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Methodology Article

Development and Verification of a MALDI-TOF MS-Based Method for Rapid and Confirmatory Identification of *Salmonella* in Feed and Foods

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Abstract: *Salmonella* infection is one of the major global public health hazards and remains as an economic burden to both developed and developing countries through the costs associated with trade banning, as well as surveillance, prevention, and treatment of the disease. Confirmatory and rapid identification of *Salmonella* in feed and foods of animal origin is crucial for mitigating the associated burdens. The conventional methods for isolation and identification of *Salmonella* species are based on culture and biochemical tests and are very time-consuming requiring 10-11 days. Hence, these drawbacks of the conventional methods warrant a rapid method for confirmatory identification of *Salmonella* in feed and foods of animal origin. Therefore, a method based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) was developed and verified for its reliable use in the laboratory. For verification, *Salmonella typhimurium* (ATCC 14028) was used as the target bacteria. *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as non-target bacteria. *Salmonella typhimurium* (ATCC 14028) produced black colonies with metallic sheen on BS agars, black colonies on XLD agars, and black-centered blue colonies on HE agars. MALDI TOF MS was found very efficient for confirmatory identification of *Salmonella* bacteria at the genus level without performing biochemical tests. Confirmatory identification of *Salmonella* could be made within four to five days after the commencement of the test. The expected limit of detection ($eLOD_{50}$) of *Salmonella* in chicken and beef was found < 1.7 ± 0, in milk 1.2 ± 0.2, in eggs < 1.2 ± 0.2, and in feed pellet < 1.6 ± 0.2 CFU/test portion. The accuracy, sensitivity, and specificity of the method were found 98%, 96%, and 100% respectively. No significant effects (p = 0.400) of sample matrices were found on test results carried out by the developed method. Likewise, in terms of ruggedness, no significant effects of analyst change (p = 0.787), incubator change (p = 0.787), and day change (p = 0.242) were found in the test results. The method was found robust and could be used in the laboratory for rapid and confirmatory identification of *Salmonella* in feeds, and foods of animal origin.

Keywords: Salmonella, Identification Method, Verification, MALDI TOF MS

1. Introduction

Salmonella bacteria are Gram-negative, facultative anaerobic, non-spore-forming, straight rods belonging to the family Enterobateriaceae [1]. *Salmonella* species are predominantly motile bacteria by peritrichous flagella with cell diameters between 0.7 and 1.5 µm, and lengths from 2 to 5 µm [2]. The bacteria may survive in variable conditions and able to grow at temperatures ranging between 8 to 45°C, at pH between 4.0 to 9.5, and in conditions of low water activity of 0.94 [3]. *Salmonella* bacteria is classified into *S. enterica* and *S. bongori* species based on the genetic diversity in their 16S rRNA. *S. enterica* is further classified into six subspecies based on their genomic relatedness and biochemical properties, which are- *S. enterica* subsp. *enterica*; *S. enterica* subsp. *salamae*; *S. enterica* subsp. *arizonae*; *S. enterica* subsp. *diarizonae*; *S. enterica* subsp. *houtenae*; and *S. enterica* subsp. *indica*. Among the subspecies, *S.*

enterica subsp. *enterica* is predominantly responsible for approximately 99% of *Salmonella* infections in humans and warm-blooded animals [4]. *S. typhi* and *S. paratyphi* serovars of *Salmonella* genus are pathogenic for humans, but non-pathogenic for animals. On the other hand, serotype *S. cholerasuis*, mostly carried by pigs, can cause salmonellosis in humans. Serovars *S. enteritidis* and *S. typhimurium* cause gastrointestinal tract infections in humans [2].

Salmonella infection is considered as one of the major global public and animal health hazards and remains as an economic burden to both developed and developing countries through the costs associated with surveillance, prevention and treatment of disease [4]. Four different clinical manifestations of *Salmonella* infection in humans are gastroenteritis, enteric fever, bacteremia and other extraintestinal complications, and chronic carrier state [5]. The organisms are transmitted via the ingestion of food or water contaminated with the wastes of infected individuals or animals [4]. Animals acquire *Salmonella* via vertical, or horizontal transmission from the contaminated environment, vehicles, feed, and vectors. After getting the bacteria, these animals become *Salmonella* disseminators through both horizontal and vertical pathways during primary production [6].

Eventually, animals are the primary source of salmonellae, and animal-based foods are the main transmission route to humans. Food contamination by *Salmonella* bacteria is a significant public health concern for consumers worldwide. The economic consequences are also crucial for the food producers and the industries. Ninety five percent *Salmonella* originated acute gastroenteritis occurs through consumption of contaminated food, especially meat and eggs [7]. Among the animal-based foods, pork is usually contaminated by Typhimurium and Derby serovars, whereas, poultry products disseminate Enteritidis, Typhimurium, and Sofia serovars to humans. Beef is contaminated predominantly by Typhimurium, Anatum and Weltevreden serovars [6]. Eggs may get *Salmonella* contamination directly during the formation of an egg in the reproductive tract of hens (including the ovary and oviduct); or, indirectly from contaminated environment where the bacteria penetrates through the shell membrane [8]. Among the different serotypes, *S. enteritidis* and *S. typhimurium* are considered to be zoonotic pathogens and responsible for non-typhoid human salmonellosis characterized by acute gastroenteritis [7]. *Salmonella* bacteria should be absent in animal-based food products [9]. Livestock carrying these bacteria rarely develop symptoms and making them almost impossible to detect, but animal-based foods get contaminated during the process of food handling, preparation and storage. Thus, identification and confirmation of salmonellae in foods play an important role for preventing food borne outbreaks [7].

