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Assessment of Modus Operandi for Phenotypic and Genotypic Recognition of *Salmonella* Species

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Abstract:

Salmonella sp. is a fastidious gram negative rod shape bacteria. These enteric bacteria are known to be pathogenic and cause

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infections to human and animals. Currently, Salmonella causing infections are mainly identified at molecular level. Many different strategies with recent improvements have been utilized to recognize different species of Salmonella such as colony morphology, biochemical tests, substrate metabolism, serotyping, phase typing, ELISA and genomic DNA probing. As in different parts of the world, various Salmonella causing diseases are rapidly elevating in both humans and animals; therefore, the need to diagnose or identify the specific Salmonella pathogen is a necessary exploitation for the specific cure or prevention approaches. Hence this succinct review as an enlightening assessment has been undertaken to be acquainted with the most reliable methods for the swift detection of contagious Salmonella species all over the world.

Key words: *Salmonella enteric*, salmonellosis, phage typing, plasmid profiling, ribotyping, multiplex PCR

INTRODUCTION

Salmonella are enteric gram negative; rod shaped flagellated, non-spore forming; aerobic and facultative anaerobic organisms (Figure 1). These are classified under the family Enterobacteriaceae. These are widely spread in nature and can reside in the gastrointestinal tracts of animals and humans (some are animal-host specific) and cause disease state that ranges from self-limited diarrhea to bacteremia with enteric fever or invasion of vascular structures, bone or other localized sites (Hook, 1990). Many bacteria species are reported to be pathogenic (Patchanee et al., 2010) or may be beneficial (Shinwari et al., 2016) but *Salmonella* is one of the most notable pathogens involved in human food-borne illness. Most of human salmonellosis cases are caused by the consumption of contaminated egg, poultry, pork, beef and milk products (Geimba et al., 2004).

Salmonella causes serious health problems in developing countries and its infective dose can be as low as 15 to 20 cells which also depend upon age and health (immunity) of the host. *Salmonella* infection of humans is a worldwide health problem through water consumption and spreads largely as epidemic (FDA, 2003).

Salmonella infection is a continuous threat to public health and various challenges have been faced to manage *Salmonella*. The first confront is to stop large spread of food contaminations; the second confront is the traceability of *Salmonella* in various food supply chains and due to lack of food markers, its origin can't be traced easily. The third defy is to be in command of the antimicrobial resistance of various *Salmonella* strains which have been found to resist drugs in many countries all over the world. Another imperative challenge is to augment the *Salmonella* detection by using advanced molecular methods and instantaneous sharing of information about different strains and also establishing great surveillance systems for tracking *Salmonella* in different vicinities of the world (Khan et al., 2007).

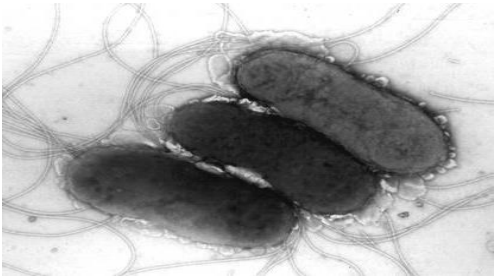

Fig 1. Microscopic image of *Salmonella*

Nomenclature of *Salmonella*

Salmonella classification has altered many times and it is still unsound. The genus *Salmonella* was formerly differentiated into two species: *Salmonella enterica* and *Salmonella bongori.*

However, a new species, *Salmonella subterranea* was identified and validated (Shelobolina et al., 2004). Among them, the species *Salmonella enterica* (*S. enterica*) is further divided into the six subspecies *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI). Fermentation of selected substances such as sorbitol, dulcitol, lactose, malonate, galacturonate and d-tartrate as well as assemblage of enzymes such as ß-glutamyl-transferase or ß-glucuronidase, gelatinase and also lysis by phage O1 allows differentiations between the different species and subspecies (Le Minor, 1984). At this time, *Salmonella* genus is composed of over 2500 serotypes differentiated according to three different types of surface antigens, 99% of these serotypes belong to *S. enterica* and nearly 60% of them are *in S. enterica* subsp. *enterica.* The average DNA sequence similarity between S*almonella* serotypes is 96-99% (Edwards et al., 2002).

