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A Review on Typing of Non-typhoidal *Salmonella* (NTS)

Mohammed S. Al-Mogbel[1], Godfred A. Menezes[1]*, Mohamed T. Elabbasy[2], Sumod P. George[1] and Mushtaq A. Khan[1]

[1]College of Applied Medical Sciences and Molecular Diagnostics and Personalised Therapeutics Unit (MDPTU).
[2]College of Public Health and Molecular Diagnostics and Personalised Therapeutics Unit (MDPTU), Hail University, Hail, Kingdom of Saudi Arabia.

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***Salmonella* infections are responsible for significant public health problem worldwide. *Salmonella* are one of the most common causes of food-borne illness in humans. Salmonellae are of two broad categories: those that cause typhoid [typhoidal *Salmonella*] and those that do not [much broader group of non-typhoidal *Salmonella*-NTS]. Infection by the NTS serovars is estimated to cause a large burden worldwide, with a higher morbidity rate than mortality. Antimicrobial agents are critical to the successful outcome of invasive and focal NTS infections. Genus *Salmonella* of family *Enterobacteriaceae* comprises of heterogenous group of bacteria. The taxonomy and nomenclature of *Salmonella* has changed over the years and is still evolving. A variety of molecular biological tools based on characterization of the genotype of the organism by analysis of plasmid and chromosomal DNA have now been developed either to complement the more traditional phenotypic methods of typing (serotyping, phage typing) or, in some cases, as methods of discrimination in their own right. This brief review tries to reiterate different methods used for typing NTS.**

Key words: Non-typhoidal *Salmonella*, Bacteriophage, Molecular Subtyping, MALDI-TOF mass spectrometry.

Genus *Salmonella* is Gram-negative, non-spore forming, rod-shaped bacteria belonging to the family *Enterobacteriaceae*. They are generally motile with peritrichous flagella, grow on nutrient agar, do not ferment lactose, and they reduce nitrate into nitrite and they are oxidase negative. Genus *Salmonella* has two species *Salmonella enterica* and *Salmonella bongori*[1]. The species, *Salmonella* enterica is further divided into six subspecies - *Salmonella enterica* subsp. *enterica* (I), *Salmonella enterica* subsp. *salamae* (II), *Salmonella enterica* subsp. *arizonae* (IIIa), *Salmonella enterica* subsp. *diarizonae* (IIIb), *Salmonella enterica* subsp. *houtenae* (IV), and *Salmonella enterica* subsp. *indica* (VI). Currently, 2,610 different *Salmonella serovars* are described according to the White-Kauffmann-Le Minor scheme[2].

Genus *Salmonella* of family *Enterobacteriaceae* comprises of heterogenous group of bacteria. The taxonomy and nomenclature of *Salmonella* has changed over the years and is still evolving. Strains of *Salmonella* spp. are classified into serovars on the basis of extensive diversity of the heat-stable lipopolysaccharide (O) antigens and heat-labile flagellar protein (H) antigens and sometimes the capsular (Vi) antigens. A variety of phenotypic methods have been used both independently and in combination for subdivision within serovars. Those currently in use include phage typing (bacteriophage typing) and antibiogram (resistance) typing.

NTS serovars (in humans) typically cause a localized disease, which manifests itself as acute gastroenteritis. On the other hand, when the macrophages are unable to limit spread,

* To whom all correspondence should be addressed.
Tel.: +966 507091946;
E-mail: godfredmenezes@gmail.com/
g.menezes@uoh.edu.sa

Salmonella can cause a systemic disease[3]. Many virulence factors have been identified in *S. enterica* serovars Typhimurium and Typhi, but the underlying reasons for the different host specificities and disease outcome of various serovars are not fully understood.

Bacterial virulence factors are predominantly encoded associated with various mobile genetic elements, including plasmids, insertion elements, transposons and a large number of such determinants situated within pathogenicity islands (PAI). To successfully colonise and cause disease in a broad range of different hosts, *Salmonella* has acquired genes which are frequently clustered at particular parts of chromosome called *Salmonella* Pathogenicity Islands (SPI)[4]. A total of 14 such islands have now been identified, termed SPI-1–SPI-14[5]. There are no less than 21 PAIs in *Salmonella*, but most attention has been paid to SPI-1 and SPI-2, which are significant for invasion of nonphagocytic cells and for replication in nonphagocytic and phagocytic cells, respectively. SPI-1 and SPI-2 encode type III secretion system-1 (T3SS-1) and T3SS-2, respectively, and play major roles in virulence[6].

