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Concurrent Detection of Foodborne Pathogens: Past Efforts and Recent Trends

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Abstract

Rapid and specific identification of foodborne pathogens is important for not only for food producers but also consumers as well as food safety authorities. Foods can harbor pathogen bacteria of different species, thus it is also important to be able to detect different pathogens simultaneously from the food samples even though they are in very low number. Detection of bacteria depends on different factors such as the initial number, food matrix, sensitivity of the method, competitor microflora and physical state of the bacteria whether they are injured or not. Simultaneous detection of different bacteria yet adds another hurdle for the level of detection, and thus, different approaches have developed and tested so far to optimize the maximum recovery.

Keywords: Concurrent detection; Foodborne pathogens; IMS; ELISA; PCR; Biosensors; MS

Introduction

Foodborne pathogens causing infections and intoxications are the big concern for food industry due to the food safety issues. Although some pathogens such as *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* spp have zero tolerance; the others such as *Staphylococcus aureus*, *Camplyobacter jejuni* and *Bacillus cereus* are allowed to be present in low number in food products. Detection of number of foodborne pathogens with higher sensitivity with the exact number is a food safety issue that still is under the development. Precise determination of the number of the cells is somewhat difficult is because it is possible that the number of cells present in food could be too low that cannot be detected by the method used. Another issue raised up with injured cells in that some cells present especially in processed foods might be injured rather that inactivated and these cell cannot be detected in regular detection methods. These cells, unfortunately, can repair their cell structure and may cause problems for food safety in consecutive stages of distribution and storage.

Monitoring the food safety is a vital aspect for the food industry. Both the security and safety of food products rely on the ability to detect identify and trace foodborne pathogens [1,2]. Foodborne pathogens detection methods have a great significance due to their biological threats to public health and economy. Current detection methods despite greater biological understanding and technological advancements have significant drawbacks. Though accurate and affordable, traditional plate counting methods are time consuming and require sample pre-enrichment. Methods such as immunomagnetic separation (IMS), enzyme linked immunosorbent assay (ELISA), DNA amplification, non-biofouling polyethylene glycol (PEG) based microfluidic chip integrated with functionalized nanoporous alumina membrane, and polymerase chain reaction (PCR) offer faster detection with good sensitivity, but some are laborious and expensive; and magnetic-based approaches are applicable to complex food samples, but require lengthy sample preparation, costly reagents, and limited sensitivity [3]. These methods are frequently tested for the detection of one pathogen at a time and there is a need to test them for simultaneous or concurrent detection of multiple pathogens (Table 1).

Techniques	Detected pathogens	Disease caused	Limit of detection	Assay time	Reference
Quantitative PCR	*L. monocytogenes*	Listeriosis	detect as few as 100 cfu/g and quantify as few as 1000 cfu/g	3 h	[34]
	Salmonella	Gastrointestinal infection	10^3 to 10^4 cfu/ml of inoculums in broth withour enrichment, <10 cfu/mL of inoculum in broth after enrichment	3 h	[35]
	Shigella	Shigellosis	0.12 to 0.74 cfu/reaction	24 h	[36]
	L. monocytogenes	Listeriosis	7 cfu/g in coleslaw	24 h	[37]
	S. aureus	Intoxication	2 cfu/g in raw minced meat	24 h	[38]