Conventional methods for isolation, identification and confirmation of *Salmonella* species in sample matrices are usually based on culturing the organism on differential agar media and subsequent biochemical tests [10]. The basic steps in conventional methods for the detection of *Salmonella* in feed and food samples include a pre-enrichment in an appropriate media followed by selective enrichment in broth media, and isolation on differential media and finally biochemical and serological confirmation [7]. The conventional methods are cumbersome, time consuming and interpretation of test results is often difficult. At least four to five days are required to obtain the initial results, and four to six additional days to confirm a positive result by biochemical and serological tests [11, 12]. During this long period of time required for completion of the tests, there is a risk for commercialization of the food stocks be forbidden. Hence, these drawbacks of the conventional methods warrant a reliable method for rapid identification and confirmation of *Salmonella* in feed and foods. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is such a rapid and reliable method that could be alternative to reference methods for confirmatory identification of *Salmonella* in feed and food matrices [13-15].

MALDI-TOF MS is a robust tool for microbial identification [16]. In this method, bacterial protein profiles obtained from ribosomes are compared to a database of bacterial reference mass spectra for rapid identification at the genus, species, and even at the subspecies level [17]. Bacterial ribosomal proteome ranging from 4000 Da to 20,000 Da are readily scanned, identified and distinguished in the system [13]. The identification is typically done from fresh cultures on agar plates. In this technique, the sample for analysis is prepared on a target plate first by mixing with a matrix and dried. Then, the sample-matrix mixture is ionized using a laser beam to convert the molecules to gas-phase ions and individually charged [M+H]+. Once the sample molecules are ionized, the ions are arranged and separated based on their m/z ratio using a TOF mass analyzer [14]. Microbial identification through MALDI-TOF MS technique has advantages over reference methods by virtue of its rapidness, efficiency and low cost operation [15]. Hence, in our laboratory we developed and verified a MALDI-TOF MS based method for rapid and confirmatory identification of *Salmonella* species in feed and foods.

2. Methods

2.1. Method Development and Verification Plan

For rapid and confirmatory identification of *Salmonella* species in feed and foods, the method under the present study was developed based on the US Food and Drug Administration's Bacteriological Analytical Manual (FDA's BAM) Chapter 5 and Association of Official Analytical Chemists (AOAC) Official Method 2017.09. In this method, the target bacteria was first isolated on selective Xylose lysine desoxycholate (XLD) agar, Hektoen enteric (HE) agar, and Bismuth sulfite (BS) agar according to the procedure described in FDA's BAM Chapter 5 followed by confirmatory identification by MALDI Biotyper (Bruker microflex LT, Germany) following AOAC Official Method 2017.09 [11, 18]. Henceforth, the method was verified in the laboratory following the plan delineated in Figure 1.

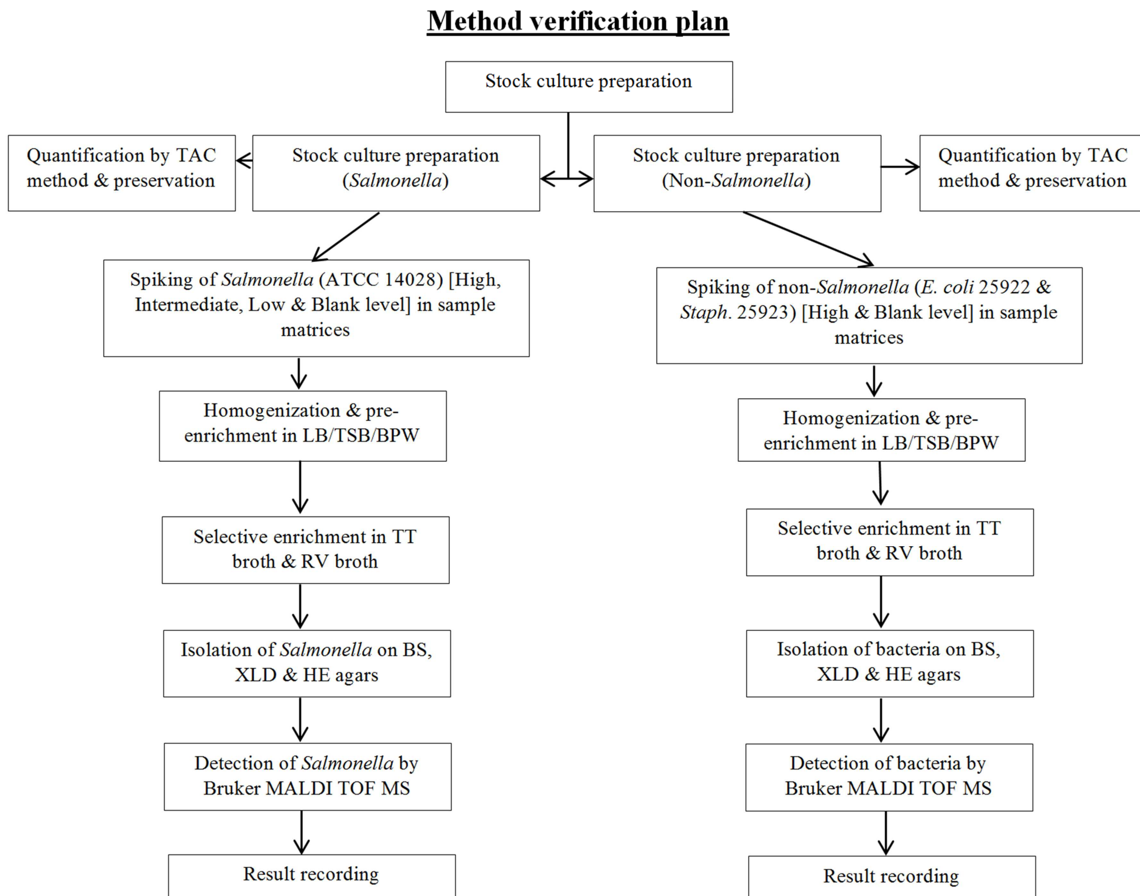


Figure 1. *Flow diagram showing the method verification plan and procedure.*

TAC= Total aerobic count, ATCC= American type culture collection, LB= Lactose broth, TSB= Trypticase soy broth, BPW= Buffered peptone water, TT= Tetrathionate, RV= Rappaport-Vassiliadis, BS= Bismuth sulfite, XLD= Xylose lysine desoxycholate, HE= Hektoen enteric, MALDI TOF MS= Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

The qualitative method developed in the present study was verified following the ISO 16140 and National Association of Testing Authorities (NATA), Australia 2012 guidelines. Hence, expected limit of detection ($eLOD_{50}$), accuracy, sensitivity, specificity, matrix effect, and ruggedness of the method were determined [19, 20].

