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Integration of MALDI TOF MS in the Most Probable Number Method for Enumeration of *Escherichia coli* Significantly Reduces the Assay Time

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Abstract: *Escherichia coli* (*E. coli*) can be associated with food contamination incidents and frequently causes serious food poisoning in humans. Detection and enumeration of *E. coli* in food matrices is crucial regarding food safety issues. Most Probable Number (MPN) assay for the enumeration of *E. coli* is widely used in laboratories. A limitation of the conventional reference MPN method is the long time required to obtain definitive results, which often sequels dissatisfaction among the customers. The aim of the current research was to mitigate the problem by the integration of a credible and rapid tool for the confirmatory identification of *E. coli* in MPN assay instead of biochemical tests. *Real-time* PCR and/or MALDI TOF MS were considered better candidates for so. The experiment was conducted in three sample matrices (beef, chicken, milk) and each was spiked with target *E. coli* (ATCC 25922) at low (47.7±4.5 CFU/g), intermediate (103.0±5.0 CFU/g), and high (204.7±2.5 CFU/g) doses. The mean *E. coli* counts by MPN method in low, intermediate, and high-level contaminated beef were 53.7±4.0, 99.3±9.2, and 216±5.8/g respectively. Those in chicken were 53.3±4.6, 110.0±0.0, and 203.3±20.8/g; and in milk 56.0±0.0, 104.7±9.2, and 213.3±5.8/ml respectively. *Real-time* PCR and MALDI TOF MS did not differ significantly ($p=0.199$) with biochemical tests in resulting MPN of *E. coli* in sample matrices. The method was found very linear within the contamination range with high R-squared values (≥ 0.99) in all three sample matrices. The mean assay time when employed biochemical tests, *real-time* PCR and MALDI TOF MS were 121.3±6.3, 77.4±6.3, and 74.2±6.1 hours respectively. Both *real-time* PCR and MALDI TOF MS significantly ($p=0.000$) reduced the assay time compared to that by biochemical tests. Significant ($p=0.003$) difference was also found between MPN assay times required by *real-time* PCR and MALDI TOF MS methods. Considering the research findings, MALDI TOF MS is recommended for integration in MPN assay for *E. coli*.

Keywords: MPN, *Escherichia coli*, PCR, MALDI TOF MS

1. Introduction

Escherichia coli (*E. coli*) is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium under the family Enterobacteriaceae [1]. *E. coli* cells are typically about 2.0 µm long and 0.25–1.0 µm in diameter, and occur as single straight rods. They are motile by peritrichous flagella, but may be nonmotile as well [2]. The bacteria is commonly found in the lower intestine of warm-blooded animals. Most of the *E. coli* strains are harmless, except some pathogenic serotypes which can cause serious food poisoning in humans, and are responsible for food contamination incidents [3]. *E. coli* is expelled into the environment with feces of the hosts and the bacterium can grow massively in fresh fecal matter under aerobic conditions [4]. Pathogenic strains of *E. coli* are usually transmitted through fecal–oral route to cause food poisoning in humans. Shiga toxin-producing *E. coli* (STEC) and O157:H7 strains, can be particularly dangerous. The primary sources of STEC outbreaks are usually raw or undercooked meat products, raw milk and cheeses [5].

E. coli cells are able to survive outside the host body, which makes them potential indicator organisms to test food samples

for fecal contamination [6]. Detection of *E. coli* in raw and processed foods indicates contamination by fecal materials from poor hygienic practices (cross contamination from food contact surfaces, raw foods or food handlers) or there has been inadequate processing. The tolerance level of *E. coli* in raw and processed foods is 0 log to 1 log CFU/g, and a count > 2 log CFU/g makes the foods unsatisfactory for consumption [7, 8]. Therefore, only detection of *E. coli* is not sufficient, rather enumeration of the bacteria in food stuffs is essential.

There are many methods for enumerating *E. coli* in use including the Most Probable Number (MPN), fluorogenic MUG, Petrifilm, ColiComplete disc, Colilert, qPCR methods, and membrane filtration assays [6, 9-11]. Among all, the MPN method is reliable, comparatively easy to execute, and widely used in laboratories [12]. The MPN method is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases. Here, serial dilutions of a sample are inoculated into different types of broth media step-by-step. Scores from the number of gas positive tubes in the first phase are used to perform the test in another broth in second phase. In third phase, the bacteria is isolated on selective agar media and confirmatory identification is made by Indole, Voges-Proskauer, and Citrate tests [6, 13]. Finally, combinations of positive results are used to estimate the number of *E. coli* present.