	E. coli O157:H7	Gastrointestinal infection, HC, HUS, TTP****	8 × 10^{-1} cfu/g (or cfu/ml) in apple cider, cantaloupe, lettuce, tomato and watermelon; 8 × 10^{1} cfu/ml in alfalfa sprouts	30 h*	[36]
	Salmonella	Gastrointestinal infection	8 × 10^{-1} cfu/g (or cfu/ml) in apple cider, cantaloupe, lettuce, tomato and watermelon; 8 × 10^{1} cfu/ml in alfalfa sprouts	30 h	[36]
	Shigella	Shigellosis	8 × 10^{-1} cfu/g (or cfu/ml) in apple cider, cantaloupe, lettuce, tomato and watermelon; 8 × 10^{1} cfu/ml in alfalfa sprouts	30 h	[36]
	Salmonella spp.	Gastrointestinal infection	10^{3} cfu/ml by pure culture, 1 cell per 25 f of inoculated pork sample	30 h	[39]
	L. monocytogenes	Listeriosis	10^{3} cfu/ml by pure culture, 1 cell per 25 f of inoculated pork sample	30 h	[39]
	E. coli O157:H7	Gastrointestinal infection, HC, HUS, TTP	10^{3} cfu/ml by pure culture, 1 cell per 25 f of inoculated pork sample	30 h	[39]
	E. coli O157:H7	Gastrointestinal infection, HC, HUS, TTP	10^{5} cfu/g	3 h	[40]
	Salmonella	Gastrointestinal infection	10^{3} cfu/g	3 h	[40]
	Shigella	Shigellosis	10^{4} cfu/g	3 h	[40]
	Salmonella spp.	Gastrointestinal infection	5 cfu/25 of inoculated sample after 20 h of enrichment	3 h	[41]
	L. monocytogenes	Listeriosis	5 cfu/25 of inoculated sample after 20 h of enrichment	3 h	[41]
	E. coli O157:H7	Gastrointestinal infection, HC, HUS, TTP****	5 cfu/25 of inoculated sample after 20 h of enrichment	3 h	[41]
Multiplex PCR	*E. coli* O157:H7, O26, O103, O111, O145 sorbitol fermenting O157 and non-sorbitol fermenting O157	Gastrointestinal infection, HC, HUS, TTP****	Minced beef and sprout seeds enrichment broths were inoculated with 5 × 10^{4} cfu/ml STEC O157 and raw milk cheese enrichment broths with 5 × 10^{3} cfu/ml STEC O157	24 h	[42]
	Shigella	*Shigellosis*	8 cfu per reaction	2 h	[42]
	Enteroinvasive E. coli	Gastrointestinal infection	8 cfu per reaction	2 h	[43]
	Streptococcus pneumonia	Pneumonia	10 or more copies of purified S. pneumoniae DNA	1 h	[44]
	Salmonellae	Gastrointestinal infection	3.4 to 34 viable Salmonella cells in pure culture and 6.1 × 10^{3} to 6.1 × 10^{4} cfu/g in spiked produce samples	3 h	[45]
LAMP**	*Vibrio paramaemolyticus*	Gastroenteritis	5.3 × 10^{2} cfu/ml	1 h	[46]
	Chlamydia pneumonia	Pneumonia	10 molecules of *in vitro* wild type C. pneumoniae RNA	1 h	[47]
	Chlamydophila pneumonia	Pneumonia	0.1 indusion-forming unit (IFU) of C. pneumoniae	1 h	[47]
NASBA***	*Mycobacterium tuberculosis*	Tuberculosis	1 × 10^{2} cfu/ml	<5h	[48]

*enrichment; **LAMP: Loop-mediated isothermal amplification; ***NASBA: Nucleic acid sequence-based amplification; ****HC: hemorrhagic colitis; HUS: hemolytic uremic syndrome; TTP: thrombotic thrombocytopenic purpura

Table 1: Nucleic acid based techniques for foodborne pathogens detection.

Enrichment procedures

The main obstacle of the detection of pathogens of being either in very low number or injured can be solved by enrichment protocols. Thus, an enrichment procedure need to be applied to increase the initial number to the detectable level and extract DNA, RNA or antigens present. Nonselective and selective enrichment are the common practices to increase the low number of bacteria in a short period of time to the desired level for detection. Depending on the