2.2. Reference Bacterial Strains and Inoculum Preparation

Three different reference bacterial strains *Salmonella typhimurium* (ATCC 14028), *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used for the verification of the method. The bacterial strains were purchased from Microbiologics, Saint Cloud, Minnesota, USA. The freeze dried bacterial strains were revived following the manufacturer's guidelines and preserved in laboratory following the procedure described in [21]. *Salmonella typhimurium* (ATCC 14028) was used as target bacteria whereas *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as non-*Salmonella* during the process of method verification (Figure 1).

For inoculum preparation, the bacterial strains were grown in tryptic soy broth (Liofilchem, Italy) and incubated at 35°C for 24 h to obtain expected bacterial concentrations of 10^9 CFU/ml. Hereafter, serial 10-fold dilutions in 10-ml volumes were prepared for each bacterial strain to obtain expected high-level (10 CFU/mL), intermediate level (5 CFU/mL), and low level (1 CFU/mL) inocula [19, 22]. To determine the actual bacterial count in inoculum levels, the high-level inoculum was enumerated in non-selective Plate Count Agar media (Liofilchem, Italy) following FDA's BAM chapter 3 [23]. The counts of the intermediate and low-levels were calculated using the counts obtained from high-level inoculum and taking into account the dilutions factors used [19]. The procedure was repeated for six times for each bacterial strain and the mean bacterial counts were used for expected limit of $detection_{50}$ ($eLOD_{50}$) calculation. The inoculums were aliquoted in 1 mL volume and preserved at -80°C until further use.

2.3. Artificial Contamination of Food and Feed Sample Matrices

Five different types of sample matrices- chicken, beef, milk, eggs, and feed pellets were used to verify the method. All the sample matrices were autoclaved prior to use to confirm sterility. For each sample matrix, eight test portions each containing 25 g were prepared. For target bacteria, test

portions were spiked as the following plan: 2 at high level (10 CFU), 2 at intermediate level (5 CFU), 2 at low level (1 CFU), and 2 at blank level (Figure 1) [19]. For non-*Salmonella* bacteria, four test portions were used for each sample matrix where 2 portions were spiked at a high level and 2 portions were kept blank.

On the first day (08/11/2021), chicken and beef sample portions were artificially contaminated by Analyst 1 and proceeded to further steps. Likewise, on the second day (09/11/2021), the experiment was conducted on milk and egg sample portions by Analyst 2 and Analyst 3 respectively. Furthermore, Analyst 2 conducted the experiment on feed pellets on the third day. (10/11/2021).

2.4. Isolation of Bacteria from Artificially Contaminated Samples

Bacteria from spiked sample matrices were isolated following the procedure described in FDA's BAM Chapter 5 [11]. Briefly, 225 mL of appropriate pre-enriched media (LB for chicken, beef, and milk; TSB for eggs, and BPW for feed pellets) was added to each spiked 25 g sample portion and thoroughly mixed by an automated homogenizer (BagMixer, Interscience, France). After homogenization, the mixture was incubated for 24 ± 2 hours at 35°C. After pre-enrichment, the mixture was subjected to selective enrichment in both TT and RV broth for 24 ± 2 hours at 35 ± 2.0°C and 42 ± 0.2°C respectively. Henceforth, mixtures from both TT and RV broth were inoculated on XLD, HE and BS agars. The agar plates were incubated for 24 ± 2 hours at 35°C and colonies were examined.

2.5. Identification of Bacteria

The colonies from XLD, HE and BS agar plates were examined by Bruker MALDI Biotyper for confirmation and identification of both *Salmonella* and non-*Salmonella* species [13, 18, 24]. Reusable steel target plates were prepared using extended direct transfer (eDT) procedure [18]. Using a sterile colony-transfer device, smear of an isolated colony of bacteria was prepared as a thin film directly onto a sample position of target plate. The smear was overlaid with 1 µl 70% aqueous formic acid and allow to dry at room temperature. A1 position on the target plate was selected for bacterial test standard (BTS) control in each run and 1 µl of BTS solution was added at that point. After samples and BTS had dried, 1 µl HCCA matrix was added to the each BTS and sample points and dried at room temperature. Thereby, the target plate was ready for examination. Target plates were read by using two software namely *flexControl* and *MBT Compass* following manufacturer's protocol. The spectrum patterns generated from bacterial ribosomal proteins were used to reliably and accurately identify the bacteria [13]. The spectrum values presented in green with scores ≥2.0 were determined to be acceptable with high confidence identification. Those presented in yellow with scores between 1.70 and 1.99 were determined to be acceptable with low confidence identification. Results presented in red with scores ≤1.70 were considered not acceptable for identification [18].

2.6. Determination of Expected Limit of Detection$_{50}$ (eLOD$_{50}$)

eLOD$_{50}$ for target bacteria (*Salmonella* spp.) was determined following ISO/DIS 16140-3: 2017 [19]. The data obtained from spiking and subsequent recovery of *Salmonella typhimurium* (ATCC 14028) in different food matrices were used for eLOD$_{50}$ determination (Table 2).

2.7. Determination of Accuracy, Sensitivity, and Specificity

Accuracy, sensitivity, and specificity of the method was calculated from the following formulae [20, 25]

$$\text{Accuracy} = \frac{TP+TN}{TP+TN+FP+FN}$$

$$\text{Sensitivity} = \frac{TP}{TP+FN}$$

$$\text{Specificity} = \frac{TN}{TN+FP}$$

Here, TP =true positive, FN=false negative, TN=true negative and FP=false positive.