Salmonella Detection Strategies

Detection strategy for *Salmonella* involves conventional laboratory techniques, biochemical tests, serological diagnosis, ELISA and DNA based molecular diagnosis. These methods are appraised in detail subsequently.

Laboratory Techniques

Conventional lab isolation of *Salmonella* is done by using cultural methods. These cultural procedures usually have four distinct phases i.e. (i) pre-enrichment in a non-selective medium (peptone buffer media), for multiplication of the target organism and others present in the sample; (ii) selective enrichment, to allow the survival or growth of the *Salmonella*, while inhibiting other accompanying organisms in the selective broth; (iii) isolation, using selective agar media (e.g. Mckonkey's

Agar) that restrict growth of bacteria other than *Salmonella*, (iv) confirmation, where isolates are subjected to a variety of biochemical and serological tests to confirm that these are *Salmonella* and to determine their serovar. In broad-spectrum, each individual step needs at least 16 h up to a maximum of 48 h. Thus, the whole procedure takes 4–7 days to complete and is therefore laborious and also requires expert staff (Waltman and Mallinson, 1995).

Biochemical Tests

Many biochemical tests can be used to detect *Salmonella* because these microbes are able to ferment many substances in media like mannitol, lactose, glucose, lysine, sorbitol, arginine, arabinose etc, their biochemical reactions are specific and give a characteristic color change for easy detection of different *Salmonella* serovars. In one of biochemical test for *S. typhi*, it is unable to ferment trehalose and so this inability is used mainly for its detection. Nowadays, special biochemical kits are available for rapid detection of many *Salmonella* spp. in a single step. Biochemical kits like API 20E is available which can detect reactions with 20 different substances in single incubation (Fig. 2). After incubation, the color reactions are read and analyzed with the kit manufacturer's software and the % probabilities of *Salmonella* are then confirmed (Salem et al., 2007).

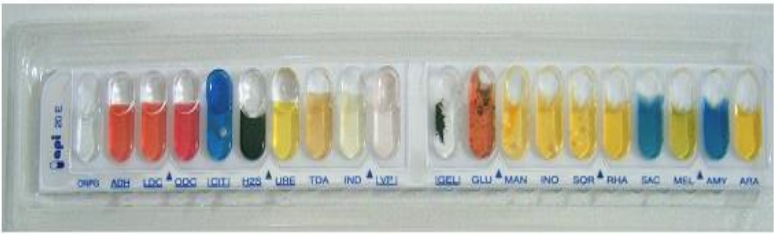

Fig 2. Typical *Salmonella* reaction of API 20E test kit

Serotyping

Serotyping is the first step for routine diagnostics of *Salmonella* strains and performed with commercially available omni, poly and monovalent antisera. Up to date, more than 2500 serotypes of *Salmonella* has been identified and classified in the Kaufmann-White scheme (Theral and Frost, 1990). This scheme differentiates between O (somatic) antigens of the cell surface, H1 and H2 (flagellar) antigens and the virulence capsular (Vi) antigens which may be present in some serotypes like such as *S. typhi* and *S. paratyphi*. Each *Salmonella* serogroup has a group specific O-antigen and within each O-group, different serovars are distinguished by the combination of O & H antigens that are present and by presence of these antigens each serotype has been given a specific antigenic (naming) formula. Serotyping is easy to perform and standardized antisera are commercially available but it only allows the grouping of *Salmonella* strains to a specific serotype and no more differentiation between strains of the same serotype is obtained (Threfall and Frost, 1990). Serological analysis holds the first step in an epidemiological investigation of *Salmonella* and is good enough for epidemiological investigations but smaller labs do not have access to the pools of anitsera required for this analysis and may need to rely on other techniques to analyze isolates (Theral and Frost, 1990).

Phage typing

Phage typing is the technique in which lysis of different *Salmonella* serotypes is done with bacteriophages. Individual isolates of many serotypes vary in their susceptibility to lysis and yield a specific lytic pattern. Therefore, a *Salmonella* strain is subjected to a specified set of typing phages and the lytic pattern obtained commonly allows the assignment to a specific phage type. Phage typing is mostly performed for serotypes such as *S. typhi, S. typhimurium*, *S. enteritidis* or *S. paratyphi*.