bacteria, the enrichment procedure is changed. For example, TSB, BHI and no. 17 broth with 0.5 g/L dextrose or modified in the dextrose concentration (0 or 2.5 g/L) was used as an enrichment medium for the simultaneous growth of *Salmonella*, *L. monocytogenes* and *E. coli* O157. Total of 125 mL milk samples were simultaneously inoculated with various contamination levels (10^2-1 cfu of each species of target pathogens), divided into 25 mL aliquots and diluted 1:10 in 225 mL of enrichment media for sample enrichment. In order to increase the number of each culture to 10^8 cfu/mL inoculated cultures were separately grown in TSB, and then they were inoculated into milk samples. After incubation at 35°C for 18 ± 2 h and 48 h, the bacterial growth was determined by plating on selective media: Listeria selective agar base (LSAB) with Listeria selective supplement (LSS), Eosin methylene blue agar (EMB) or Xylose Lysine Dextrose agar (XLD) before real time PCR test [4].

In order to simultaneously detect *S. typhimurium*, *E. coli* O157:H7 and *L. monocytogenes* in mixed vegetable salad, bacteria were contaminated with 500 mL water having 1 cells/mL to 1000 cells/mL. Inoculated samples were incubated at 37°C for 16 h for pre-enrichment. After 16 h of pre-enrichment the samples were stomached and one mL of the suspension was transferred to 9 mL of prewarmed (37°C) UPB. Samples were further incubated for 4 h at 37°C, and aliquots were withdrawn for determining viable cell count, specific viable count of individual pathogens, and DNA isolation [5].

After the viability and enumeration of the microbiological ISO recommended methods for *S. enterica*, *L. monocytogenes* and *E. coli* O157:H7, the plates were incubated at 37°C for 24 to 48 h in order to allow for the microbial growth. Before DNA extraction, each bacterial strain was grown in TSB at 37°C. Serial dilutions were prepared from this TSB bacterial cultures after bacterial growth was assessed by the increase in the turbidity of the solution observed for all the target microorganisms and confirmed by microbial count (>10^9 cfu/mL for all the pathogens).

The overnight (18 h) grown cultures of *E. coli*, *L. monocytogenes*, and *Salmonella spp.* were used to contaminate the eggs, raw milk, Raw kobide, salad, chicken and cheese samples to a final concentration of 100 cfu of each pathogen/mL or g. These contaminated samples were taken as the positive controls to eliminate the effect of PCR inhibitors existing in food matrixes to detect three pathogens simultaneously by traditional microbiological methods (such as culture and serology) and multiplex PCR method [6].

Although pre-enrichment and selective enrichment is an initial step for some studies, it could be part of the whole procedure for simultaneous detection of pathogens. One of the earliest attempts for the concurrent detection of *Salmonella spp.* and *E. coli* O157:H7 was made to develop a single assay protocol from a single sample grown in a single enrichment in 24 h by using the combination of IMS and ELISA. Twenty-five and 375 g of ground beef, nonfat dry milk, and dry pet food samples inoculated with low (10 cfu/sample) and high (100 cfu/sample) levels of *E. coli* O157:H7 and *Salmonella* cultures incubated at 35 and 41°C for 18 h for nonselective pre-enrichment. After the incubation period, the samples were analyzed by IMS following a 6-h incubation for selective enrichment at 37°C using M-broth and ELISA. Depending on the food samples and the inoculation level, the minimum concurrent detection level of *E. coli* O157:H7 and *Salmonella* was <1 cfu/g in the samples at the competitor flora level of 105 cfu/g or less in ground beef samples. On the other hand, when higher competitor loads and low target inoculations were the testing conditions, *E. coli* O157:H7 could not be detected in the presence of the *Salmonella* [7].

Development of a protocol by using the different culture methods with several enrichment conditions was also tested for the concurrent recovery of *E. coli* O157:H7 and *Salmonella* from bovine carcass, hide and fecal samples. Incubation in tryptic soy broth for 2 h at 25°C and then for 6 h at 42°C was the protocol selected for use. Selective agars for plating after IMS were chosen on the basis of ease of target colony identification. While Sorbitol MacConkey agar supplemented with cefixime and potassium tellurite and Rainbow agar supplemented with novobiocin and potassium tellurite were chosen for the recovery of *E. coli* O157:H7, brilliant green agar with sulfadiazine and Hektoen enteric agar supplemented with novobiocin were selected for the recovery of *Salmonella*. The meats research unit (MRU) methods performed, except that a secondary enrichment in tetrathionate (TT) broth prior to IMS was required for the optimal recovery of *Salmonella* from feces. The MRU and MRU-TT methods are found to be effective in the recovery of both *E. coli* O157:H7 and *Salmonella* from a single bovine carcass, hide, or fecal sample [8].