Values of TP, FN, TN and FP were determined from spiking and recovery data of all three bacteria used in the study.

2.8. Matrix Effect and Ruggedness Calculation

Effects of sample matrices on test results were determined; and ruggedness in terms of effects of analyst change, incubator change, and day change were determined as well. Chi-square test was used to determine the sample matrix effects and the ruggedness of the test method. Associations were compared in IBM SPSS Statistics 20 software. Analyses were carried out at a 95% confidence level and *p*-values less than 0.05 were considered significant [20].

3. Results

3.1. Inoculums Preparation

Inoculums at high, intermediate, and low-levels were prepared for each bacterial strains used in this method verification procedure. The actual bacterial counts determined at different inoculum levels are delineated in Table 1.

3.2. Isolation of Salmonella and Non-salmonella Bacteria

A total of 70 individual sample portions were spiked to verify the method (Table 2 and Table 3). Target organism (*Salmonella typhimurium*, ATCC 14028) was spiked in 30 sample portions; whereas, non-*Salmonella* bacteria (*E. coli*, ATCC 25922 & *Staphylococcus aureus*, ATCC 25923) were spiked in other 20 sample portions. A total of 20 sample portions were kept blank. *Salmonella typhimurium* (ATCC 14028) bacteria produced black colonies with metallic sheen on BS agars, black colonies on XLD agars and black centered blue colonies on HE agars (Figure 2).

Table 1. *Actual bacterial counts at high, intermediate, and low-level inoculums prepared during method verification.*

Name of Bacteria	Days	Bacterial count (CFU)		Mean CFU±SD		
		1	2	High-level	Intermediate-level	Low-level
Salmonella typhimurium (ATCC 14028)	Day 1	17	17	17±0	8.5±0	1.7±0
	Day 2	13	10	11.5±2	5.8±1	1.2±0.2
	Day 3	17	14	15.5±2	7.8±1	1.6±0.2
E. coli (ATCC 25922)	Day 1	15	17	16±1	ND	ND
	Day 2	12	9	10.5±2	ND	ND
	Day 3	17	14	15.5±2	ND	ND
Staphylococcus aureus (ATCC 25923)	Day 1	11	16	13.5±4	ND	ND
	Day 2	11	8	9.5±2	ND	ND
	Day 3	12	10	11±1	ND	ND

CFU=Colony forming unit, SD= Standard deviation, ND= Not done.

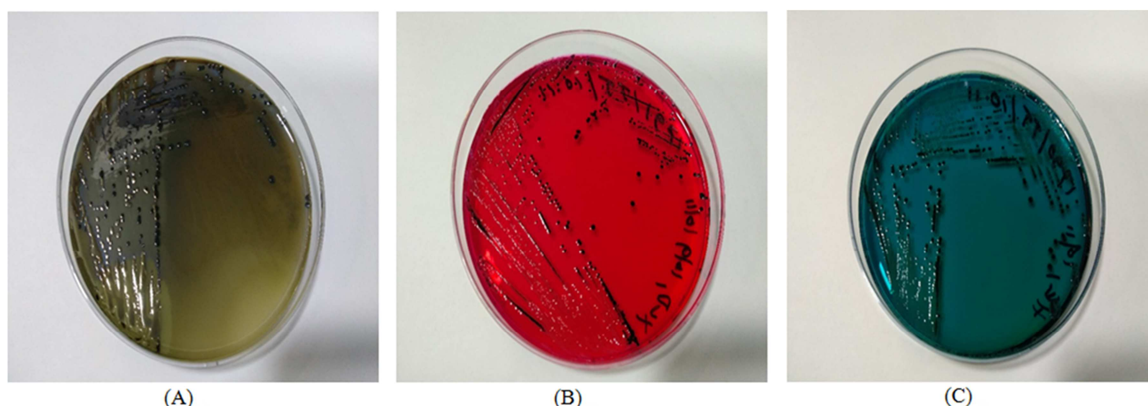


Figure 2. *Isolation of Salmonella typhimurium (ATCC 14028) on different isolation agar media. Black colonies with metallic sheen on BS agar (A), black colonies on XLD agar (B), and black centered blue colonies on HE agars (C).*

E. coli (ATCC 25922) produced yellow colonies on XLD agar, brownish colonies on BS agar, and pink colonies on HE agar. *Salmonella typhimurium* could be isolated from 29 out of 30 spiked sample portions (Table 2), whereas, *E. coli* could be isolated from all 20 spiked sample portions (Table 3). *Staphylococcus aureus* (ATCC 25923) could not be isolated on BS, XLD and HE agars. All the blank sample portions were found negative to any bacteria.

Table 2. *Expected limit of detection ($eLOD_{50}$) for Salmonella typhimurium (ATCC 14028) in different sample matrices.*

Sample matrix type	Positive/Spike ratio				$eLOD_{50}$/test portion (CFU) [19]
	High-level	Intermediate-level	Low-level	Blank	
Chicken	2/2	2/2	2/2	0/2	<1.7±0
Beef	2/2	2/2	2/2	0/2	<1.7±0
Milk	2/2	2/2	1/2	0/2	1.2±0.2
Egg	2/2	2/2	2/2	0/2	<1.2±0.2
Feed pellet	2/2	2/2	2/2	0/2	<1.6±0.2
Total=	10/10	10/10	9/10	0/10	-

Table 3. *Results of non-Salmonella (E. coli, ATCC25922 & Staphylococcus aureus, ATCC 25923) bacteria detection.*

Spiked bacteria	*Positive/Spike ratio					Total
	Chicken	Beef	Milk	Egg	Feed pellet	
E. coli (ATCC 25922)	2/2	2/2	2/2	2/2	2/2	10/10
Staphylococcus aureus (ATCC 25923)	2/2	2/2	2/2	2/2	2/2	10/10
Blank	0/2	0/2	0/2	0/2	0/2	0/10

*Positive as non-*Salmonella*.

