Salmonella Serotyping in US Public Health Laboratories

WHITE PAPER

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APHL ASSOCIATION OF PUBLIC HEALTH LABORATORIES™

Cover Photo: This Centers for Disease Control and Prevention (CDC) microbiologist was in the process of preparing Streptococcus agalactiae (Group B Streptococci) for serotyping and antimicrobial susceptibility testing for CDC's Active Bacterial Core surveillance (ABCs). Credit: CDC/Melissa Brower

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Overview

Recent foodborne disease burden estimates rank *Salmonella* among the top four pathogens associated with foodborne illness, causing an estimated 1.0 million illnesses and over 23,000 hospitalizations annually in the United States (1). Moreover, the economic costs associated with *Salmonella* illnesses are staggering, ranging from $4 billion to $11 billion dollars annually (2).

National surveillance data for *Salmonella* based on serotype designation has existed for nearly 50 years (3), and is collected through various passive and active, laboratory-based surveillance systems. *Salmonella* serotypes are designated by an international standardized nomenclature according to the Kauffmann-White (K-W) scheme which is maintained by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella*, located at the Pasteur Institut in Paris, France (4). There are currently more than 2,500 serotypes (serovars) of *Salmonella* (4).

Serotype-specific *Salmonella* surveillance data is essential for monitoring burden of disease and trends in antibiotic resistance, detecting outbreaks, informing and evaluating programmatic activities and national policies aimed at reducing the burden of salmonellosis, and conducting attribution and other special studies. A recent report issued by the Centers for Disease Control (CDC), *An Atlas of Salmonella in the United States, 1968-2011*, nicely illustrates how comprehensive surveillance data for the 30 most prevalent serotypes can be used to better define and understand the epidemiology of salmonellosis (3). Federal regulatory agencies rely on serotype data for the control of *Salmonella* in meat and poultry establishments and for animal and environmental surveillance activities such as those through the National Veterinary Services Laboratories within USDA-FSIS (5).

I. Serotype-specific Surveillance in the US

A number of issues are threatening to derail our current public health laboratory-based surveillance systems. The rapidly increasing availability of culture-independent diagnostic (CID) methods for foodborne pathogens poses several, serious challenges for public health. National surveillance programs for *Salmonella* including serotype-specific surveillance are wholly dependent on the receipt of bacterial isolates at state and local public health laboratories for confirmation and further characterization. CIDs do not produce isolates. Without isolates, information on pathogen serotype, subtype, virulence, and antimicrobial susceptibility will be scant, if available at all. State-specific isolate submission laws for *Salmonella* vary considerably, and in some cases are non-existent. In a survey conducted by the Association of Public Health Laboratories (APHL) in 2011, only 31 state public health laboratories reported having a mandatory isolate submission law in place for non-typhoidal *Salmonella*. In CDC's 2011 *National Salmonella Surveillance Annual Report*, the number of laboratory-confirmed human *Salmonella* isolates reported to the Laboratory-based Enteric Disease Surveillance (LEDS) system was estimated to be less than 20% of the number of Salmonellosis cases reported to the National Notifiable Diseases Surveillance System (NNDSS) (6). APHL is collaborating closely with CDC and other federal partners and associations regarding the rapid implementation of culture-independent diagnostics and has convened a CID Subcommittee to address the impact of the increased use of such technologies.

Budget cuts to staffing and resources have limited the ability of the state and local public health laboratories to fully characterize all of their enteric pathogens. In 2011, approximately 13% of laboratory-confirmed human *Salmonella* isolates reported to LEDS were either partially serotyped or not serotyped at all (6). With the advent of CIDs, public health laboratories will likely be receiving stool specimens and other clinical materials in addition to isolates, making their work load even more burdensome.

Quality *Salmonella* antisera are in short supply. Since the 1960's, CDC has maintained *Salmonella* antisera for traditional serotyping in the public health laboratories. Over the years, expertise and facilities for maintaining antisera production have diminished at CDC leaving limited amounts of antisera available for public health laboratory use. CDC contracted to produce a fairly large supply of antisera in the late 1990s; but, some reagents have been depleted from the national stockpile. To help mitigate the problems associated with maintaining high quality antisera, the national *Salmonella* laboratory at CDC developed a molecular serotyping method based on targets specific to the O and H antigens (7) (8). This method is able to serotype up to 90% of the most prevalent *Salmonella* isolates within the US while maintaining the conventional Kaufmann-White Scheme. Isolates which are un-typable are forwarded to the National *Salmonella* Reference laboratory at CDC for confirmation and further characterization. At times, the number of isolates forwarded to the National *Salmonella* Reference Laboratory at CDC has exceeded capacity leading to backlogs and delays in reporting to submitters.