A remarkable drawback of the MPN method is the long time required to obtain definitive results from time consuming biochemical tests [14]. The aim of the current research was to address the problem by integration of a rapid and reliable method replacing biochemical tests for confirmatory identification of *E. coli* in MPN assay. Among the rapid and confirmatory methods, *real-time* PCR assay is reliable with high sensitivity and accuracy for *E. coli* detection [15]. Likewise, MALDI TOF MS is also a very rapid and credible method for microbial detection with high accuracy and specificity [16, 17]. Thus, the hypothesis of the current research was that integration of *Real-time* PCR and/or MALDI TOF MS might significantly reduce the MPN assay time for *E. coli*.

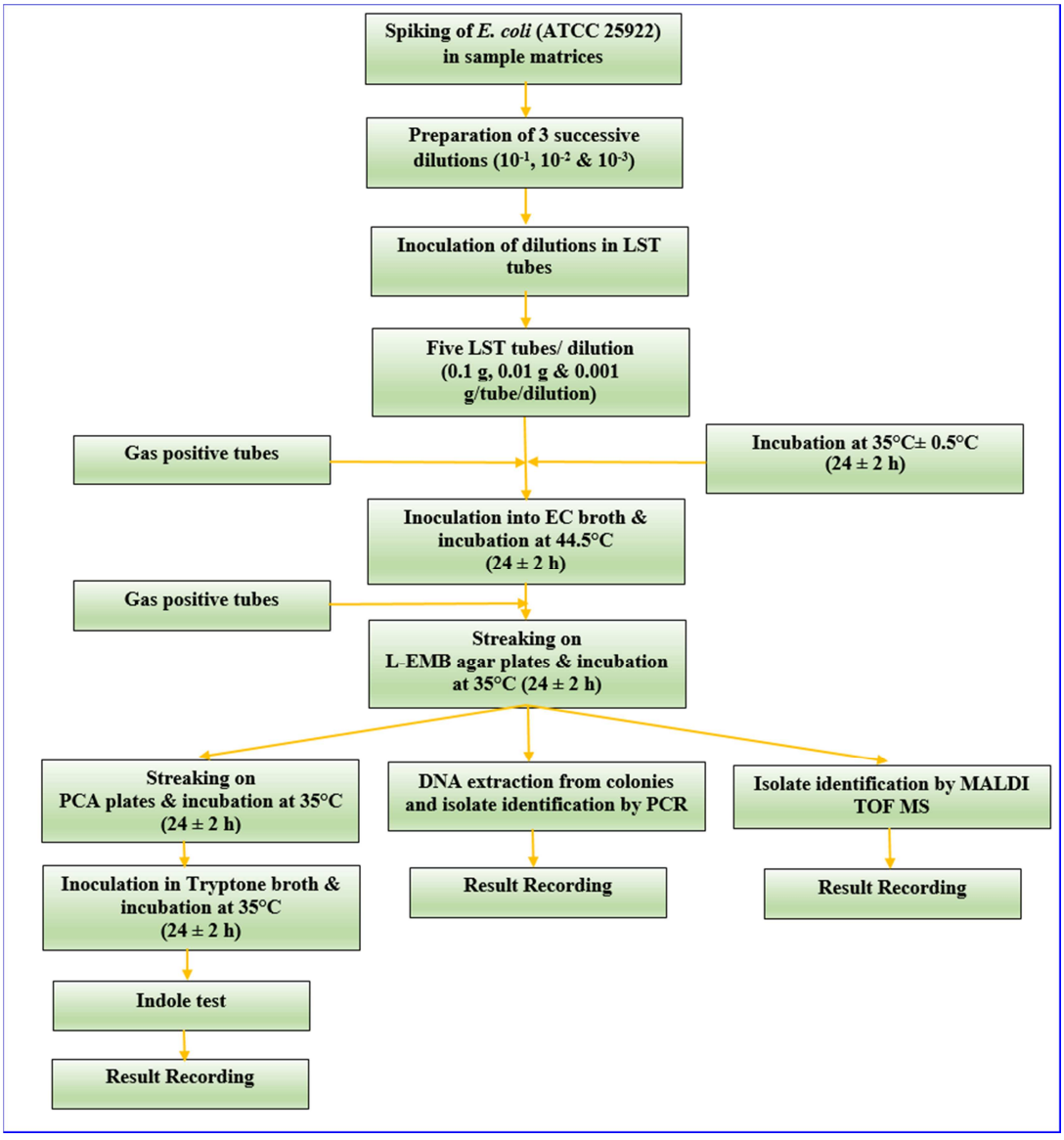


Figure 1. *Research plan for the integration of MALDI TOF MS in the MPN method for enumeration of E. coli.*