3.3. Confirmatory Identification of Isolated Bacteria

In each run, Bruker MALDI biotyper was calibrated at BTS position (A1) and proceeded to testing samples after successful completion of MS calibration at assigned eight points ranging between 4000 Da to 20000 Da. Successful completion of MS calibration was marked by appearance of a dialogue box on *flexControl* window stating specific molecular weight of the individually charged molecules, peak error levels and standard deviation of the spectra (Figure 3). In each successful MS calibration, standard deviation of the spectra was ±300 ppm.

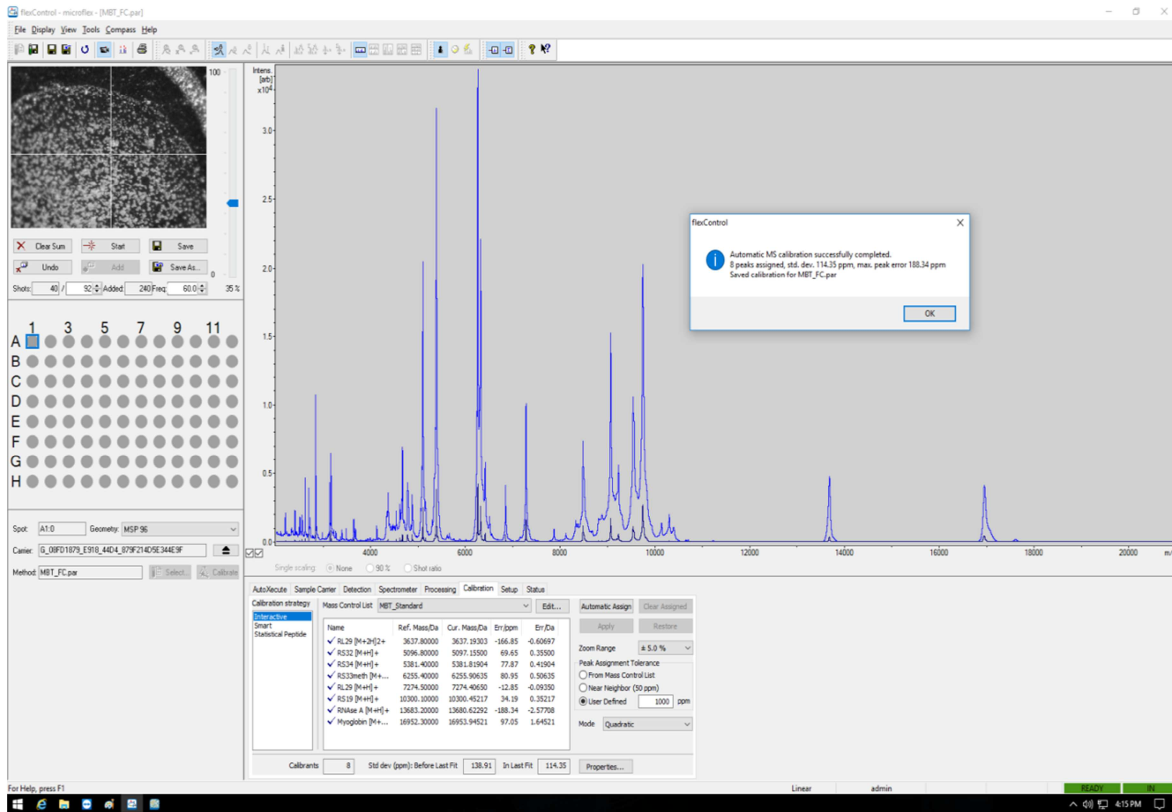


Figure 3. *Mass spectra acquired from BTS (A1) position indicating successful MS calibration.*

Mass spectra acquired from both test and BTS control positions were processed, and the resulting peak patterns were matched against reference patterns in the MALDI Biotyper reference library (Figure 4).