Given the resource limitations at both the national and local levels, CDC along with state and local public health laboratories must explore ways to sustain a national *Salmonella* surveillance system including fully serotyping all *Salmonella* isolates received at public health laboratories in a timely manner and which does not exclusively depend on CDC as the primary reference laboratory for un-typable isolates. In 2012, APHL convened a work group to develop a sustainability model for *Salmonella* serotyping based on current and up-and-coming methods. This white paper serves as an introduction to the sustainability model and examines currently acceptable methods for *Salmonella* serotyping including advantages, disadvantages and cost considerations for each.

II. Overview of *Salmonella* Serotypes[1]

The National *Salmonella* Reference Laboratory at CDC focuses primarily on supporting national *Salmonella* surveillance systems by: performing identification and serotyping of atypical or difficult-to-identify isolates, providing technical assistance and training to state public health laboratories that serotype *Salmonella*, and supporting implementation of molecular methods for determination of serotype in *Salmonella* in interested laboratories. The nomenclature for *Salmonella* serotypes has evolved from the initial one serotype-one species concept proposed by Kauffmann on the basis of the serologic identification of O (somatic) and H (flagellar) antigens. The O antigens are composed of lipopolysaccharide, and are designated by number. H antigens are proteins found on the flagella. *Salmonella* often express two of these flagellar proteins, referred to as phase 1 and phase 2. Additional antigens, such as the Vi capsular antigen found in *Salmonella* Typhi, can help confirm serotype determination. Many O and H antigens can be present in multiple different

subspecies; therefore, identification of *Salmonella* to the subspecies level is required for serotype determination.

The genus *Salmonella* contains two species, each of which contains multiple serotypes. The two species are *S. enterica*, the type species, and *S. bongori*, which was formerly subspecies V. *Salmonella enterica* is divided into six subspecies which are referred to by a Roman numeral and name:

— I, *S. enterica* subsp. *enterica*
— II, *S. enterica* subsp. *salamae*
— IIIa, *S. enterica* subsp. *arizonae*
— IIIb, *S. enterica* subsp. *diarizonae*
— IV, *S. enterica* subsp. *houtenae*
— VI, *S. enterica* subsp. *indica*

The K-W Scheme uses names for serotypes in subspecies I (e.g. Enteritidis, Typhimurium, Typhi and Choleraesuis) and antigenic formulas for other *Salmonella*, though all serotypes can be described by an antigenic formula. For named serotypes, to emphasize that they are not separate species, the serotype name is not italicized and the first letter is capitalized. After the first citation of a serotype including the genus-species name, serotype is typically abbreviated as *Salmonella* serotype Typhimurium, *Salmonella* Typhimurium or simply serotype Typhimurium.

Antigenic formulae include the following: (i) subspecies designation for *S. enterica* (subspecies I through VI), (ii) O (somatic) antigens and Vi antigen (if present) followed by a colon, (iii) H (flagellar) antigens (phase 1) followed by a colon, and (iv) H antigens (phase 2, if present) (for example, *Salmonella* serotype IV 45:g,z51:−. For formulae of serotypes in S. bongori the subspecies is omitted, (for example, *S. bongori* serotype 61:z35:−). Antigenic formulas are written as follows:

Subspecies O antigen,Vi (if present):phase 1 H antigen:phase 2 H (if present)

[1]Information for this section was pulled from the Manual of Clinical Microbiology, 10th Edition (9)

III. Traditional *Salmonella* Serotyping[1]

Brief Description

Salmonella is O group serotyped using O group antisera. The most common approach is to test O groups A to E1 since 95% of the known *Salmonella* fall into one of these groups. Once agglutination has been observed in a group, the individual O antigens represented in that group are tested. If no agglutination occurs in one of these groups, the lab should then proceed to test antisera for groups O:11 through 67 (higher groups). Antisera pools that detect multiple O groups are commonly used to reduce the number of reagents tested. When an isolate is positive in a specific pool, it can then be tested with the individual components of that pool.