2. Materials and Methods

2.1. Research Plan

The current research was conducted during the period of July 2021 to May 2022. *Real-time* PCR and MALDI TOF MS was integrated in the reference MPN method for the rapid enumeration of *E. coli* in different sample matrices. Following the ISO 7251:2005, and the US Food and Drug Administration's Bacteriological Analytical Manual (FDA's BAM) Chapter 4 reference methods, *E. coli* was isolated on Levine's eosin-methylene blue (L-EMB) agar (Liofilchem, Italy) plates and identified by biochemical tests [6, 13]. Alongside the biochemical tests, isolates were also identified simultaneously by *real-time* PCR using CFX96™ Real-Time system (Bio-Rad, USA) and MALDI TOF MS using Daltronic Microflex LT MALDI Biotyper (Bruker®, Germany) (Figure 1) [16, 18]. The MPN of *E. coli* was determined based on the number of the confirmed EC tubes contained the bacteria in each dilution. Each test was repeated three times to reduce manual errors. Finally, results obtained from the stated three methods were compared to establish the rationales for the integration of MALDI TOF MS in the reference MPN method for enumeration of *E. coli*.

2.2. Reference Bacterial Strains and Inoculum Preparation

E. coli (ATCC 25922) purchased from Microbiologics, Saint Cloud, Minnesota, USA, was used in the research. For inoculum preparation, the bacterial strain was cultured in Tryptic Soy Broth (Liofilchem, Italy) and incubated at 35°C for 24 h to obtain expected bacterial concentrations of 10^9 Colony Forming Unit (CFU)/ml. A serial 10-fold dilution of the stock was prepared in 100-ml volume to obtain low-level (50 CFU/ml), intermediate level (100 CFU/ml), and high-level (200 CFU/ml) inocula. To determine the actual bacterial counts in all the inocula levels, *E. coli* was enumerated in plate count agar (PCA) media (Liofilchem, Italy) following FDA's BAM chapter 3 [19]. The enumeration procedure was repeated three times for each level inoculum and the mean bacterial count was determined. The inocula were aliquoted in 25 ml volume and preserved at -80°C until further use.

2.3. Artificial Contamination of Sample Matrices

The experiment was conducted in three different types of sample matrices- beef, chicken, and milk. The sample matrices were autoclaved prior to use to confirm sterility. From each sample matrix, 25 g was aseptically weighed and artificially contaminated with 25 ml of respective stock culture to obtain expected 200 CFU (high-level), 100 CFU (intermediate level), and 50 CFU (low-level) *E. coli* load per gram or milliliter of sample portion. Thereafter, 200 ml of Butterfield's phosphate-buffered water (pH 7.2) was added to each artificially contaminated sample portion and homogenized to obtain 10^{-1} dilution [6].

2.4. Presumptive Enumeration of E. coli

E. coli in artificially contaminated sample matrices was enumerated following the most probable number (MPN) method described in FDA/BAM chapter 4 [6]. A serial decimal dilutions of 10^{-2} and 10^{-3} were prepared in Butterfield's phosphate-buffered water from 10^{-1} dilutions of the artificially contaminated sample matrices. One ml aliquot from each of 10^{-1}, 10^{-2}, and 10^{-3} dilutions was inoculated into 5 Lauryl tryptose (LST) broth tubes for a 5 tube MPN assay. LST tubes were incubated at 35°C± 0.5°C for 24 to 48 h and examined for gas formation. Gas formation in LST tubes indicated presumptive *E. coli* positivity and confirmed test was performed on such tubes. A loopful of suspension from each gassing LST broth tubes of the presumptive test was transferred to EC broth and incubated for 24 to 48 h at 44.5°C and examined for gas production.