DNA extraction methods

Methodologies targeting the DNA require extraction of genomic DNA of bacteria from food matrix. A study focused on simultaneous detection of *E. coli*, *L. monocytogenes* and *Salmonella spp* from different food matrices includes a phenol-chloroform genomic DNA extraction and boiling methods followed by the extraction and purification which are analyzed by 1% agarose gel and by means of spectrophotometric analysis as UV absorption at 260 nm (A260) and A260/A280 ratio [6].

Isolation of DNA from *S. typhimurium*, *E. coli* O157:H7 and *L. monocytogenes* started with the mixing of 400 Al of UPB with 1.3 mL of saline and centrifuged at 14000 g for 10 min to pellet bacteria. The pellet was suspended in 400 Al of BAX DNA lysis reagents and DNA was isolated. The cells were lysed first at 55°C for 60 min, followed by 95°C for 10 min. Obtained lysate was chilled on ice for 10 min and clarified by centrifugation at 12000 g for 10 min. For each pathogen 49 µL of DNA preparation were transferred to BAX PCR tubes specific for the individual pathogen. The BAX tubes contained lyophilized pellet of all PCR reagents except target DNA. After the addition 1 µL of SYBR Green I dye, PCR was initiated. The thermocycler programme started at 93°C for 2 min (94°C for 35 s, 69°C for 3 min) x37, and 71°C for 5 min. Data were collected after each annealing step, i.e. after 69°C for 3 min using emission and excitation wavelengths (530 nm and 490 nm, respectively). All the samples were automatically processed for melt-curve analysis of amplified DNA. PCR was also carried out on "lysates" obtained by combining the appropriate volume of UPB and produce with the lysis buffer. These samples served as controls to determine if the UPB or the produce were contaminated with amplifiable target DNA [5].

DNA isolation and multiplex Real-Time PCR, enabling the detection of as few as 1 cfu of each pathogen in 125 mL milk in order to detect *Salmonella* spp., *L. monocytogenes* and *E. coli* O157 concurrently was applied. Primer and probe specific sequences for each target were designed and three LNA (Locked Nucleic Acids) modified bases were incorporated in different positions into *Salmonella* 756R and 956R primers (L756R, L956Ra and L956Rb). Two multiplex real-time PCR assays, with the same primer sequences but different detection chemistries, were carried out with one with dual-labelled probes (mRT-PCR) and another, that included a final step of

high resolution melting analysis, with an intercalating dye (mHRM). Both reactions were optimized in a RotorGene 6000 using the Hot-Rescue Real-Time PCR kit-FLUO PROBE with 0.8 units of DNA PCR per reaction, and additional 1XEva- Green in the mHRM assay. The final optimized primer and probe concentrations were: 600 nM for 664F-L756R and 75 nM for 712T probe (*Salmonella* spp.); 1200 nM for 903F-1004R and 150 nM for 927T probe (*E. coli* O157); 900 nM for 634F-770R and 200 nM for 713T probe (*L. monocytogenes*). In the mHRM assay, primer concentrations were 1000 nM for 664F-L756R and 634F-770R; 800 nM for 903F-1004R. The thermal protocol of mRT-PCR was: 10 min at 95°C, 40 cycles of 20 s at 95°C and 1 min at 63°C. In the mHRM assay it was: 10 min at 95°C, 40 cycles of 20 s at 95°C, 20 s at 64°C, 30 s at 72°C, with a final melting analysis rising from 79°C to 90°C by 0.1°C/5 s. For optimal acquisition of fluorescence data, the gain was set to 20% of saturation in the highest fluorescent signal. For amplification inhibition, pDEF recombinant plasmid (104 copies) with its primers (600 nM in mRT-PCR and 350 nM in mHRM) and probe (50 nM) set was included in each PCR reaction [4].