Determination of H antigens occurs similarly to O antigens in that first pooled H antisera are tested. Once a pool has been found to be positive by tube or slide agglutination, the individual factors represented in that pool are tested. Often times a *Salmonella* will have 2 H antigens but only express one at a time. In this case, the organism must be passed through semisolid agar containing antibodies to the known antigen to select for cells expressing the other antigen. This process is known as a phase reversal. In order to achieve this, the organism must be actively motile. It can take several repeated passes to reverse the organism.

Cost

Traditional *Salmonella* serotyping can be fairly inexpensive though this is dependent on a number of factors. The costs associated with traditional serotyping can be broken down into three main categories- consumables, antisera, and hands-on time. A sample cost analysis worksheet tool itemizing costs is attached as Appendix A. The average cost for consumables is estimated at approximately $8.22/ isolate. The average cost for antisera is estimated at $8/isolate with a range of $6–$30 depending on the source of the antisera. The average estimated hands-on time required for a single isolate is one hour.

Advantages

The cost for identifying common serotypes is fairly inexpensive. Additionally, there is no need to batch isolates in order to keep costs down. The time required to manually serotype can be fairly quick with some isolates being completed in a matter of minutes. In theory, an experienced microbiologist with a fully array of antisera can determine the serotype of any *Salmonella* isolate.

Disadvantages

Traditional *Salmonella* serotyping can also be labor intensive and time-consuming depending on the isolate being processed. Some isolates require several passes through semi-solid media to enhance motility and flagellar antigenic expression. Also, some isolates do not express serotype antigens thus limiting the utility of traditional serotyping. Delayed serotyping results of such isolates can be problematic for detecting and investigating clusters of illness due to salmonellosis. With traditional serotyping, isolates can be in various stages of the typing process leading to confusion on the bench. Manual serotyping requires a number of tubes and slides to complete the whole process which can take up a lot of room in a laboratory. Maintaining all of the necessary reagents can be cumbersome and quality control can be difficult when managing a full array of antisera. As antisera stocks run out, CDC will eventually discontinue providing antisera leaving public health laboratories with the task of finding and purchasing high-quality antisera which could prove challenging.

[1]Information for this section was pulled from the Manual of Clinical Microbiology, 10th Edition (9)

IV. Molecular *Salmonella* Serotyping

Brief Description

To avoid the difficulties of traditional serotyping, a molecular assay was developed on the Luminex platform for serotype determination based on DNA markers within genes responsible for O and H antigen expression. The Luminex technology has been licensed to several different companies. Currently the Luminex 100 and 200 instruments from Luminex (www.Luminex.com) and the BioPlex™ instrument from Bio-Rad (www.bio-rad.com) are two Luminex platforms in popular use.

The Luminex xMAP technology allows detection of nucleic acids by combining PCR with a multiplexed detection system. The format is a suspension array that utilizes a flow cytometry-like instrument with two lasers; a classification laser and a reporter laser. Analyte-specific probes are covalently linked to one of 100 different fluorescently-labeled microspheres (bead) sets. Each of 100 bead sets are internally labeled with a different proportion of red and infrared fluorophores, resulting in 100 "different colored" bead sets. When excited by the classification laser, each bead set fluoresces differently and the bead set is recognized by its unique spectral address. When labeled with a specific probe, that spectral address defines the probe that is covalently bound to the bead's surface. Because the bead sets can be distinguished by their unique spectral address, they can be combined, allowing up to 100 different targets to be detected in a single reaction. Biotinylated PCR products amplified from DNA region(s) of interest are hybridized to bead sets that have been covalently labeled with antigen-specific probes. The spectral address of each bead plus any bound PCR fragment is then detected in the flow cytometer.

