www.iso.org/iso/

Jasson V.; Jacxsens L.; Luning P.; Rajkovic A.; Uyttendaele M. (2010). Alternative microbial methods: An overview and selection criteria. Food Microbiol. 27: 710-730.

Jeong, S.K.; Gang, G.L.; Jong, S.P.; Yong, H.J.; Hyo, S.K.; Soo, B.K.; Young, S.N.; Kwon, S. (2007) .A novel multiplex PCR assay for rapid and simultaneous detection of five pathogenic bacteria: Escherichia coli O157:H7, Salmonella, Staphylococcus aureus, Listeriamonocytogenes, and Vibrio parahaemolyticus. J. Food Protect. 70: 1656–1662.

Jesumirhewe, C.; Ogunlowo, P.O.; Olley, M.; Springer, B.; Allerberger, F. and Ruppitsch, W. (2016) Accuracy of conventional identification methods used for *Enterobacteriaceae* isolates in three Nigerian hospitals. *Peer J*, 4: e2511.

Johnson, J.R. (2000). *Shigella* and *Escherichia coli* at the crossroads: Machiavellian masqueraders or taxonomic treachery? J. Med. Microbiol. 49: 583–585.

Karger, A.; Stock, R.; Ziller, M.; Elschner, M.; Bettin, B.; Melzer, F.; Maier, T.; Kostrzewa, M.; Scholz, H.C.; Neubauer, H. and Tomaso, H. (2012). Rapid identification of Burkholderia mallei and Burkholderiapseudomallei by intact cell matrix-assisted laser desorption/ ionisation mass spectrometric typing. BMC Microbiol. 12: 229.

Kiranmayi, C.B.; Krishnaiah, N. and Mallika, E.N. (2010). Escherichia coli O157:H7 - An emerging pathogen in foods of animal origin. Veterinary World, 3: 382-389.

Lai, K.; Twine, N.; O'Brien, A.; Guo, Y.; and Bauer, D. (2016). "Artificial intelligence and machine learning in bioinformatics," in Encyclopedia of Bioinformatics and Computational Biology, ed M. Gribskov (Elsevier). doi: 10.1016/B978-0-12-809633-8.20325-7

Lawsoni, A.J.; Shafi, M.S.; Pathak, K.; Stanley, J. (1998). Detection of campylobacter in gastro-enteritis; comparison of direct PCR assay of fecal samples with selective culture. Epidemiology Infection Journal. (121): 547-553.

Leuschner, R.G.; Beresford-Jones, N. and Robinson, C. (2004). Difference and consensus of whole cell Salmonella enterica subsp. enterica serovars matrix-assisted laser desorption/ionization time-of-flight mass spectrometry spectra. Lett Appl Microbiol; 38: 24–31.

Liu H.; Du Z.; Wang J.; Yang R. (2007). Universal sample preparation method for characterization of bacteria by assisted laser desorption ionization-time of flight mass spectrometry. Appl. Environ. Microbiol. 73: 1899-1907.

Liu, B.; Zhou, X.; Zhang, L.; Liu, W.; Dan, X.; Shi, C. and Shi, X. (2012). Development of a novel multiplex PCR assay for the identification of Salmonella enterica Typhimurium and Enteritidis. Food Control 27: 8-93.

Mandal, P.K.; Biswas, A.K.; Choi, K. and Pal, U.K. (2011). Methods for rapid detection of foodborne pathogens: an overview. Am. J. Food. Technol. 6: 87–102.

Moreno, Y.; Ballesteros, L.; García-Hernández, J.; Santiago, P.; González, A.; Ferrús, M.A. (2011). Specific detection of viable Listeria monocytogenes in spanish wastewater treatment plants by fluorescent in situ hybridization and PCR. Water Res. 45: 4634–4640.

Naiara, M.B.R.; Greiciane, F.B.; Gabrielli, S.S.; Larissa, A.B.B.; Beatriz, M.M.; Irene, D.C.; Miliane, M.S.D. and Shana, D.D.C. (2017). The matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) identification versus biochemical tests: A study with enterobacteria from a dairy cattle environment. *Braz. J. Microbiol*.; 48: 132-138.

Nassif, X. (2009). Editorial commentary: A revolution in theidentification of pathogens in clinical laboratories.Clinical Infectious Diseases, 49(4): 552–553.

Okoh, A.I.; Odjadjare, E.E.; Igbinosa, E.O.; Osode, A.N. (2007). Wastewater treatment plants as a source of microbial pathogens in receiving watersheds. Afr. J. Biotechnol. 6: 2932–2944.

Oliver, S.P.; Jayarao, B.M. and Almeida, R.A. (2005). Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. Foodborne Pathog. Dis. 2: 115–129.

Olivera, S.D.; Rodenbusch, C.R.; Ce, M.C.; Rocha, S.L.S. and Canal, C.W. (2003): Evaluation of selective and non-selective en-richment PCR procedures for Salmonella de-tection. Lett. Appl. Microbiol.; 36: 217-221.

Popoff, M.Y. (2001):Antigenic formulas of the Salmonella Serovars, WHO Collaborating Center for reference and research on Salmonella, Paris. (8th edition).

Rešetar, D.; KraljevićPavelić S. and Josić, D. (2015). Foodomics for investigations of food toxins, Curr. Opin. Food Sci., 4: 86–91.