In addition to possessing pathogenicity islands certain serovars harbor serovar-specific plasmids. A cluster of genes positioned on a large plasmid, termed the *Salmonella* plasmid virulence (*spv*) cluster has been identified in certain serovars[7]. The emergence of multidrug-resistant (MDR) strains such as *S.* Typhimurium DT104 is a major problem. Characterization of the resistance factors of such isolates led to the identification of a genomic island in MDR strains of *S. enterica* serovars Typhimurium and Agona. This locus is termed *Salmonella* Genomic Island 1 (SGI-1)[8].

The capacity to distinguish between genomes is necessary to various disciplines of microbiology research including taxonomy, phylogenetic relationships, population genetics of microorganisms, and microbial epidemiology. A variety of molecular biological tools based on characterization of the genotype of the organism by analysis of plasmid and chromosomal DNA have been developed over time either to complement the more traditional phenotypic methods of typing (serotyping, phage typing) or, in some cases, as methods of discrimination in their own right[9]. This brief review tries to reiterate different methods used for typing NTS.

Literature review

Non-typhoidal *Salmonella* (NTS) - Identification and Typing

Phenotypic Characteristics

Salmonella is a genus of rod-shaped, Gram-negative, non-spore forming, predominantly motile (peritrichous) bacteria belonging to the family '*Enterobacteriaceae*.' Salmonellae are aeroanaerobes and grow on simple media such as nutrient agar. The following characteristics are used for the *Salmonella* identification: oxidase negative; reduce nitrate into nitrite; ferment glucose, mannitol and maltose, forming acid and gas; lactose, sucrose and salicin are not fermented; indole not produced; MR positive; VP negative; citrate positive; urea not hydrolysed; H_2S is produced and decarboxylates lysine and ornithine[10].

Serotyping

Strains of Salmonella spp. are classified into serovars on the basis of extensive diversity of the heat-stable lipopolysaccharide (O) antigens and heat-labile flagellar protein (H) antigens and sometimes the capsular (Vi) antigens[11,12,13]. The Kauffmann and White scheme[14] is recognized worldwide and remains the definitive method for the serological identification of salmonellae.

Based on the similarities in content of one or more antigens O antigens, members of Salmonella, are placed in groups designated O:2, O:4, O:6,7 and so on. For further classification, the H antigens are employed. These antigens are divided into 2 groups: specific phase or phase 1 and group phase or phase 2. The H antigens of phase 1 are named with small letters, and those of phase 2 are designated by Arabic numerals (**Table 1**). Any given culture may consist of organisms in only phase or of organisms of both flagellar phases.

Subdivision within Serovars: Phenotypic Subtyping

A variety of phenotypic methods have been used both independently and in combination for subdivision within serovars. Those currently in use include phage typing (bacteriophage typing) and antibiogram (resistance) typing.

Bacteriophage Typing

Phage typing of *Salmonella* is a valuable typing tool for subcategorising the more common

Salmonella enterica serovars, such as, *S.* Typhimurium and *S.* Enteritidis. It can be helpful to ascertain the epidemiology of the isolates.

The fundamental principle of phage typing is the host specificity of bacteriophages and on this basis several phage-typing schemes have been developed for serovars of clinical or epidemiological importance[15]. The most important schemes are those for *S.* Enteritidis, *S.* Typhimurium, and *S.* Virchow.

In comparison to the phage typing scheme for Typhi, phage typing schemes for other serovars rely on, to a limited extent, phage adaptability and, for the most part, are based on patterns of lysis produced by serologically distinct phages isolated from a variety of sources. More than 70 phage types (PTs) are now documented in the Enteritidis scheme, the significance of which was realized on an international scale following the global pandemic of Enteritidis. This scheme was prominent in identifying the global pandemic PT 4, and more recently, in detecting and monitoring the emergence of non-PT 4 associated with eggs from poultry flocks in several different European countries. A major accomplishment has been the optimising of phage typing for *S.* Enteritidis throughout Europe. It is in use in reference laboratories for human salmonellosis in 22 European countries as well as in Australia, Japan, and Canada[16,17].

For *S.* Typhimurium, more than 300 PTs have been recognized and designated using the most commonly used worldwide scheme of Anderson and colleagues[18]. The significance of this scheme is well demonstrated by the universal recognition of the multiple drug-resistant (MDR) epidemic clone of *S.* Typhimurium DT 104.

Salmonella phage-typing is economical and requires no expensive equipment. Nevertheless, PTs cannot always be considered as indicative of clonality because PT conversions may result from the acquisition of both plasmids and bacteriophages. Further, problem with phage typing is that because of the requirement to propagate and preserve bacteriophage stocks and to execute strict quality control procedures, the procedure is best executed by highly trained staff in reference laboratories.