In order to detect *S. enterica*, *L. monocytogenes* and *E. coli* O157:H7 by four different DNA extraction methods, the liquid cultures were removed with centrifugation at 8700 g for 15 min and resuspended in 1 mL of sterile water. The second centrifugation was performed at 12500 g for 15 min, and the pelleted were then used for DNA extraction. The cells were resuspended in 200 µL of Tris-EDTA (TE) buffer and then kept in a boiling water bath for 10 min. After cooling on ice for 5 min, the samples were centrifuged at 12500 g for 5 min, and 2 µL of the supernatant was used directly for the PCR for the boiling method. For the alkali lysis, the cells were resuspended in 200 µL of 3% KOH solution by vigorous mixing and the supernatant was used directly for the PCR after centrifugation at 12500 g for 5 min. The cells were resuspended in 500 µL of 4 M guanidine isothiocyanate solution containing 2% (w/v) of Tween 20 for guanidine isothiocyanate method. Solubilized cell components were then treated with isopropanol solutions in order to precipitate DNA and wash DNA pellet. Two µL of the final DNA solution were used directly for the PCR. For the 10% Chelex100 resin method, a 245 µl of 0.1 M TE (pH 8) were added to the cells, and the solution was centrifuged at 4280 g for 5 min, and this procedure was repeated once for the broth cultures and twice for food samples. Cell pellets were then mixed with 245 µL TE and 5 µL lysozyme 50 mg/mL for cell cultures or 500 µL TE and 10 µL lysozyme 50 mg/mL for spiked food. Samples were then incubated at 56°C for 45 min. After that, 250 µL of extraction solution 20 µL of 0.25 M EDTA pH 8, 25 µL of 10% (w/v) sodium dodecyl sulfate and 3.8 µL of proteinase K (20 mg/L) were added and the samples left at 37°C for 1 h. After the incubation, 500 µL of thoroughly mixed 10% Chelex100 was then added, and the samples were incubated at 56°C for 15 to 30 min. After vortexing of the samples for 10 s and following incubation at 100°C for 8 min, the samples were then centrifuged at 8700 g for 2 min. Pellet was removed, and 2 µL of this solution were used directly in PCR [9].

Boiling method was also used for the extraction of DNA from the overnight cultures of *B. cereus* and *S. aureus* grown in tryptic soy yeast extract (TSBYE) broth. Multiplex PCR was carried out in 20 µL reaction containing 400 nM of nheAF and nheAR, 200 nM of forward and reverse primers of cyt K and hly, 300 nM of hbl A and ent B primers, 160 nM of iap and nuc primers, 200 µM concentrations of dNTP mix, 105 copies of IAC DNA, 1.2 unit of Taq polymerase, 2.0 mM MgCl2 in 1 X PCR with 1.5 µL of template DNA. Amplification consisted of initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 57°C for 1 min and extension at 72°C for 1.30 min followed by final 6 min extension at 72°C. The PCR products were analyzed on 2% (wt/vol) agarose gel [10].

For the detection of *Cronobacter sakazakii*, *S. aureus* and *B. cereus*, propidium monoazide (PMA) was dissolved in 20% dimethyl sulfoxide (DMSO) to obtain a stock solution at 1 mg/mL and stored at 20°C in the dark, and 10 mL stock solution of PMA was added to 1 mL of sample solution in a light transparent 1.5 mL microcentrifuge tube to create a final concentrations of 10 µg/mL. Following an incubation period of 5 min in the dark, the samples were placed on ice and were light exposed for 5 min using a 500-Whalogen light source. After light-exposed PMA cross-linking, the samples were centrifuged at 12000 g for 5 min and washed three times with equal volumes of PBS to get rid of the free PMA. Genomic DNA extraction of cells was prepared as follows: the bacterial pellets were resuspended in 180 mL of enzymatic lysis buffer containing Tris (20 mM), Na_2 -EDTA (2 mM), TritonX-100 (1.2%), and lysozyme (20 mg/mL) and incubated at 37°C for 30 min. Subsequently, bacterial DNA was extracted using DNeasy blood and tissue kit and the extracted genomic DNA was used in mPCR assay immediately [11].

