Journal of Animal and Veterinary Advances 8 (9): 1723-1728, 2009
ISSN: 1680-5593
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Modern Trends to Investigate Salmonella in Foods

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Abstract: In recent years, modern diagnostic laboratories have been concerned with reducing the time required for diagnosis of Salmonellae infections. The current standard laboratory procedures to culture and identify Salmonella serovars requires 3-4 days to obtain a negative result and upto 4-7 days to get a confirmed positive result. Conventional isolation protocols tend to prolong the time needed for the detection of Salmonellae making it inconvenient and expensive as a routine method for screening out negative samples. There is an urgent need for molecular methods which would allow food industry to respond quickly to raw material and product contamination and which would also help with the early release of tested stock of raw materials and finished products.

Key words: Salmonella, modern, food, diagnosis, investigation, India

INTRODUCTION

The genus of Salmonella is a gram negative rod shaped bacteria in the family of Entrobacteriacea. Meat, chicken and poultry products have been implicated as a major source of Salmonella infections in human. Salmonella infections still occur at high frequencies in industrialized nations and developing countries as well. Infections due to Salmonella serotypes continue to be a major public health concerned (Ferreti *et al*., 2001; Malorny *et al*., 2003).

Salmonella infection is important for food processing industries. Established conventional methods to detect and identify Salmonella are time consuming and include selective enrichment and plating followed by negative controls and bio-chemical tests. The availability of rapid, reliable methods to detect salmonella in food is becoming increasingly important for food producers, for legislators and for international trade to ensure consumer microbiological safety.

Salmonellosis in humans: Non-typhoidal Salmonellosis is usually manifested in humans in the form of acute, enteric infection with diarrhea, abdominal pain and nausea (Humphrey, 2000; Threlfall *et al*., 1992) have reported incidences of extra-intestinal infections associated with *S. dublin*, *S. choleresuis* and some strains of *S. virchow*.

Salmonellosis associated with food commodities: Salmonellosis has most often been associated consumption of contaminated foods of animal origin such as poultry, eggs and milk, meat and dairy products. Several epidemiological studies conducted globally indicate the association of wide variety of foods related to outbreaks of Salmonellosis (Hedberg *et al*., 1993) demonstrated that larger outbreaks can result even if a small percentage of the implicated food products are contaminated and extremely low levels of Salmonella are present in the food.

In 1888, the 1st report of food poisoning outbreak linked to Salmonella was reported from Germany in which 50 persons who consumed raw ground beef became ill and one person died (Krysinski and Heimsch, 1977).

Conventional isolation method for Salmonella: It has been applied for the rapid and efficient detection of Salmonella from different type of food commodities.

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These involve cultural, biochemical and immunological assays that rely on phenotypic characterization. It is not only labour intensive but also time consuming when handling many samples in legislative and food production control and also Salmonellae may be injured by the different food processing methods (Prusak-Sochaczewski and Luong, 1989).

In recent years, diagnostic laboratories have been concerned with reducing the time required for diagnosis of Salmonellae infections. The current standard laboratory procedures to culture and identify Salmonella serovars requires 3-4 days to obtain a negative result and upto 4-7 days to get a confirmed positive result (Carter and Chengappa, 1991; Stone *et al*., 1994).

In addition, Salmonellae are not detectable in certain clinical samples that contain small numbers of organisms.

Conventional isolation protocols tend to prolong the time needed for the detection of Salmonellae, making it inconvenient and expensive as a routine method for screening out negative samples (Cudoje *et al*., 1994). Therefore, there is an urgent need for molecular methods which would allow food industry to respond quickly to raw material and product contamination and which would also help with the early release of tested stock of raw materials and finished products (Lee *et al*., 1990).

A four steps cultural method has been developed to determine the presence of Salmonellae in foods (Varnam and Evans, 1991). These steps include pre-enrichment (Buffered peptone water for 16-20 h at 37°C) to allow the resuscitation and multiplication of sub-lethally damaged Salmonella cells.

Secondly, selective enrichment (Rapport-vassiliadis broth or Tetrathionate-bile-brilliant green broth or Selenite cystine broth at 42°C for 18-48 h) to allow the survival or growth of Salmonella at the same time as reducing the number of non-Salmonellae in the broth. Followed by plating on selective (Brilliant green agar, Bismuth sulphite agar and Hektoen enteric agar) or Differential agar (MacConkey agar) for 24-48 h to produce presumptive isolates and to enable the recognition of Salmonella colonies while suppressing the background microflora.