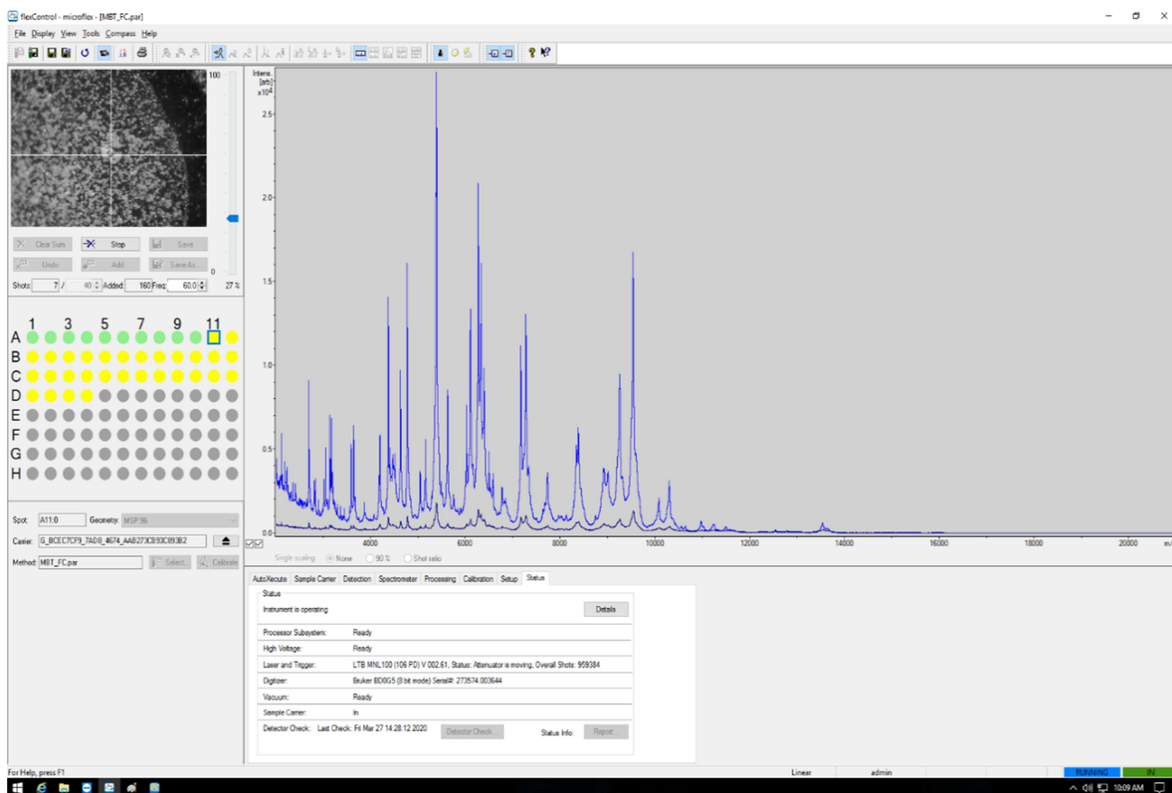
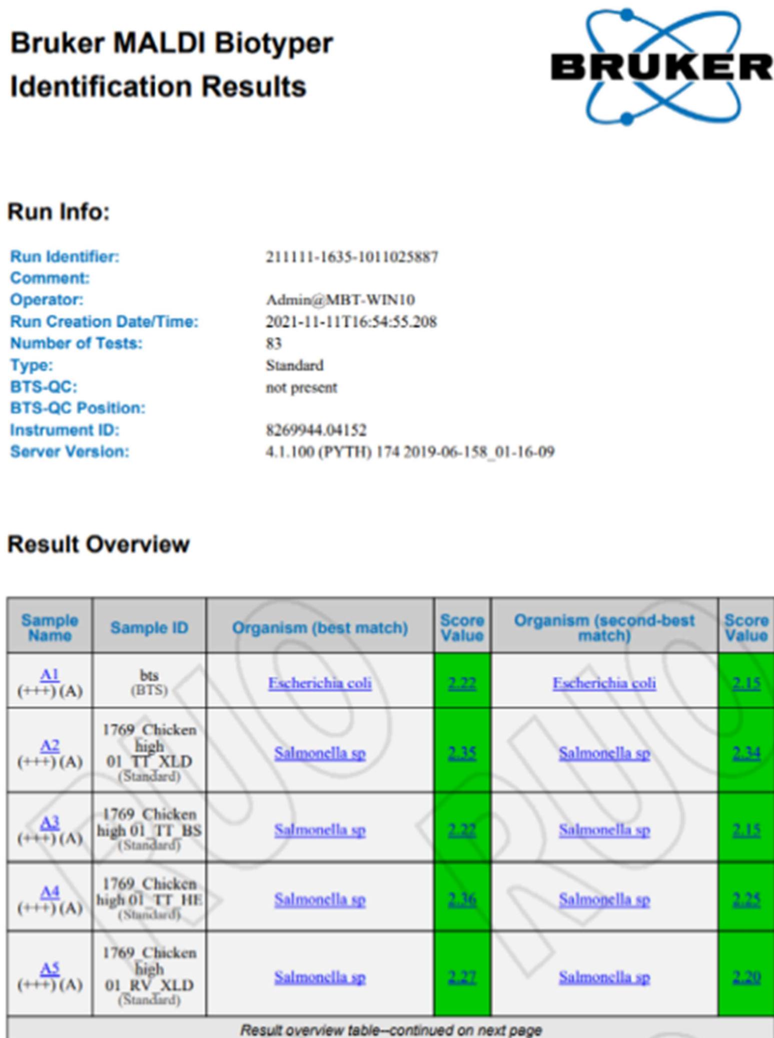


Figure 4. *Mass spectra acquired from Salmonella sp.*

After matching of peak patterns, log scores were generated based upon which bacterial confirmatory identification were made and results were reported (Figure 5).



Bruker MALDI Biotyper
Identification Results

BRUKER

Run Info:

Run Identifier:	211111-1635-1011025887
Comment:	
Operator:	Admin@MBT-WIN10
Run Creation Date/Time:	2021-11-11T16:54:55.208
Number of Tests:	83
Type:	Standard
BTS-QC:	not present
BTS-QC Position:	
Instrument ID:	8269944.04152
Server Version:	4.1.100 (PYTH) 174 2019-06-158_01-16-09

Result Overview

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A1 (+++) (A)	bts (BTS)	Escherichia coli	2.22	Escherichia coli	2.15
A2 (+++) (A)	1769 Chicken high 01 TT XLD (Standard)	Salmonella sp	2.35	Salmonella sp	2.34
A3 (+++) (A)	1769 Chicken high 01 TT BS (Standard)	Salmonella sp	2.22	Salmonella sp	2.15
A4 (+++) (A)	1769 Chicken high 01 TT HE (Standard)	Salmonella sp	2.36	Salmonella sp	2.25
A5 (+++) (A)	1769 Chicken high 01 RV XLD (Standard)	Salmonella sp	2.27	Salmonella sp	2.20

Result overview table—continued on next page

Figure 5. *Reported results after successful identification run in MBT Compass software. Score values >2.0 are presented by green, and scores between 1.70 and 1.99 are presented by yellow.*

All the organisms isolated from the sample portions spiked with *Salmonella typhimurium* were identified as *Salmonella* bacteria, and all the isolated non-*Salmonella* bacteria were identified as *E. coli.* In most of the cases, score values were >2.0 for both *Salmonella* and non-*Salmonella* bacteria.