Concurrent detection of foodborne pathogens

Detection of bacteria is performed either by single or multiple gene identification. Single gene PCRs have been developed to detect pathogens bacteria individually by targeting toxin-specific or genus specific genes. However, if other organisms are present, detection of only one organism by single PCR often misinterprets presence of another organism; thereby delaying treatment procedures, misleading food safety precautions need to be taken. Detection of pathogens simultaneously by a single multiplex PCR (mPCR) would have advantages in terms of rapidity, convenience and cost saving [10]. Studies involved in multiplex PCR mostly focused on concurrent detection of different pathogens with different enrichment protocols or different primers. Simultaneous detection of major food-borne pathogens (*L. monocytogenes*, *Salmonella* spp. and *E. coli* O157:H7) in Iranian food materials including eggs, raw milk, Raw kobide, salad, chicken, and cheese was studied for the comparison of traditional microbiological methods (such as culture and serology) and multiplex PCR method. Two primer pairs were selected according to the former works for *E. coli* O157:H7 and *L. monocytogenes* and other pairs of primers were used for *Salmonella* [4,10,12,13]. Due to the requirements to set each uniplex in optimal conditions, the same T_a temperature for all these primers on 58°C was used for multiplex PCR setting up. Structural interferences of primers were also tested including secondary structure like dimmer and hairpins. Because of all different sizes of the amplified fragments for each uniplex reaction, concentration of the each primer was optimized to achieve a clearly visible band pattern of Agarose gel (210 bp for *Listeria*, 556 bp for *E. coli* and 942 bp for *Salmonella*). Data obtained for simultaneous detection of these three pathogens were also compared with other methods including differential culturing and biochemical analysis, but this method was found to be faster and more specific [14,15]. Besides rapid and specific detection of pathogens via multiplex PCR (mPCR) method, stepwise protocols need to be considered to achieve a reliable data including optimized multiple primer concentrations, DNA extraction and PCR reaction conditions [6].

Detection of *B. cereus*, *L. monocytogenes* and *S. aureus* simultaneously was conducted in food samples by a single mPCR

standardized by targeting three major diarrheal enterotoxin genes of *hbl* A, *cyt* K and *nhe* A for *B. cereus*, virulence associated *nuc* and *Ent* B genes for *S. aureus* and virulence associated *hly* and *iap* genes for *L. monocytogenes* along with internal amplification control (IAC). The results showed that mPCR accurately identified all the three organisms individually or in combination without non-specificity. As low as 10 to 100 organisms per mL of growth following overnight enrichment of spiked food samples (vegetable biriyani and milk), and their presence in naturally contaminated samples was detected by mPCR [10].

Evaluation of four different DNA extraction procedures for the simultaneous detection of *S. enterica serovar Typhimurium*, *L. monocytogenes* and *E. coli* O157:H7 in a liquid whole egg revealed that chelex resin combined with a DNA purification step has better performance on the food system considered. Primer sets were developed based on the evaluation and combination of published primer sets. Developed protocol involved an overnight enrichment step followed by DNA isolation and mPCR, and detection limit was as low as 10 cells/25 g in liquid whole egg samples [9].

A protocol for the simultaneous detection of *E. coli* O157:H7, *L. monocytogenes* and *S. typhimurium* using artificially contaminated fresh vegetables of green cabbage, broccoli, cauliflower, and cilantro tested by association of official analytical chemists (AOAC) approved PCR method including a melting-curve analysis of PCR products. Minimum detected number of cells was 1 to 10 cells/mL for both *E. coli* O157:H7 and *S. enterica* and 1000 cells/mL for *L. monocytogenes* [15].