2.5. Isolation, Identification and Confirmed Enumeration of E. coli

For isolation of *E. coli,* a loopful of broth from each gassing EC tube was streaked on L-EMB agar plates and incubated for 18-24 h at 35°C ± 0.5°C. The colonies on L-EMB agar plates were observed for suspected *E. coli.* For confirmatory identification, suspected colonies were examined by Indole tests, *real-time* PCR, and MALDI TOF MS. Confirmed MPN of *E. coli* was calculated based on the proportion of EC tubes in 3 successive dilutions that contained *E. coli* [6, 16, 18]. The total assay time from commencement of the test to the acquisition of the final results were recorded in each case.

2.5.1. Confirmatory Identification of E. coli by Indole Test

For Indole tests, suspected single colonies of *E. coli* on L-EMB agar plates were streaked on non-chromogenic PCA media and incubated for 18-24 h at 35°C± 0.5°C. Henceforth, the single colonies from PCA plates were inoculated in Tryptone broth and incubate 24 ± 2 h at 35°C ± 0.5°C. Indole tests were conducted by adding 0.3 mL of Kovacs' reagent in the Tryptone broth tubes. Appearance of distinct red color in upper layer of the Tryptone broth was considered positive [6, 13].

2.5.2. Confirmatory Identification of E. coli by Real-Time PCR

Suspected colonies from L-EMB agar media were subjected to *real-time* PCR for confirmatory identification of *E. coli*. DNA from *E. coli* suspected colonies was extracted in automated Maxwell® RSC nucleic acid extraction system (Promega, USA) using Maxwell® RSC tissue DNA kits (Promega, USA) following manufacturer's protocol. Briefly, 1-2 *E. coli* suspected colonies were suspended in 500 µl nuclease free water in a sterile 1.5 ml centrifuge tube. The suspension was centrifuged for 5 minutes at 13000 rpm and the supernatant was discarded. The pellet was re-suspended in 500 µl nuclease free water

and 350 µl bacterial suspension was loaded in the appropriate well of the Maxwell® RSC tissue DNA kit. The kit was inserted in the automated Maxwell® RSC nucleic acid extraction system and after 42 minutes run, pure DNA was obtained in 50 µl elution buffer.

The *real-time* PCR assays were performed in CFX96™ thermal cycler (Bio-Rad, USA). Each reaction mix was prepared in 20 µl volume by adding 10 µl Go Taq® 2X qPCR sybr green master mix (Promega, USA); 1 µl *yaiO* forward primer (5′ TGATTTCCGTGCGTCTGAATG 3′) and 1 µl *yaiO* reverse primer (5′ ATGCTGCCGTAGCGTGTTTC 3′); 3 µl extracted DNA template; and 5 µl nuclease free water. PCR assays were conducted under the following conditions: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing at 58°C for 30 s, and primer extension at 72°C for 1 min. Melt curve step from 65°C to 95°C was added to observe the specificity of amplification in each PCR run [18]. Confirmatory identification of *E. coli* were made observing amplification curve and respective *CT* values in each PCR reaction.

2.5.3. Confirmatory Identification of E. coli by MALDI TOF MS

The colonies on L-EMB agar plates were examined by MALDI TOF MS using Daltronic Microflex LT MALDI biotyper (Bruker®, Germany) for confirmatory identification of *E. coli*. Smears of bacterial colonies were prepared on spots of reusable steel target plate following extended direct transfer (eDT) procedure [16]. The smears were overlaid with 1 µl 70% aqueous formic acid and dried at room temperature. A1 position on the target plate was selected for bacterial test standard (BTS) control in each run. After smears and BTS dried, 1 µl HCCA matrix was added to the each BTS and smear position, and dried at room temperature. Thus, the target plate was ready for examination. Target plate was read by *flexControl* and *MBT Compass* softwares following manufacturer's protocol. The spectrum patterns acquired from bacterial ribosomal proteins were used to identify the bacteria [20]. The spectra generated log scores between 2.0 and 3.0 were considered acceptable with high confidence identification. Those presented with log scores between 1.70 and 1.99 were considered acceptable with low confidence identification. Results presented with log scores ≤1.70 were considered not acceptable for identification [16].

2.6. Linearity of the MPN Method for Enumeration of E. coli

Linearity of the MPN method was calculated to determine the ability of the procedure to obtain test results in different sample matrices within the given range [21]. For linearity calculation, the mean MPNs of *E. coli* obtained in different sample matrices were plotted against their respective inoculum dose and regression line was built in Microsoft Excel 2013 [22]. Three regression lines were built separately for MPNs of *E. coli* found in beef, chicken, and milk. R-squared values (coefficient of determination) were used to determine the differences between the observed data and the fitted values.