Antibiogram (Resistance) Typing

An antibiogram is the result of a laboratory testing for the susceptibility of an isolated bacterial strain to different antibiotics. The susceptibility or resistance patterns to selected antimicrobial drugs can be a very valuable screen for epidemiological investigations. Because of mutation and/or plasmid acquisition such patterns cannot be regarded as definitive. Nevertheless, patterns such as ACSSuT (Ampicillin, chloramphenicol, streptomycin, sulphonamides and teteracycline) for *S.* Typhimurium DT 104 and for the identification of *Salmonella* Genomic Island 1 (SGI-1), and ACSSuTTm (Ampicillin, chloramphenicol, streptomycin, sulphonamides, teteracycline and trimethoprim) for *S.* Typhi have become very useful markers and have been used on a global basis to assist in the identification of epidemic clones or drug resistance islands. Of particular importance in the use of susceptibility or resistance patterns (R-types) as epidemiological markers, and also in the international surveillance of antimicrobial drug resistance in *Salmonella* is the standardization of methodologies coupled with the interpretation of results. For human *Salmonella* isolates within Europe this has been achieved, in the first instance, by the standardization of methods of susceptibility testing in all reference laboratories. Following international agreement on the definitions of resistance and susceptibility based on various methodologies, it has been possible to combine and analyze the antimicrobial drug resistance data originating from all countries within Europe who participate in the surveillance network[19].

Currently, the drugs of choice for empiric treatment of acute infectious diarrhea, in which *Salmonella enterica* are etiologically implicated, are FQs in adults and third generation cephalosporin's in children. Alternative treatment may use azithromycin and imipenem in life-threatening systemic *Salmonella* infections. Aminoglycosides are considered ineffective in gastrointestinal salmonellosis. The emerging resistance to FQs, production of ESBL, and the increase of MDR *Salmonella* strains are major problems in the efficient antimicrobial therapy of *Salmonella* infection[20,21]. However, decline in MDR among typhoidal salmonellae, probably due to the loss of a high molecular-weight self-transferable plasmid encoding chloramphenicol, ampicillin, and cotrimoxazole resistance should lead to the

cautious reuse of classical first-line antibiotics, such as chloramphenicol[22].

Subdivision within Serovars: Molecular Subtyping

The capacity to distinguish between genomes is necessary to several disciplines of microbiology. A range of molecular biological tools based on characterization of the genotype of the organism by analysis of plasmid and chromosomal DNA have now been developed either to supplement the more traditional phenotypic methods of typing and in some cases as methods of discrimination in their own right[9,23].

Plasmid Typing

Isolates from same epidemic will have plasmids with identical sizes. But, this test requires the presence of at least one plasmid type. Some phage types in serovars Enteritidis and Typhimurium can be subdivided by plasmid profiling.

Salmonellae carry plasmids differing in both molecular mass and number. Plasmid typing depending on the numbers and molecular mass of plasmids after extraction of partially purified plasmid DNA has been used for differentiation within serovars. Plasmid typing is therefore limited to serovars containing plasmids and is of restricted use in those serovars in which the greater part of isolates contain only one plasmid or are without plasmids. The sensitivity of the plasmid profile typing may be increased by cleaving plasmid DNA with a limited number of restriction endonucleases and the resultant plasmid 'fingerprint' may be used to discriminate between plasmids of similar molecular mass. Identical plasmids should have the same restriction pattern[24]. More precise results are obtained when plasmid DNA is extracted, digested by a restriction endonuclease and the fragments separated by agarose gel electrophoresis. More recently the characterization of plasmids, developed by Carattoli and colleagues (2005) for identification of specific replicon areas has added a new dimension to plasmid typing[25]. This method has considerable potential not only for the identification of plasmid incompatibility groups but also for investigating the spread of such plasmids, and the resistance genes encoded thereon.

Identification of Chromosomal Heterogeneity

Molecular typing methods based on the characterization of plasmid DNA include, plasmid fingerprinting, plasmid profile typing and the identification of plasmid borne virulence genes.

Chromosomally based methods have sought to identify small regions of heterogeneity within the bacterial chromosome. Of these the most commonly used have been, a. ribotyping, b. pulsed field gel electrophoresis (PFGE), c. insertion sequence (IS) 200 typing, d. Amplification methods: RAPD; ERIC-PCR; REP-PCR; AFLP & VNTR, and e. Multilocus sequence typing (MLST)

Ribotyping (rRNA gene restriction patterns)

When bacterial DNA is extracted, purified, digested by a restriction endonuclease and the fragments separated by agarose gel electrophoresis, fragments in restriction patterns are too numerous for these patterns to be compared. Instead, the fragments in the gel are transferred to a nylon membrane, to retain their relative positions, and hybridized with a labeled mixture of 16S and 23S rRNA, which can be detected by immunoenzymatic reaction or autoradiography yielding simpler patterns, termed ribotypes[26].