Lastly, conformation is achieved by subjecting the isolates to various biochemical and serological tests to confirm and determine it serotype.

Theoretically, this method provides a level of sensitivity of detecting one Salmonella cells per 25 g of food sample examined. However, this level of detection can be hampered by the presence of other micro-organisms that compete with Salmonella during cultural enrichment and also due to the presence of various inhibitory substances present in the food commodity.

Immunomagnetic separation (Ims): These methods have been used alone or with combination of other methods such as plating agars, ELISA and PCR. Cudjoe *et al.* (1995) reported the use of immunomagnetic separation techniques for the detection of pathogenic bacteria in foods (Mansfield and Forsythe, 2000) recommended immunomagnetic separation as an alternative to enrichment broths for detection of food borne Salmonella.

Electrical conductance/impedance technology: Impedance technology is a rapid, automated and qualitative technique which measures the conductance change in the medium induced by bacterial metabolism. Several medium have been proposed for the detection of Salmonella using impedence technology but they continue to give false positive results due to presence of other bacteria(s) such as Citrobacter freundii (Easter and Gibson, 1985), *Escherichia coli* (Ogden and Cann, 1987), *Enterobacter cloacae* and *Klebsiella oxytoca* and *Pseudomonas* sp.

Serological tests: A number of serological tests have been devised so far for the detection of invasive serotypes of salmonellae, the most successful being slide agglutination test which uses whole blood or serum for detection of poultry flocks infected with *S. gallinarum* and *S. pullorum* (Barrow *et al*., 1994). Although, this test is relatively crude and the frequency of false positive results are more.

Serological assays, mainly ELISA are now routinely used in a number of countries. Recently ELISA has been used in combination with immunomagnetic particle (Cudjoe *et al*., 1995; Mansfield and Forsythe, 2000), chemiluminescent immunoassay (Zamora and Hartung, 2002) and PCR (Luk, 1994) for the rapid and effective detection of food borne micro-organisms compared of PCR, electrochemical enzyme-linked immunosorbent assays and the standard culture method (International Organization for Standardization) for detecting Salmonella in meat products.

They reported that both ELISA-FIA and PCR allowed detection of Salmonella in a product

contaminated with a low number of the micro-organisms as compared to standard cultural methods.

MOLECULAR DETECTION METHODS

Techniques not implying DNA-hybridization

Plasmid profile analysis: Most of the research on epidemiological sub-typing of Salmonella is concentrated on plasmid profiles. This is of special importance, since some plasmids in Salmonellae exhibit a strong influence on its virulence (Helmuth and Schroeter, 1994). Plasmid profiling is a typing method in which extra-chromosomal DNA is isolated and subjected to agarose gel electrophoresis.

However, this approach is simple but the instability of plasmids, rigidity of bacteria and degradation of large plasmids due to rough handling interferes with the appropriate identifications.

Restriction Fragment Length Polymorphism (RFLP): Restriction Enzyme Analysis (REA) which is based on targeting the whole genome probe useful in the typing (Stahl *et al*., 1990).

However, complexity of the patterns often showing from hundreds to over thousands bands, hinders proper evaluation and therefore, the suitability of REA for routine identification purpose is very doubtful.

Finger printing of total genomic DNA: This method is based on the distribution of restriction sites along the bacterial chromosomes. They are defined by a unique DNA sequence on the chromosomal DNA which is recognized by a corresponding restriction endonuclease. These enzymes cut the DNA into various fragments which subsequently can be analysed by gel electrophoresis according to their size.

Pulsed field gel electrophoresis: In Pulsed Field Gel Electrophoresis (PFGE) large DNA molecules are resolved by continuous reorientation of electric field (Schwartz and Cantor, 1984), this provides new possibilities for typing and subsequent identification.

Techniques based on DNA-hybridization: In the basic application of DNA-hybridization technique, DNA is fixed to a solid phase and a labeled DNA probe is added and allowed to react with its counterstrand (Mathews and Kricka, 1988). Due to lack of common genes for toxin or other virulence factors, the approach for isolation of specific DNA probes for Salmonella has been to select and test randomly cloned chromosomal fragments (Olsen *et al*., 1995).

Polymerase Chain Reaction (PCR): PCR technology is one of the most promising tools for the rapid, specific and sensitive detection bacteria in a wide variety of samples. Several PCR methods for detecting Salmonella have been published targeting various specific gene sequences.