2.7. Data Analysis

Raw data of all experiments were subjected to statistical analyses. Regression lines and graphs were built in Microsoft Excel 2013, mean values and associations were compared in IBM SPSS Version 20.0 software. Analyses were carried out at 95% confidence level and *p*-values less than 0.05 were considered significant.

3. Results

3.1. Actual E. coli Counts at Different Inoculum Levels

Inocula were prepared at low, intermediate, and high-levels and the actual *E. coli* counts determined at different levels are delineated in Table 1. The mean *E. coli* counts in low, intermediate, and high-level inocula were 47.7±4.5, 103.0±5.0, and 204.7±2.5 CFU/ml respectively.

Table 1. *Actual E. coli counts at low, intermediate, and high-level inocula.*

Inoculum level	Counts in test replicates (CFU)			Mean count (CFU±SD)
	1	2	3	
Low-level	48	52	43	47.7±4.5
Intermediate-level	108	103	98	103.0±5.0
High-level	205	207	202	204.7±2.5

CFU=Colony forming unit, SD= Standard deviation.

3.2. Gas Formation in LST and EC Tubes

Gas formation in both LST and EC tubes were marked by the accumulation of gas in Durham tubes, displacement of medium in tubes or effervescence when tubes were gently agitated. Total numbers of gassing EC tubes per inoculation level in each test replicate in different sample matrices are presented in Table 2, Table 3, and Table 4.

Table 2. *Determination of the MPN of E. coli in beef.*

Inoculation level	Gas positive /dilution level			MPN of *E. coli*/g	Mean MPN of *E. coli*/g
	10^{-1} (0.1g/tube)	10^{-2} (0.01g/tube)	10^{-3} (0.001g/tube)		
	4/5	5/5	2/5	56	
Low	4/5	5/5	2/5	56	53.7±4.0
	5/5	2/5	0/5	49	
	5/5	2/5	2/5	94	
Intermediate	5/5	2/5	2/5	94	99.3±9.2
	5/5	3/5	1/5	110	

Inoculation level	Gas positive /dilution level			MPN of *E. coli*/g	Mean MPN of *E. coli*/g
	10^{-1} (0.1g/tube)	10^{-2} (0.01g/tube)	10^{-3} (0.001g/tube)		
	5/5	4/5	2/5	220	
High	5/5	4/5	2/5	220	216±5.8
	5/5	3/5	4/5	210	

Table 3. *Determination of the MPN of E. coli in chicken.*

Inoculation level	Gas positive /dilution level			MPN of *E. coli*/g	Mean MPN of *E. coli*/g
	10^{-1} (0.1g/tube)	10^{-2} (0.01g/tube)	10^{-3} (0.001g/tube)		
	4/5	5/5	2/5	56	
Low	4/5	5/5	2/5	56	53.3±4.6
	4/5	5/5	1/5	48	
	5/5	3/5	1/5	110	
Intermediate	5/5	3/5	1/5	110	110.0±0.0
	5/5	3/5	1/5	110	
	5/5	4/5	2/5	220	
High	5/5	3/5	4/5	210	203.3±20.8
	5/5	3/5	3/5	180	

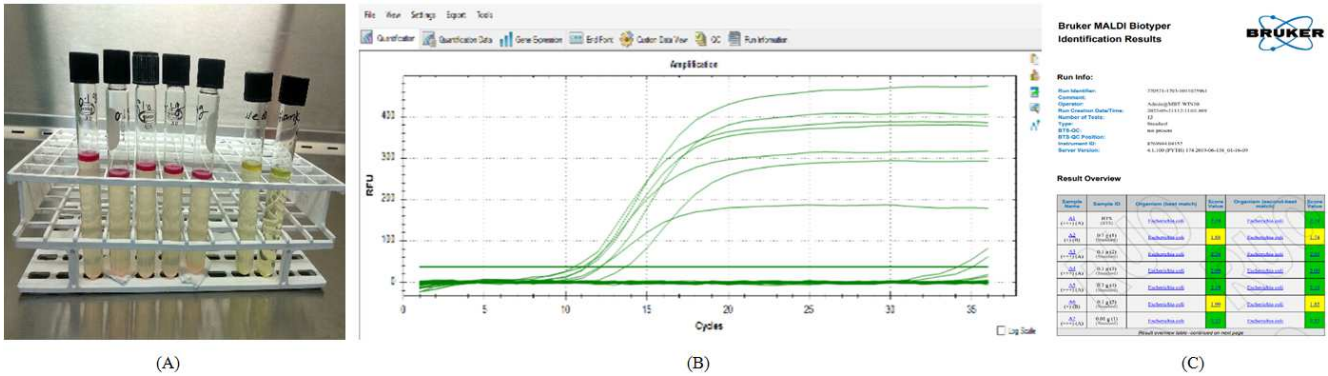
Table 4. *Determination of the MPN of E. coli in milk.*