Phage typing has led to the identification of over 200 *S. typhimurium* phage types (Threlfall & Frost, 1990). Phage typing has proved to be an important tool for strain characterization, surveillance, source attribution and outbreak investigations (Hald et al., 2007). Phage typing is not a common lab practice; it is only performed at research centers with trained individuals.

ELISA

Immunological tests like ELISA have been advanced specifically for *Salmonella* detection. Nowadays it involves automation and miniaturization technology along with accessibility of commercially equipped high eminence reagents. The application of ELISA has led to the detection of *Salmonella* infection in humans, poultry, pigs and cattle. ELISA based on lipopolysaccharide (LPS) and virulence (Vi) antigens have been used to detect *S. typhimurium* and *S. typhi* infection in rodents and humans respectively (Nicholas and Cullen, 1991). Both indirect and sandwhich ELISA methods are used to check agglutation reaction of IgG, IgM and IgA antibodies with above mentioned antigens. The ELISA is an easy and rapid approach but it suffer from limited sensitivity and a considerable chance to produce both false-positive and false-negative results (Nicholas and Cullen, 1991). Widal test a form an old ELISA method still is used widely for diagnosis of *S. typhi* and confirmation of typhoid fever. The Widal kit includes binding of dead cells onto a microtiter plate and is dyed red, when blood of patient is added to microtiter plate, an immunologic response occur due to binding of antibodies which result in color change and confirmation of typhoid fever (King, 2000).

DNA Detection Methods

Phenotypic typing methods requiring large time, personnel and reagents have led to the development of typing methods based

on genotypic information. Numerous genotyping methods have been applied to the typing of *Salmonella*. Two of the main advantages of these methods are that they do not depend on the expression of phenotypic properties and that all strains of *Salmonella* can be typed by the same method with only small changes for use with a particular serovar (Olsen, 2000). Few DNA diagnostic methods have been used largely but no single method can be recommended for all typing purposes for *Salmonella*. The choice of method depends on the specific case and sometimes more than one method is applied to improve the quality of the typing. Some important molecular methods are explained further.

Plasmid Profiling

Plasmid profile analysis was one of the first DNA-based typing schemes. It is particularly important, since most of the plasmids have virulence and antimicrobial resistance properties in *Salmonella*. Plasmid content of the host within the same serotype shows the differentiation according to the profile i.e. the number and molecular sizes of plasmids obtained. The different plasmid profiles within a serotype indicate the lateral transfer by gaining or losing the plasmid(s). The plasmids found in *Salmonella* differ in size 2–200 kb with different functions (Rychlik et al., 2006). The detection method is based on the isolation of plasmids followed by agarose gel electrophoresis and to view the plasmid pattern, agarose gel must be stained with ethidium bromide solution and then visualized under UV light. Plasmid profiling is most useful in an outbreak which is limited temporally and geographically (Mendoza & Landeras, 1999). Plasmid analysis has some limitations i.e. plasmids can rapidly be acquired or lost by conjugation or horizontal plasmid transfer to other serotypes in *Salmonella*.

PFGE (Pulsed Field Gel Electrophoresis)

PFGE has been considered as the "gold standard" among other molecular typing methods. By cutting the bacterial DNA with rare-cutting restriction endonucleases and running with special electrophoresis separation technique which use pulsed currents that change polarity at defined intervals, it separates the large fragments of DNA up to 12000 kb and yields strain specific patterns. The most commonly used restriction enzymes in *Salmonella* are XbaI, SpeI and NotI. Comparisons of patterns from multiple enzymes can determine new subtypes and increase the distinguishing power of this technique (Liebisch & Schwarz, 1996). PFGE of 60 *S. enteritidis* isolates revealed 28 different XbaI restriction profiles and 26 with SpeI but when the patterns generated from both enzymes were combined, 32 different pulsed-field types could be identified (Ridley et al., 1998). PFGE has also been used to track outbreak strains within and across boundaries of other countries. Despite that PFGE is method of first choice but this method is relatively slow, often takes three days to complete, and requires the presence of expensive specialized equipment, high quality chemicals and a good experience in the preparation of the DNA-containing agarose gels. Moreover, single genetic events, such as point mutations, integration, deletion or recombination events can result in differences in the fragment patterns (Herschleb et al., 2007).