The various gene sequences which have been targeted for designing primers are replicons, gene coding for abequose and paratose synthase (Luk, 1994), gene coding for aggregative fimbriae, agfA (Donaghy and Madden, 1993), genes from spvR of the virulence plasmid (Mahon and Lax, 1993), *H-li* and *H-in* flagellin genes (Vugia *et al*., 1993), histidine transport operon, gene coding for DNA binding protein, *16s rRNA* gene, three pairs of 18-mer oligonucleotides targeting the *rfbJ* gene, gene coding for Vi antigen (Hanes *et al*., 1995), 15-mer oligonucleotide, *ompC* gene, *16s* and *23s rRNA* gene (Lagatolla *et al*., 1996), *16s rDNA* gene, rRNA operon, fur regulated gene *iroB*, random genomic fragments (Aabo *et al*., 1993), junction of *sipB* and *sipC* genes, enterotoxin gene *stn* (Makino *et al*., 1999) and *hilA* gene, fimc, InvA (Rahn *et al*., 1992; Galan *et al*., 1992) identified a highly conserved genetic locus, Inv which allows *Salmonella* sp., to enter epithelial cells.

A group of genes, *InvABC* and *InvD* allow Salmonella to penetrate in cultured epithelial cells. InvA is the member of this locus and it is the 1st gene in the operon (InvABC) which consist two additional invasion genes, viz. *InvB* and *InvC*.

This gene is present and functional in most (if not all) Salmonella serotypes. Two strains each of the sub-species 1 serovars: *S. Senftenberg* and *S. Litchfield* gave false negative results. Members of *S. arizonae* are deficient of InvD operon (Galan and Curtiss, 1991).

Recently (Ziemer and Steadham, 2003) evaluated the specificity of 9 sets of primers for the specific detection of Salmonella and found that only 3 primer sets were specific for Salmonella highlighting the need for good internal lab validation of the specificity of species specific primers. As a part of major international project for the validation and standardization of PCR for the detection of major food borne pathogens (Malorny *et al*., 2003), selected four primer sets (oriC, ompC, Random fragment ST11 and ST15 and InvA) specific for Salmonella for evaluation of their analytical

accuracy (selectivity and detection limit) in identifying 43 *Salmonella* sp. and 47 non-Salmonella bacteria. Primers specific to *InvA* gene was found to be the most selective primer set. An extended determination of selectivity showed an inclusivity of 99.6% and exclusivity of 100% for the InvA primer set. This primer set was further validated in an international collaborative study that included 16 participating laboratories which revealed the analytical accuracy of 98% with 28 coded (blind) DNA samples.

Henceforth, a simple PCR assay that is specific for *Salmonella* sp. and amplifies a chromosomal DNA fragment was established through extensive validation and is thus proposed as an international standard.

REAL TIME PCR

Compared to conventional PCR, it has the advantage of providing integrated amplification and detection in a single reaction, eliminating the need for post-PCR analysis while significantly reducing the risk of contamination.

It is more sensitive than conventional PCR which is valuable in diagnostic applications where pathogen numbers in sample can be low.

Real time PCR assays have been developed for identification of Salmonella using molecular bacon technology (Chen *et al*., 1997; Wan *et al*., 2004). There are two types of flurogenic PCR based methods. One of the assay utilizes the 5'nuclease activity of Taq DNA polymerase to hydrolyse an internal flurogenic probe for monitoring amplification of DNA target while the other like iq-check system utilizes a flurogenic probe which has flanking GC-rich arm sequences complementary to one another.

The detection sensitivity of real time PCR to detect Salmonella in artificially inoculated poultry samples are 6 cfu mL^{-1} (Isacsson *et al*., 2000).

CONCLUSION

Over the last 5 years, the development of molecular diagnostics assays for the identification of Salmonella has seen significant research efforts and advancement in rapid diagnostic methods. PCR is still popular choice among researchers with the bias currently towards the development of real time PCR based assays.

IMPLIMENTATIONS

The current market for food diagnostics is much smaller than other market segments. While the business of food testing is expected to grow in response to consumer demands in relation to food safety, the implementation of HACCP syatems where the emphasis is on ensuring that the process of food production is safe thereby assuring that the final product is safe will impact the amount of final product testing required.

The development of high throughput systems for the extraction of high quality DNA from foods is the next major challenge that needs to be addressed to maximize the application of the modern technologies for Salmonella detection and characterization.

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