Inoculation level	Gas positive /dilution level			MPN of *E. coli*/ml	Mean MPN of *E. coli*/ml
	10^{-1} (0.1g/tube)	10^{-2} (0.01g/tube)	10^{-3} (0.001g/tube)		
	4/5	5/5	2/5	56	
Low	4/5	5/5	2/5	56	56.0±0.0
	4/5	5/5	2/5	56	
	5/5	3/5	1/5	110	
Intermediate	5/5	3/5	1/5	110	104.7±9.2
	5/5	2/5	2/5	94	
	5/5	4/5	2/5	220	
High	5/5	3/5	4/5	210	213.3±5.8
	5/5	3/5	4/5	210	

Table 2 shows that in beef, total number of gas positive EC tubes in all three test replicates at low-level inoculation was 29, those in intermediate and high-levels were 27 and 34 respectively. Likewise, in chicken, those were 32, 27 and 34 (Table 3), and in milk 33, 27 and 35 respectively (Table 4). No significant ($p=0.223$, Pearson Chi-Square test) matrix effects were found based on gas production by the contaminating bacteria.

3.3. Isolation and Confirmatory Identification of E. coli

E. coli could be isolated on L-EMB agar media from all gas positive EC tubes shown in Table 2, Table 3, and Table 4. *E. coli* produced black colonies with metallic sheen on the L-EMB agar plates. In Indole tests, suspected *E. coli* isolates produced distinct red color in upper layer of Tryptone broth, whereas, no such color development was observed in negative control (*Salmonella typhimurium*, ATCC 14028) and blank tubes (Figure 2). Thus, Indole tests confirmed all the isolates in gas positive EC tubes from beef, chicken, and milk sample matrices as *E. coli*. A total of 90 isolates from beef, 93 isolates from chicken, and 95 isolates from milk samples in three replicates of the test were confirmed as *E. coli*.



(A) (B) (C)

Figure 2. *Production of distinct red color by suspected E. coli on upper layer of Tryptone broth in Indole tests (A), Graphs in real-time PCR confirming isolates as E. coli (B), and confirmatory identification of the isolates by MALDI TOF MS (C).*

The same isolates from beef, chicken, and milk confirmed as *E. coli* in Indole tests were also identified as *E. coli* in *real-time* PCR

and MALDI TOF MS. Cycle threshold (Ct) values for *E. coli* positive cases in *real-time* PCR ranged from 14.0 to 19.0 and Melt temperatures for both *E. coli* positive control and recovered isolates were found between 85.5°C and 86°C. In MALDI TOF MS, *E. coli* could be identified with high confidence in most of the cases (log scores ≥2.0) [Figure 2]. No significant ($p=0.199$, Pearson Chi-Square test) differences were found among biochemical tests, *real-time* PCR, and MALDI TOF MS in the efficiency of identifying *E. coli* in the current research.

3.4. Confirmed MPN of E. coli

Confirmed MPNs in test replicates in different sample matrices were determined upon confirmatory identification of presumptive *E. coli* by Indole tests, *real-time* PCR, and MALDI TOF MS. In all three methods, the mean MPNs of *E. coli* in low, intermediate, and high-level contamination in beef were found 53.7±4.0, 99.3±9.2, and 216±5.8/g respectively (Table 2). Those in chicken were 53.3±4.6, 110.0±0.0, and 203.3±20.8/g; and in milk 56.0±0.0, 104.7±9.2, and 213.3±5.8/ml respectively (Table 3 and Table 4). Integration of *real-time* PCR and MALDI TOF MS in the MPN method resulted similar *E. coli* counts with insignificant ($p=0.199$, Pearson Chi-Square test) differences compared to biochemical tests.