Rettinger, A.; Krupka, I.; Grunwald, K.; Dyachenko, V.; Fingerle, V.; Konrad, R.; Raschel, H.; Busch, U.; Sing, A.; Straubinger, R.K. and Huber, I. (2012). *Leptospira* spp. strain identification by MALDI TOF MS is an equivalent tool to 16S rRNA gene sequencing and multi locus sequence typing (MLST). BMC Microbiol. 12: 185-199

Ronholm, J.; Nasheri, N.; Petronella, N. and Pagotto, F. (2016). Navigating microbiological food safety in the era of whole-genome sequencing. Clin. Microbiol. Rev. 29: 837–857.

Rosello, M.R. and Amann, R. (2001). The species concept for prokaryotes. FEMS Microbiol. Rev. 25: 39-67.

Sambrook, J.; Fritscgh, E.F. and Mentiates (1989). Molecular coloning. A laboratory manual. Cold spring Harbor Laboratotry press, New York.

Sandrin, T.R.; Goldstein, J.E. and Schumaker, S. (2013). MALDI-TOF MS profiling of bacteria at the strain level: a review. Mass Spectrometry Reviews, 32(3): 188-217.

Sayour, A.E. and Sayour, H.E. (2015). Binomial identification of Brucella isolates by MALDI-TOF mass spectrometry Animal Health Research Institute, 3-7 September 2015 pp. 28-49.

Scallan, E.; Hoekstra, R.M.; Angulo, F.J.; Tauxe, R.V.; Widdowson, M.A.; Roy, S.L. (2011). Foodborne illness acquired in the United States- major pathogens. Emerg. Infect. Dis. 17: 7–15.

Scallan, E.; Hoekstra, R.M.; Angulo, F.J.; Tauxe, R.V.; Widdowson, M.-A.; Roy, S.L. (2011). Foodborne illness acquired in the United Statesdmajor pathogens. Emerging Infectious Diseases, 17(1): 7e15.

Seng, P.; Drancourt, M.; Gouriet, F.; La Scola, B.; Fournier, P.-E.; Rolain Jean, M. *et al*. (2009). Ongoing revolution in bacteriology: Routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.Clinical Infectious Diseases, 49(4): 543–551.

Shannon, K.E.; Lee, D.; Trevors, J.T.; Beaudette, L.A. (2007) Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. Sci. Total Environ. 382: 121–129.

Shell, W. S.; Sayed, M. L.; Gad Allah, F.M.; Elzahraa, F.; Afaf, G.; Khed, A.; Samy, A.A. and Ali, A.M. (2017) Matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry as a reliable proteomic method for characterization of *Escherichia coli* and *Salmonella* isolates Veterinary World, 10: 1083-1093.

Shi, Q.; Zhang, Y.; Wang, Q.Y.; Gao, G.; Fang, H.; Miao, F.; Yuan, Z. and He, H. (2012). Phylogenetic analysis of virulence factor gene of *Salmonella* isolated from clinically symptomatic Chickens. African Journal of Microbiology Research, 6(8): 1718-1722.

Swayne, D.; Charman, E.; Glisson, J.R; Jackwood, M.W; Pearson, J.E. and Reed, W. . (1998) Colibacillosis: A laboratory manual for the Isolation and identification of avian pathogens, fourth edition. Am. Assoc. Avian Pathol. 14-16.

Ulrich, W.; Katrin, S.; Christiane, B.; Leith, F. and Markus, K. (2011) Rapid detection of *Salmonella* from clinical specimen by MALDI-TOF MS. *Pathology,* 43 Supp1: S74.

van Belkum, A.; Welker, M.; Erhard, M. and Chatellier, S. (2012). Biomedical mass spectrometry in today's and tomorrow's clinical microbiology laboratories. J. Clin. Microbiol. 50: 1513–1517.

van Duijkeren, E.; Wannet, W.J.; Houwers, D.J.; van Pelt, W. (2002). Serotype and phage type distribution of Salmonella strains isolated from humans, cattle, pigs, and chickens in The Netherlands from 1984 to 2001. J. Clin. Microbiol. 40: 3980–3985.

Welker, M. and Moore, E.R.B. (2011). Application of whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in systematic microbiology. Syst. Appl. Microbiol. 34: 2-11.

Wenning, M.; Breitenwieser, F.; Konrad, R.; Huber, I.; Busch, U.; Scherer, S. (2014). Identification and differentiation of food-related bacteria: A comparison of FTIR spectroscopy and MALDI-TOF mass spectrometry. J. Microbiol. Methods. 103: 44-52.

WHO (2015). WHO Estimates of the Global Burden of Foodborne Diseases. WHO 46: 1–15.

Wieser, A.; Schneider, L.; Jung, J. and Schubert, S. (2012). MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond (minireview). Appl. Microbiol. Biotechnol. 93: 965-974.

Wray, C. and Wray, A. (2000). Salmonella in Domestic Animals. CABI Publishing, New York, USA.

Zhao, X.; Lin, C. W.; Wang, J.; and Oh, D. H. (2014). Advances in rapid detection methods for foodborne pathogens. J. Microbiol. Biotechn. 24: 297–312.