Ribotyping

Ribotyping is the fingerprinting of rRNA coding sequences which describes the hybridization of restriction-digested DNA fragments with probes specific for rDNA. Multiple copies of the rRNA operon are present within the *Salmonella* chromosome (Mendoza & Landeras, 1999). Although rRNA genes themselves are homologous among many serotypes but differences occur in the intervening sequences length and nucleotide composition.

Ribotyping is important for making differentiations of serotypes (like *S. enteritidis*) in case of recurrent epidemics and outbreaks at particular areas (Ridley et al., 1998). Ribotyping method begins with separating endonucleases digested chromosomal DNA on agarose gels. DNA is then transferred to a nylon membrane and fragments are hybridized to a probe that recognizes 16S and 23S rRNA. Analysis of multiple restriction endonucleases can improve the discriminatory powers of ribotyping (Mendoza & Landeras, 1999). Automated ribotyping has also been developed and the banding pattern can be compared to data stored in computer databases. Ribotyping is better than PFGE because it takes less time and drawback is that its reagents are very costly and also the riboprinter equipment is very expensive (Fontana, 2004).

IS (Insertion Sequence) Typing

IS200 is a mobile (transposable) element found in a variety of eubacterial genera such as *Salmonella, Escherichia* and *Shigella*. IS200 elements are very small (707-711 bp) and contain a single gene present in chromosome with multiple copies. IS200 transposes rarely and is very stable therefore; this stability makes IS200 a suitable molecular marker for epidemiological and ecological studies especially when the number of IS200 copies is high. IS200 typing has been used to evaluate the molecular relationships between *Salmonella* isolates e.g. in *S. enterica*, IS200 fingerprinting is extensively used for strain identification. The method involves hybridization of digested chromosomal DNA with an IS200 probe. IS200 typing is sometimes considered as superior to PFGE and ribotyping (Jeoffreys et al., 2001).

PCR Methods & Multiplex PCR

Commonly used PCR based detection methods involve RAPD technology which uses short synthetic 10 bases long

primers which can amplify *Salmonella* genomic DNA by PCR and then the banding pattern of amplified product is observed on gels. AFLP is also used for *Salmonella* diagnosis which involves use of restriction enzymes to form fragments then ligation of adapters to genomic restriction fragments followed by PCR amplification with adapter specific primers. The optimal number of scorable bands (50–100) can easily be set by selection of the suitable AFLP primers and restriction enzymes (like EcoR1 & MseI). These characteristics make AFLP a powerful fingerprinting technique which can be used in identification, epidemiology and taxonomy (Folkerstma et al., 1996).

Multiplex polymerase chain reaction (mPCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. During the last dercade, a lot of research has shown the practicality of identifying *Salmonella* serovars using mPCR (Kim et al., 2006). In addition, the technique has been shown to be a powerful and cost-effective tool for *Salmonella* detection. This method is based on detection of genes present in specific *Salmonella* serotypes and these genes are selected from analysis of whole-genome sequencing. Usually set of 3-5 primers are designed for virulence genes of infectious agents like *S. typhi, paratyphi, enteritidis* and primers for surface antigen genes are also designed to detect intra serovar variation. The specific virulence genes gryA, gryB and cry1 of *S. typhi, paratyphi* and *enteritidis* respectively are commonly used in primer designs and their presence in mPCR result help in easy detection of *Salmonella* from other pathogenic microbes. In order to carry out the mPCR, it has to be optimized i.e. primers should not dimerize with self and other primers and the melting temperature should not vary otherwise unwanted amplification might occur which will affect the mPCR results. Due to the rapidness, cost effectiveness, high

specificity and sensitivity, it has become the most used method nowadays (Kim et al., 2006).

CONCLUSION

Salmonella illustrate large phenotypic diversity and several phenotypic typing techniques like serotyping and phage typing have been largely developed and used for many years but they have some drawbacks. An ideal typing method should fulfill the following criteria: type ability, reproducibility and discriminatory power, ease of interpretation, easy to use and low cost. Any method used currently for typing of *Salmonella* strains is an ideal method alone in terms of these criteria, but all methods exhibit benefits and also limitations. So far PCR based diagnosis are the best available methods and the most preferred for detection of *Salmonella* sp.

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