Recently, nanoporous membrane based electrochemical sensor has been used in biosensing areas due to its enhanced sensitivity and easy fabrication process [16-18]. Many nanoporous membrane based electrochemical sensors have been used in various applications including ion channel detection [19], DNA hybridization sensing [20,21], virus detection [22], cell based biosensing [23] and bacteria detection [24,25]. However, the current nanoporous membrane based methods were only used for single type of bacteria detection and could not realize the simultaneous detection of multiple types of bacteria from the mixed bacteria samples. The integration of suitable microfluidic chip with functionalized nanoporous alumina membranes is necessary to provide a multi-functional platform for multiple types of bacteria detection at the same time [26]. Surface biofouling, on the other hand, is always a challenge for traditional PDMS microfluidic devices. Recent efforts have been made to use photocurable polyethylene glycol (PEG) polymer with low molecular weight to fabricate microfluidic devices because of its high resistance to swelling in aqueous environment [27-29]. PEG based microfluidic chip can significantly reduce sample loss especially compared with PDMS based microfluidic device [22] for the detection at low concentration [30,31].

A non-biofouling polyethylene glycol (PEG) based microfluidic chip integrated with functionalized nanoporous alumina membrane from the mixed samples demonstrated the specificity for target bacteria detection and the low cross-binding of non-target bacteria with the simultaneous detection of mixed bacteria sample of *E. coli* O157:H7 and *S. aureus*. Developed sensor had a linear detection range from10^2 cfu/mL to 10^5 cfu/mL with the limit of detection (LOD) around 10^2 cfu/mL [26].

Final remarks

Even though molecular detection methods are in high demand for the detection of foodborne pathogens, the traditional methods for the simultaneous detection of foodborne pathogens which include culture-based techniques and phenotypic characterization based on morphological and metabolic characteristics are still valid. Due to their disadvantages of being time-consuming and laborious as well as the lack of effective selective media for every bacterium including *Aeromonas salmonicida* they are not preferred by some researchers.

Efforts for concurrent detection of foodborne pathogens mostly involve PCR based methods with different primers or genes, different enrichment protocols and amplification conditions. mPCR and PMA-PCR assays combined with IAC was found specific, sensitive, and accurate for the simultaneous detection of different foodborne pathogens for pure cultures or inoculated bacteria into food samples. Generally, both false negative and false positive results were eliminated with PMA treatment before DNA extraction. In addition, the application of an efficient DNA extraction method for the mPCR assay effectively eliminated PCR inhibitors.

Even though these studies provide a possible concurrent detection mechanism of different foodborne pathogens; they also cause a huge vague in terms of what protocol need to be followed since there is a lack of standardization. Because most of the studies has the detection limit of ca. 10^2 cfu/mL, level of detection needs to be diminished for <1 cfu/mL for zero tolerance foodborne pathogens as they are a great concern in food industry. On the other hand, these methods require culture-independent molecular methods. Many DNA probes and PCR primers have been designed for rapid and specific detection of pure culture. Moreover, most of these methods are based on the use of 16S rDNA and the vapA gene, a gene encoding a subunit protein of the A-layer, as target genes but these molecular methods suffer from the disadvantage that they do not distinguish living and hence pathogenic cells from dead cells [32]. Therefore, a molecular method with discriminative power as between living and dead cells remains to be described. In addition, bacterial mRNA has a very short half-life, usually measured in minutes, and detection of it decreases comparatively quickly with loss of bacterial cell viability. Therefore, mRNA based detection methods should provide a sensitive indicator of cell viability as compared with methods that rely on the amplification of DNA and rRNA, which is extremely stable [33].

Thus, the potential of different approaches such as mass spectrometry since it has the high sensitivity and high resolution to differentiate microbial species based on subcellular variations, the application of either matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) mass spectrometry (MS) need to investigated for the concurrent detection and identification of foodborne pathogens.

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