Pulse-field gel electrophoresis (PFGE)

PFGE technique uses restriction endonucleases having infrequently occurring restriction sites in a bacterial genome. A small number of fragments of a much larger size are produced which are separated using specialized electrophoresis, termed PFGE. The most refined configuration for this technique is called clamped homogeneous electric field (CHEF) electrophoresis, which uses an array of hexagonally arranged electrodes to generate uniform electric fields at an angle of 120° to each other, thus confirming that large DNA fragments migrate through the gel in a straight line. Although PFGE is highly discriminative for *Salmonella* (gold standard) with the endonucleases *Xba*I, *Bln*I or *Spe*I, it is expensive and time-consuming. Further, its standardization, analysis and comparison of restriction profiles require effort[27]. This method is used as the basic method of subtyping of *Salmonella* in the United States, and for subdivision within PTs in those countries which use phage typing as the primary method for the discrimination of epidemiologically important serovars. The method has become standardized and networks have been developed- PulseNet in the United States and SalmGene in Europe- to

provide common, unified molecular typing methods, and to facilitate the rapid electronic transfer of the images captured by them in a digitized format[28,29]. More recently the SalmGene database of PFGE types has been expanded to form the basis of PulseNet Europe, which is fully compatible with PulseNet USA and other PulseNet networks, thereby providing an encompassing network for the molecular subtyping of *Salmonella* worldwide.

IS200 typing

DNA of most Salmonella serovars contains several copies of a 708-base-pair insertion sequence, IS200. Stanley et al.[30] and Calva et al.[31] found that isolates could be differentiated by comparing the restriction patterns of bacterial DNA after hybridization with an IS200 probe. Strains differ by the number of visualized fragments (IS200 number of copies) and the size of fragments. IS200 has been proposed as a good typing scheme, but IS200 profiling has been found to have low discriminatory power[32].

Amplification methods: RAPD; ERIC-PCR; REP-PCR; AFLP and VNTR

Even within a particular species the genetic composition of many microorganisms is highly variable. This difference is used for subtyping or distinguishing the strains by polymerase chain reaction (PCR) amplification methods. Such methods include random amplified polymorphic DNA typing (RAPD), enterobacterial repetitive intergenic consensus typing (ERIC-PCR), repetitive extragenic palindromic element typing (REP-PCR), amplified fragment length polymorphism fingerprinting (AFLP), and variable number of tandem repeats (VNTR) fingerprinting.

RAPD is a rapid genomic typing method of broad application. This technique uses a single arbitrarily chosen primer, which anneals at multiple sites throughout the genome. The profiles of amplified products are characteristic of the template DNA. A RAPD method has been developed to differentiate *S.* Enteritidis isolates, providing more discrimination than any other subtyping method[33]. This method proved to discriminate between isolates of different *Salmonella* serovars[34]. However, it lacks the reproducibility between laboratories[35].

In ERIC-PCR, ERIC sequences are 126-base pair conserved motifs seen in enteric bacteria.

Table 1. Antigenic formulae of some common salmonellae

Group	Serovars	O antigens	H antigens	
			Phase 1	Phase 2
O:2*	*S.* Paratyphi A	1, 2, 12	a	(1, 5)
O:4	*S.* Schottmuelleri(*S.* Paratyphi B)	1, 4, (5), 12	b	1, 2
	S. Typhimurium	1, 4, (5), 12	i	1, 2
	S. Agona	1, 4, (5), 12	f, g, s	(1, 2)
O:7	*S.* Hirschfeldii(*S.* Paratyphi C)	6, 7, (Vi)	c	1, 5
	S. Choleraesuis	6, 7	c	1, 5
	S. Oranienburg	6, 7	m, t	-
	S. Montevideo	6, 7, (54)	g, m, s, (p)	(1, 2, 7)
O:8	*S.* Newport	6, 8	e, h	1, 2
O:9	*S.* Typhi	9, 12, (Vi)	d	-
	S. Enteritidis	1, 9, 12	g, m	-
	S. Gallinarum	1, 9, 12	-	-
O:3,10	*S.* Anatum	3, 10	e, h	1, 6
	S. Lexington	3, 10	z_{10}	1, 5
O:1,3,19	*S.* Senftenberg	1,3,19	g, (s), t	-
O:17	*S.* Kirkee	17	b	1, 2

( ) = May be absent.