The molecular determination of *Salmonella* serotypes, or "molecular serotyping," is currently accomplished using a series of four BioPlex assays: i) the O-grp-1 assay detects the six most common O groups of *Salmonella* and serovar Paratphi A; ii) the O-grp-2 assay detects five additional O groups; iii) the H-ag assay detects the five recognized H antigen complexes, secondary antigens within the complexes, and additional H antigens; iv) the AT assay detects targets that assist with the identification of serovars Enteritidis (sdf) and Typhi (viaB), and a conserved region of fljB that serves as a control for the presence of this gene. Molecular determination of serotype consists of three main steps: i) Preparation of *Salmonella* template DNA; ii) Amplification of PCR fragments from the target regions within the fliC, fljB, the rfb region, sdf, and viaB; iii) Identification of the antigen that is encoded by the PCR fragment by hybridizing with antigen specific-DNA probes.

The assay is currently being produced for sale by Luminex Inc. as a kit including only the O-grp-1, H-ag, and AT assays. An additional O-grp-2 assay standalone kit is currently in the production process. The following cost analysis is estimated based on the current Luminex production kit including the O-grp-1, H-ag, and AT assays.

Cost

The costs associated with the molecular assay can be broken down commensurate with the three main steps. CDC estimates the reagent cost of the DNA extraction of one isolate is $0.12. The reagent cost of three PCR reactions for one isolate is estimated to be $2.62. The reagent cost of three hybridization reactions for one isolate is estimated to be $10.84. The total estimated reagent cost to perform the Luminex Molecular Serotyping Assay on one isolate is $13.58. The addition of the O2 assay would add minimal cost per isolate if the O2 assay is only performed on O1 negative samples. It should also be noted that after initial instrument purchase a maintenance agreement is recommended to ensure proper instrument operation. A typical yearly maintenance agreement is estimated to cost $8,000. A sample cost analysis worksheet tool itemizing costs is attached as Appendix A.

Advantages

The *Salmonella* Molecular Serotyping Assay largely circumvents the problems of antisera production and quality control. Basing the molecular technology on the genes responsible for serotype antigens provides a correlation between serotype determined by traditional and molecular methods. This methodology preserves the integrity and value of decades of past surveillance data. It also provides serotype information for many isolates which are identified as rough, mucoid, and nonmotile through traditional serotyping.

The Luminex system is multi-purposed and already present in many laboratories for detection of other agents of public health concern. The high throughput of the Luminex system allows 30 molecular serotypes to be performed on a single 96 well plate in under five hours total time with approximately 2 hours of technician hands on time. One trained technician is estimated to have the capacity to perform 90–120 molecular serotypes in a single work shift.

Disadvantages

The current *Salmonella* Molecular Serotyping Assay does not detect all serotype antigens; it focuses primarily on the most common serotypes reported for human clinical specimens. A list of the 100 most common clinical serotypes of *Salmonella* representing 98% of all clinical isolates seen by the public health surveillance system and their characterization by the molecular assay can be found in Appendix B. The assay should fully characterize approximately 90% of all clinical isolates seen by the public health surveillance system, though this number may be lower for regions where atypical serotypes are common. It provides partial serotype information for most other isolates. Since the assay does not fully identify all serotypes, a reserve supply of some specific traditional antisera as well as the expertise to properly use and interpret traditional serotyping methods is still required. For those sites who are not set up for molecular serotyping, the investment in new equipment may not be cost effective with next generation sequencing methods being implemented in the near future. Additionally, the Luminex system is better suited for high-throughput labs. Those sites that receive fewer isolates may not be able to justify the larger setup for a handful of isolates per week.

V. Considerations for Both Traditional and Molecular *Salmonella* Serotyping Methods

Public health laboratories weighing their serotyping options should consider the needs and the characteristics of their laboratory. The number of *Salmonella* isolates a public health laboratory receives will impact molecular serotyping test cost; also, turnaround time may be reduced if molecular serotyping is batched. Sending isolates to another institution for serotyping may require a memorandum of understanding (MOU) or contract between the institutions and will incur transport cost. The speed necessary to attain a serotype may be different between laboratories depending on the *Salmonella* workflow for each laboratory. For instance, if PFGE is performed concurrent to serotyping, timely serotyping results may not be as crucial as PFGE can be used to monitor for clusters. Both molecular and traditional serotyping requires significant expertise. There are currently four options for serotyping *Salmonella* at public health laboratories:

1. **Perform traditional serotyping**. This option is slower than molecular serotyping but cheaper, requires little equipment and nearly always is effective at identifying a serotype.
2. **Perform molecular serotyping followed by traditional serotyping on isolates that are not fully serotyped by molecular methods**. This option ensures that each isolate is able to be fully serotyped but also requires expertise of traditional serotyping.
3. **Perform molecular serotyping followed by sending isolates not fully serotyped by molecular methods to another laboratory**. This option ensures that each isolate is fully serotyped; however this requires collaboration between public health laboratories and incurs costs for shipping.
4. **Send out all *Salmonella* isolates to another public health laboratory for serotyping**. This option ensures that all *Salmonella* are serotyped however it will increase the amount of time until serotyping is complete, will incur shipping costs, and requires agreements with another public health laboratory to perform the serotyping. Costs would be affected by the serotyping methods used by the testing laboratory.

Table 1 provides a comparison of the various serotyping methods with regard to some key factors.

Table 1. Comparison of *Salmonella* serotyping options

Factors	Conventional	Molecular/ traditional	Molecular/ Send Out	Send Out
Cost	$	$$	$$$	$$
Time to result	Slow to fast	Fast*	Fast*	Slow
Equipment	Little	Luminex	Luminex	None

**Results can be attained quickly for most serotypes however some serotypes require additional information to complete serotyping*

VI. Incorporating PFGE into *Salmonella* Serotyping Workflow

Pulsed-field gel electrophoresis (PFGE) provides further subtype information which has enhanced epidemiological surveillance studies and outbreak detection and investigations. This method has proven critical for detecting outbreaks of Salmonellosis, and with careful validation and use of conservative algorithms, can be used to predict many *Salmonella* serotypes, although this is not a recommended method of *Salmonella* serotyping.

It is recommended that all laboratories PulseNet-certified for *Salmonella* analysis, subtype all *Salmonella* isolates by Pulsed Field Gel Electrophoresis (PFGE) and upload the resulting band patterns to the PulseNet national database in real time (within four working days of receipt in the PulseNet Laboratory). Laboratories should also make every effort to conduct serotyping and PFGE simultaneously.

VII. The Future of *Salmonella* Serotyping

Advances in molecular subtyping and characterization have led the way for better diagnostics and understanding of the epidemiology and biology of *Salmonella*. Both multi-locus variable — number tandem repeat (VNTR) analysis (MLVA) and multi-locus sequence typing (MLST) have been demonstrated to be suitable typing methods for *Salmonella*; however, need for the development of serotype specific protocols and lack of discriminatory power when compared with PFGE have been limitations in adoption of these methods for a nationwide laboratory-based surveillance network.

Next generation sequencing methods appear to be a promising alternative for identification and characterization of bacterial isolates. Rapid advances in the technology have paved the way for public health laboratories to consider whole genome sequencing as an alternative subtyping method due to the decreasing costs in sequencing technologies for entire genomes. The ability to generate whole genome sequencing (WGS) data on pathogens can allow for rapid serotype and strain identification, virulence characterization and identification of antimicrobial resistance markers which has the ability to radically transform reference activities for public health laboratories. Use of WGS technology has been demonstrated in epidemiological studies and outbreak detection for *Salmonella* and other enteric pathogens ([10], [11], [12], [13]). Routine use of this technology for routine public health surveillance of *Salmonella*, including serotype determination, is estimated to be in place within a few years.

VIII. Summary

Traditional and molecular *Salmonella* serotyping methods will continue to sustain public health surveillance systems until next generation sequencing (NGS) methods have been implemented and are well-established. NGS technologies will offer greater discrimination with higher automation and throughput. Because NGS technologies provide phylogenetic data, microbiologists and bioinformaticists will have to work closely with their colleagues in epidemiology to set up new information pipelines and redefine many aspects of surveillance such as disease clusters. APHL will continue to work closely with CDC to develop an interim model for serotyping sustainability over the next three to five years until NGS technologies are implemented and well-established.

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Association of Public Health Laboratories

The Association of Public Health Laboratories (APHL) is a national nonprofit dedicated to working with members to strengthen laboratories with a public health mandate. By promoting effective programs and public policy, APHL strives to provide public health laboratories with the resources and infrastructure needed to protect the health of US residents and to prevent and control disease globally.

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