3.4. Expected Limit of Detection (eLOD$_{50}$) of the Method

Salmonella typhimurium (ATCC 14028) could be isolated from all portions of each sample matrix spiked with different doses, except for one milk sample contaminated with a low-level dose. Hence, the eLOD$_{50}$ for chicken and beef has been found <1.7±0, for milk 1.2±0.2, for egg <1.2±0.2, and for feed pellet <1.6±0.2 CFU/test portion (Table 2).

3.5. Accuracy, Sensitivity, and Specificity of the Method

In this verification experiment, based on the *Salmonella typhimurium, E. coli, and Staphylococcus aureus* spiking data, the values of TP, FN, TN, and FP were found to be 29, 1, 20, and 0 respectively [Table 2 and Table 3]. The positive non-*Salmonella* readings were considered TN readings. Therefore, the accuracy, sensitivity, and specificity of the method were found 98%, 96%, and 100% respectively.

3.6. Matrix Effect and Ruggedness of the Method

No significant effects (p=0.400) of sample matrices were found on test results carried out by the developed method. Likewise, in terms of ruggedness, no significant effects of analyst change (p=0.787), incubator change (p=0.787), and day change (p=0.242) were found in the test results.

4. Discussion

High demand of animal products has provoked intensive animal production and processing of products, with an increased movement of foods globally. This situation may conduce to defective processing practices and an augment of

the risk of contamination of foods by foodborne pathogens at any point of the farm to fork chain [26]. Therefore, identification of *Salmonella* spp. in both feeds, and foods of animal origin is crucial regarding public health and food commercialization issues. However, in our Quality Control Laboratory, this method was developed to provide faster and reliable test results to our customers. Subsequently, the developed method was verified for ensuring its confident use in the laboratory. As the new method was developed based on already two validated methods, only full-filling verification parameters were considered sufficient for its use in the laboratory [19, 20].

In this method, *Salmonella* bacteria were isolated on selective agar media following reference FDA's BAM method [11]. The growth of *Salmonella typhimurium* (ATCC 14028), and *Escherichia coli* (ATCC 25922) in both pre-enrichment and enrichment media was marked by turbidity in the media. Production of characteristic colony BS, XLD, and HE agar plates indicated the suitability of the media for isolation of *Salmonella* bacteria from feed and foods of animal origin. Confirmatory identification was made by MALDI-TOF MS technology instead of biochemical and serological tests [11, 18]. Integration of MALDI-TOF MS reduced testing time from 10-11 days to 4-5 days. Successful completion of MS calibration prior to each run indicated high accuracy of the MALDI Biotyper machine used in the study (Figure 3). Moreover, identification of the bacteria in most of the cases with high confidence (scores ≥2.0) indicated the robustness of the method (Figure 4).

Five types of sample matrices were included in the verification procedure, among which one was poultry feed pellet. Inclusion of feed pellet enables the method testing any kind of feeds. Likewise, inclusions of chicken, beef, milk, and eggs enable the methods to test any kind of foods produced based on the stated animal products. Among the qualitative method verification criteria, $eLOD_{50}$ is the most important parameter, the acceptable value of which is ≤3.00 CFU/test portion [19]. In our verification study we found $eLOD_{50}$ of spiked *Salmonella typhimurium* (ATCC 14028) significantly below ($p<0.05$) in different sample matrices than the acceptable highest limit (Table 2). These findings indicate the high capability of the method to detect *Salmonella* bacteria in different sample matrices even in presence of very low numbers. Our findings are also in alignment with those found by Hantash *et al*., 2020 [22]. The high accuracy (98%) of the method confirmed its high capability to differentiate the *Salmonella* and non-*Salmonella* cases. The ability of the method to determine the *Salmonella* cases correctly (sensitivity) was found very high (96%) also. Similarly, its ability to determine the non-*Salmonella* cases correctly (specificity) was found very high (100%). The findings further confirmed the high strength of the method.

Changes in sample matrices could not influence over the test results acquired by the method. This finding indicates the absence of matrix effects in the method, and any type of relevant samples can be tested by the method. Besides, minor changes like analyst change, incubator change or time change also do not influence over the test results. Therefore, the method is a robust one and could be used even some changes take place in the laboratory.

5. Recommendations

(1) MALDI TOF MS can be used for rapid and confirmatory identification of *Salmonella* spp.
(2) MALDI TOF MS can be used as alternative to cumbersome biochemical and serological tests required for confirmatory identification of *Salmonella* spp.
(3) The method can be further verified including more sample matrices and bacterial strains for demonstrating its greater robustness and validity.

6. Conclusion

The method verified in the study was developed based on the two validated reference methods with a view to deliver rapid and confirmatory *Salmonella* identification results to the customers. All the necessary steps suggested by International Organization for Standardization, and National Association of Testing Authorities, Australia were followed for the verification of the method. The method has been found very robust with its high accuracy, sensitivity, specificity, insignificant matrix effects and ruggedness. Thus, the method can be used for the rapid and confirmatory identification of *Salmonella* in feed and foods.

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References

[1] McSorley, S. J. (2014) Immunity to intestinal pathogens: lessons learned from Salmonella. *Immunological Reviews*. 260 (1): 168-182.

[2] Chlebicz, A. and Śliżewska, K. (2018) Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. *International Journal of Environmental Research and Public Health*. 15 (5): 863.

[3] Rönnqvist, M., Välttilä, V., Ranta, J., and Tuominen, P. (2018) Salmonella risk to consumers via pork is related to the Salmonella prevalence in pig feed. *Food microbiology*. 71: 93-97.

[4] Eng, S.-K., Pusparajah, P., Ab Mutalib, N.-S., Ser, H.-L., Chan, K.-G., and Lee, L.-H. (2015) Salmonella: a review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in Life Science*. 8 (3): 284-293.

[5] Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., Jones, T. F., Fazil, A., Hoekstra, R. M., and Studies, I. C. o. E. D. B. o. I. (2010) The global burden of nontyphoidal Salmonella gastroenteritis. *Clinical Infectious Diseases*. 50 (6): 882-889.