*Historically, O groups were first identified by letters. Since there were not enough letters, it was necessary continue with numbers 51 to 67. It is now more logical to designate each O group using the characteristic O factor. Letters are provisionally kept into brackets. Ex. O:4 (B); O:18 (K). It is advisable to abadon designation-by-letter which is unnecessary[1].

ERIC-PCR has been used productively for typing a number of foodborne pathogens, including salmonellae. Though, the results usually are less discriminatory than PFGE[26].

AFLP analysis combines the beneficial characters of PCR amplification and restriction digest analysis for genotyping. Both AFLP (by fluorescent primers) and PFGE demonstrate similar abilities to distinguish salmonellae[36].

VNTR fingerprinting is based on the presence and subsequent identification of units of repeated DNA elements in the genome. Such elements, known as VNTRs, range from about 10 to 100 base pairs (bps). VNTR fingerprinting has been applied to the subtyping of *S.* Typhimurium[37]. The approach of using multiple VNTR loci for typing is referred to as multiple locus VNTR analysis (Multi-Locus Variable number of tandem repeats Analysis- MLVA)[38]. Researchers have reported genotyping using MLVA to be more discriminatory than PFGE in typing of *S.* Typhimurium[39]. A major drawback of the method is that for meaningful results VNTRs for typing should be based on the published genome sequence of a serovar. As only a limited number of serovar sequences have been published, the applicability of this method is somewhat limited[19].

Multilocus sequence typing (MLST)

Microbial genomes are subject to sequence variability due to mutation or recombination. The sequence variability within particular genes can be used in molecular typing schemes to determine the relatedness of bacteria. MLST utilizes specific nucleotide base changes rather than DNA fragment size to determine genetic relatedness. Hence, MLST relies totally on quality sequencing results. MLST utilizes specific nucleotide base changes rather than DNA fragment size to determine genetic relatedness. Because MLST relies on these nucleotide base changes for typing, high quality sequencing results are essential[26].

Of the total 79 MLST databases that are available for public use (http://pubmlst.org/databases.shtml), the MLST database for *S. enterica* (http://mlst.ucc.ie) grades fourth in number of isolates. This publicly accessible web-based MLST database enables the global exchange of information[40]. However, MLST does not provide the fine resolution needed for outbreak analysis and short-term epidemiology, unlike the resolution obtained in phage typing, PFGE, and MLVA.

Matrix Assisted Laser Desorption Ionization Time-of-Flight mass spectrometry (MALDI-TOF-MS) for identification and Subtyping

The mass spectrometric approach may complement traditional approaches, such as a method for rapid pre-screening of isolates. However, subsequent identification of the majority of serovars still relies on conventional serotyping. MALDI-TOF-MS can be used as a pre-screening procedure for identifying *Salmonella* serovars[2].

Whole-cell MALDI-TOF-MS based on intact protein profiling is increasingly been studied for bacterial species identification[41]. It has been shown to provide reliable and reproducible results[42]. A suitable number of stable mass signals of major housekeeping proteins (largely ribosomal proteins) can reproducibly be detected and employed for identification of bacterial species by using simple mass pattern-matching approaches or more sophisticated algorithms. It relies on the characteristic mass profile produced by a set of ion peaks that constitute a bacterial "fingerprint." Researchers have demonstrated that whole-cell MALDI-TOF-MS can be a rapid method for pre-screening *S. enterica* isolates to identify epidemiologically important serovar and to reduce sample numbers that have to be subsequently analyzed using conventional serotyping by slide agglutination techniques[2].

A larger number of reproducible peaks are required for subspecies identification. Hence, laborious control of the sample preparation and optimization of testing parameters is crucial for strain typing with MALDI-TOF-MS, which is not practical in clinical laboratories[41].

CONCLUSION

Various typing methods have been established and are widely used to differentiate and type pathogens for efficient outbreak investigations or epidemiological studies. The technique to be employed for typing the NTS depends mainly on its strength and the weakness. The techniques for subtyping relies on factors such as reproducibility, time, cost involved and the discriminatory power. The techniques such as MLST can provide results in a shorter period of

time, in comparison to PFGE. Further, in restricted disease outbreaks PCR based methods are sufficient to distinguish the isolates. Conversely, in foodborne disease outbreaks of *Salmonella*, PFGE is preferred due to its higher discriminatory power. MALDI-TOF-MS can be a rapid method for pre-screening *S. enterica* isolates to identify epidemiologically important serovar. Depending on the accessible laboratory resources, a combination of typing methods could assist in genotyping the strains involved.

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