3.5. Linearity of the MPN Method

Higher R-squared values were found in linear regression lines built based on the determined MPNs of *E. coli* against respective inoculum dose. The R-squared values in regression lines of beef, chicken, and milk were 0.9936, 0.9992, and 0.9978 respectively (Figure 3).

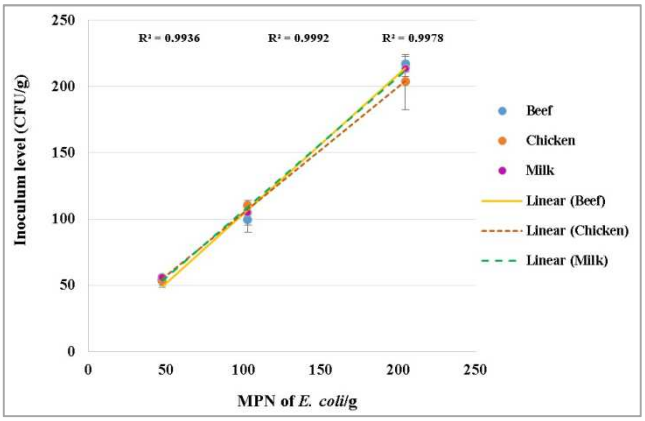


Figure 3. *Regression lines of the MPN method of enumeration of E. coli in beef, chicken, and milk. High R-squared values (≥0.99) are indicating strong linearity of the method.*

3.6. Total Assay Time

The total assay times required to obtain the confirmed MPNs of *E. coli* following biochemical test, *real-Time* PCR, and MALDI TOF MS methods for confirmation of bacteria were recorded and delineated in the Table 5. Using the biochemical procedures for the confirmatory identification of *E. coli*, the MPN assays could be completed in 121.3±6.3 h, whereas in 77.4±6.3 h and 74.2±6.1 h using *real-time* PCR and MALDI TOF MS respectively (Figure 4). *Real-time* PCR and MALDI TOF MS significantly ($p=0.000$) reduced the assay time compared to that by biochemical tests. Significant ($p=0.003$) difference was also found between MPN assay times required by *real-time* PCR and MALDI TOF MS.

Table 5. *Time required to complete the MPN assay for the enumeration of E. coli.*

Matrix	Contamination level	Test replicate	Time to complete the assay (h)		
			Biochemical tests	*Real-Time* PCR	MALDI TOF MS
Beef	Low	1	120	76	73
		2	119	75	71
		3	143	98	95
	Intermediate	1	118	74	71
		2	120	77	73
		3	122	78	74
	High	1	119	75	71
		2	119	74	71
		3	118	74	71
Chicken	Low	1	122	78	74
		2	120	77	73
		3	120	77	73
	Intermediate	1	118	75	72
		2	122	79	76
		3	122	78	74
	High	1	118	75	72
		2	118	74	72
		3	118	74	71
Milk	Low	1	121	77	74
		2	119	76	74
		3	142	99	95
	Intermediate	1	120	75	73
		2	122	77	73
		3	119	74	72
	High	1	118	76	73
		2	118	74	71
		3	119	75	71

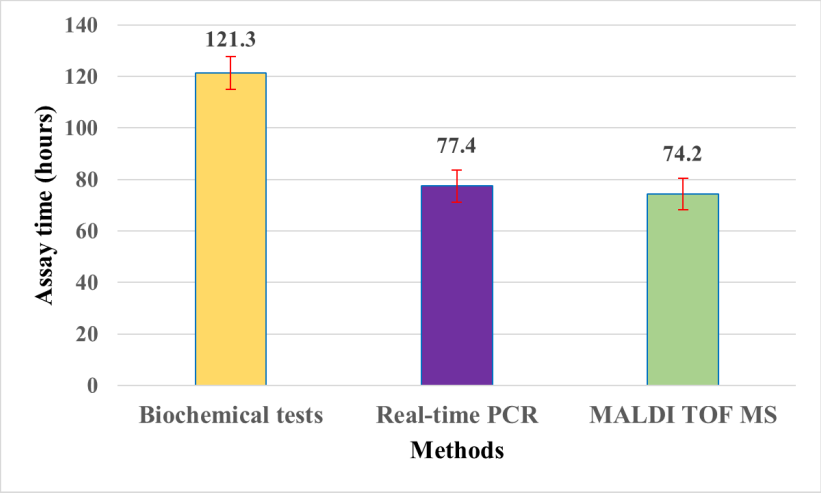


Figure 4. *Mean assay times required to complete the MPN method for enumeration of E. coli.*