[6] Ferrari, R. G., Rosario, D. K., Cunha-Neto, A., Mano, S. B., Figueiredo, E. E., and Conte-Junior, C. A. (2019) Worldwide epidemiology of Salmonella serovars in animal-based foods: a meta-analysis. *Applied and Environmental Microbiology*. 85 (14): e00591-00519.

[7] Paniel, N. and Noguer, T. (2019) Detection of Salmonella in food matrices, from conventional methods to recent aptamer-sensing technologies. *Foods*. 8 (9): 371.

[8] Whiley, H. and Ross, K. (2015) Salmonella and eggs: from production to plate. *International Journal of Environmental Research and Public Health*. 12 (3): 2543-2556.

[9] Ncoko, P., Jaja, I. F., and Oguttu, J. W. (2020) Microbiological quality of beef, mutton, and water from different abattoirs in the Eastern Cape Province, South Africa. *Veterinary world*. 13 (7): 1363.

[10] de Boer, E. and Beumer, R. R. (1999) Methodology for detection and typing of foodborne microorganisms. *International Journal of Food Microbiology*. 50 (1-2): 119-130.

[11] Andrews, W. H., Jacobson, A., and Hammack, T., *Bacteriological Analytical Manual Chapter 5: Salmonella*. 2021, AOAC International Gaithersburg. p. 5.01-05.020. https://www.fda.gov/food/laboratory-methods-food/bam-chapter-025-salmonella [retrieved on 2/11/2021].

[12] ISO 6579-1: 2017, (2017) Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of Salmonella — Part 1: Detection of Salmonella spp. *International Organization for Standardization. ISO Central Secretariat, Chemin de Blandonnet 8, CP 401-1214 Vernier, Geneva, Switzerland*: https://www.iso.org/standard/56712.html [retrieved on 5/11/2021].

[13] Kang, L., Li, N., Li, P., Zhou, Y., Gao, S., Gao, H., Xin, W., and Wang, J. (2017) MALDI-TOF mass spectrometry provides high accuracy in identification of Salmonella at species level but is limited to type or subtype Salmonella serovars. *European journal of mass spectrometry*. 23 (2): 70-82.

[14] Pavlovic, M., Huber, I., Konrad, R., and Busch, U. (2013) Application of MALDI-TOF MS for the identification of food borne bacteria. *The open microbiology journal*. 7: 135.

[15] Torres-Sangiao, E., Leal Rodriguez, C., and García-Riestra, C. (2021) Application and Perspectives of MALDI–TOF Mass Spectrometry in Clinical Microbiology Laboratories. *Microorganisms*. 9 (7): 1539.

[16] Angeletti, S. (2017) Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) in clinical microbiology. *Journal of Microbiological Methods*. 138: 20-29.

[17] Gaudreau, A., Labrie, J., Goetz, C., Dufour, S., and Jacques, M. (2018) Evaluation of MALDI-TOF mass spectrometry for the identification of bacteria growing as biofilms. *Journal of Microbiological Methods*. 145: 79-81.

[18] Bastin, B., Bird, P., Benzinger, M. J., Crowley, E., Agin, J., Goins, D., Sohier, D., Timke, M., Shi, G., and Kostrzewa, M. (2018) Confirmation and Identification of Salmonella spp., Cronobacter spp., and Other Gram-Negative Organisms by the Bruker MALDI Biotyper Method: Collaborative Study, First Action 2017.09. *Journal of AOAC International*. 101 (5): 1593-1609.

[19] 16140-3: 2017, I. (2017) Microbiology of the food chain — Method validation — Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory. *International Organization for Standardization*. ISO Central Secretariat, Chemin de Blandonnet 8, CP 401-1214 Vernier, Geneva, Switzerland: https://www.iso.org/standard/66324.html [retrieved on 66305/66311/62020].

[20] National Association of Testing Authorities, Australia. (2012) Guidelines for the validation and verification of quantitative and qualitative test methods. http://www.demarcheiso17025.com/document/Guidelines%17020for%17020the%17020validation%17020and%17020verification%17020of%17020quantitative%17020and%17020qualitative%17020test%17020methods.pdf [retrieved on 5/11/2020].

[21] Simione, F. P. (2009) Thermo scientific nalgene and nunc cryopreservation guide. *Thermo Fisher Scientific Incorporation, New York*. https://tools.thermofisher.com/content/sfs/brochures/D17044.pdf [retrieved on 5/11/2020].

[22] Hantash, T., Nauta, M., Al-Sabi, M. N., Al-ali, W. Q., and Vigre, H. (2020) The "Rapid'Salmonella" Method: Estimation of the Limit of Detection for Salmonella Strains Typhimurium and Enteritidis Isolated from Frozen Poultry Meat. *Modern Applied Science*. 14 (12): 43-51.

[23] Maturin, L. and Peeler, J. (2001) Bacteriological Analytical Manual (BAM) Chapter 3: Aerobic plate count. *Bacteriological analytical manual (BAM), US Food and Drug Administration*. https://www.fda.gov/food/laboratory-methods-food/bam-chapter-3-aerobic-plate-count. [retrieved on 15/3/2021].

[24] Shell, W. S., Sayed, M. L., Allah, F. M. G., Gamal, F. E. M., Khedr, A. A., Samy, A., and Ali, A. H. 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 (9): 1083.

[25] Baratloo, A., Hosseini, M., Negida, A., and El Ashal, G. (2015) Part 1: simple definition and calculation of accuracy, sensitivity and specificity. *Emergency*. 3 (2): 48-49.

[26] Dhama, K., Rajagunalan, S., Chakraborty, S., Verma, A., Kumar, A., Tiwari, R., and Kapoor, S.(2013) Food-borne pathogens of animal origin-diagnosis, prevention, control and their zoonotic significance: a review. *Pakistan journal of biological sciences: PJBS*. 16 (20): 1076-1085.

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