4. Discussion

Raw or undercooked meat products, raw milk and cheeses can get *E. coli* from contamination with animal or human fecal materials [5]. Detection and enumeration of the bacteria in such food matrices are crucial regarding food safety issues. In our laboratory, raw meat, milk, and various types of meat/milk based products are received from stakeholders requested for enumeration of *E. coli*. Samples are tested by reference MPN method in which biochemical tests are used for definitive identification of the bacteria [6]. But the extended time required to complete the assay frequently becomes a cause of dissatisfaction among the stakeholders. Thus, the current research was envisaged to reduce the MPN assay time for *E. coli* by replacing biochemical tests with a credible rapid bacterial identification tool.

In the current research, reference method was followed up to the isolation of *E. coli*. For confirmation of *E. coli*, along with biochemical tests, two rapid methods *real-time* PCR and MALDI TOF MS were used to obtain comparable results. Among the biochemical tests, Indole test was employed in the current research following ISO 7251: 2005 [13]. In Indole tests, development of characteristic red layer over the broth in positive cases was found, whereas not in negative and blank cases [Figure 2 (A)]. The isolates recovered from the spiked sample matrices could be accurately identified as *E. coli* based on the findings in Indole tests [23]. In *real-time* PCR assays low Ct values and unique Melt temperature indicated high specificity and absence of non-specific amplification by the *E. coli* species specific *yaiO-F* and *yaiO-R* primers [24]. Successful completion of MS calibration prior to each run indicated high accuracy of the MALDI Biotyper machine and *E. coli* identification with log scores >2.0 in most of cases indicated the high efficiency of the tool [16]. Based on the number of isolates identified as *E. coli* by all three methods, insignificant ($p=0.199$) differences were found in efficiencies of biochemical tests, real-time PCR, and MALDI TOF MS. Employment of the *real-time* PCR and MALDI TOF MS did not change the MPN of *E. coli* found by biochemical tests in sample matrices. Therefore, *real-time* PCR and MALDI TOF MS did not differ significantly ($p=0.199$) with biochemical tests in resulting MPN of *E. coli*.

High R-squared values (≥0.99) found in the regression lines of the MPN method of enumeration of *E. coli* in beef, chicken, and milk indicated strong linearity of the method within the range of 50 to 200 CFU/g or ml of sample matrices. Higher R-squared values represent smaller differences between the observed data and the fitted values [22]. Therefore, the method can be used confidently employing either biochemical tests, or *real-time* PCR, or MALDI TOF MS for the enumeration of *E. coli* within the range. The findings were similar with those found by Prats *et al*., 2007 [25].

The main objective of the current research was to reduce the MPN assay time for the enumeration of *E. coli* in different food samples by the integration of a credible and rapid microbial identification tool to replace time consuming biochemical tests. Noticeably, both *real-time* PCR and MALDI TOF MS significantly ($p=0.000$) reduced the assay time compared to that by biochemical tests (Figure 4). Interestingly, significant ($p=0.003$) difference was also found between MPN assay times by *real-time* PCR and MALDI TOF MS. Moreover, some extra steps in *real-time* PCR like DNA extraction, DNA quantification, and master mix preparation require more expertise and make the procedure cumbersome. Whereas in MALDI TOF MS, sample preparation step is very simple and requires minimal expertise and time [17]. Considering the research findings and test associated matters both in *real-time* PCR and MALDI TOF MS, the later one is recommended for integration in MPN assay for *E. coli*.

5. Conclusion

The current research was envisaged to suggest an appropriate tool alternative to biochemical tests for rapid

enumeration of *E. coli* by MPN assay. Research findings suggest that MALDI TOF MS is better alternative than *real-time* PCR for the purpose. Thus, the integration of MALDI TOF MS will reduce the MPN assay time for *E. coli* enumeration and will enable the laboratory to faster delivery of the test report to the stakeholders. However, the current research is limited by inclusion of small number of sample matrices and small enumeration range due to resource constraints. Therefore, an elaborate research with inclusion of more sample matrices and broad enumeration range is suggested.

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