



## WHO Global Foodborne Infections Network

(formerly WHO Global Salm-Surv)

"A WHO network building capacity to detect, control and prevent foodborne and other enteric infections from farm to table"

# Laboratory Protocol: "Serotyping of *Shigella* spp."

van der Ploeg, Claudia A. (INPB)  
Viñas, María R. (INEI)  
Terragno, Raquel (INEI)  
Bruno, Susana B. (INPB)  
Binsztein, Norma (INEI)

Instituto Nacional de Enfermedades Infecciosas (INEI)  
Instituto Nacional de Producción de Biológicos (INPB)  
ANLIS – "Dr. Carlos G. Malbrán"- Argentina

With acknowledgments for significant technical and editorial contributions to the:  
WHO Global Foodborne Infections Network Laboratory Sub-Committee  
&

The U.S. Centers for Disease Control and Prevention's National *Escherichia* and *Shigella* Reference Laboratory

<b>Title: Laboratory protocol: Serotyping of <i>Shigella</i> spp</b>	
<b>Protocol Number: 2010GFNLAB003</b>	<b>Effective Date: 30-06-2010</b>
	<b>Revision Number:</b>

**REVISION HISTORY**

<b>HISTORY OF CHANGES</b>				
<b>Rev. Level</b>	<b>Sections Changed</b>	<b>Description of Change (From—To)</b>	<b>Date</b>	<b>Approval</b>
0				
1				

## I. PURPOSE

A standardized process for the serotyping of *Shigella* spp.

## II. TEST PRINCIPLES & BACKGROUND

Recent phylogenetic studies indicate that the shigellae are not a unique genus; rather they are an immunologic and biochemically distinct lineage of *Escherichia coli*. However, for medical purposes and to maintain historical consistency, the shigellae have not been merged with *E. coli*.

The genus *Shigella* is comprised of four species: *Shigella dysenteriae* (also referred to Group A), *Shigella flexneri* (also referred to Group B), *Shigella boydii* (also referred to Group C), & *Shigella sonnei* (also referred to Group D). Included among these organisms, is the etiologic agent of epidemic dysentery, *S. dysenteriae* serotype 1. With the exception of *S. sonnei*, each species may be further divided into serotypes on the basis of reactivity with hyperimmune serum: *S. dysenteriae* (15 serotypes), *S. flexneri* (6 serotypes and 2 variants), & *S. boydii* (20 serotypes).

*Shigella* group and serotype are identified by slide agglutination using antisera. Both polyvalent and monovalent *Shigella* antisera are available. Polyvalent antisera, is used to determine group and contains antibodies to multiple serotypes of *Shigella*. For example, most commercially available polyvalent antisera for *Shigella flexneri* will react with all serotypes of *S. flexneri*. Monovalent antisera, is used to determine serotype. For example, *S. dysenteriae* Type 1 antiserum will only react with isolates of *S. dysenteriae* Type 1.

A final, confirmatory, identification of *Shigella* spp. is based on immunologic and biochemical profile (Appendix A: Tables 1, 2 & 3). Serotyping results must be interpreted in the context of biochemical data and the serologic and biochemical identification must be consistent prior to reporting. Inconsistent / discordant results must be confirmed by a reference centre. Additional background information on *Shigella* is provided in Appendix A.

## III. RESPONSIBILITIES

### III.1 Staff Responsibilities

*This is a general overview. Each institution must specify what responsibility their staff members will have in the execution of this protocol. Please refer to applicable manuals within the facility/locality for complete set of responsibilities to properly conduct this procedure.*

### III.2 Specific Safety Requirements and Responsibilities

Universal biosafety precautions must always be employed when processing any sample of human origin.

Specific regulations will vary by country and may change over time. The reader is advised to consult local authorities.

Biosafety level 2 (BSL-2 / RG-2) practices and procedures must be utilized when working clinical isolates of unknown *Enterobacteriaceae*, non-typhoidal *Salmonellae*, and *Shigella* spp.

Biosafety level 3 (BSL-3/ RG-3) practices and procedures should be utilized when performing procedures likely to generate aerosols of *S. dysenteriae* Type 1 or invasive serovars of *Salmonella* spp.

Organism specific information may be obtained at:

<http://www.phac-aspc.gc.ca/msds-ftss/>

Additional safety information may be obtained at:

<http://www.cdc.gov/OD/ohs/biosfty/bmbl5/bmbl5toc.htm>

<http://www.hse.gov.uk/biosafety/biologagents.pdf>

The laboratory director is responsible for insuring staff compliance with all safety requirements. All personnel shall be instructed to report any unsafe conditions to their supervisor or laboratory safety officer.

For detailed, institution specific safety requirements and responsibilities, the reader should refer to facility specific safety manuals.

#### **IV. SAMPLE COLLECTION/TRANSPORT/STORAGE**

Only pure cultures should be used for serotyping. Serotyping results must be interpreted in conjunction with biochemical testing. Biochemical testing may be performed prior to serotyping or biochemical testing and serotyping may be performed simultaneously; however, a confirmatory identification cannot be made until BOTH biochemical and serological testing is complete.

**NOTE: All testing must be performed using pure cultures. To insure accurate results, it is essential to select a single, well isolated colony for biochemical testing (“single colony pick”) and serotyping. Using a single, isolated colony helps to insure that the media will be inoculated with a pure culture of a single bacterial organism. If you are uncertain about the purity of your single colony pick, you should transfer the colony to a selective agar (e.g. MAC) to verify purity and then continue with biochemical and serotyping tests**

For additional details describing the identification of *Shigella* spp., please refer to the WHO GFN Laboratory Protocol: “Biochemical Identification of Salmonella/Shigella Using an Abbreviated Panel of Tests”.

For short term storage and for transport to the laboratory, *Shigella* cultures may be stored in semi-solid agar at room temperature. For long term preservation and to best maintain isolates for future studies (i.e. PFGE), isolates should be frozen at -70°C in a cryopreservation medium (see appendix E).

Sample Collection/Transport/Storage must be recorded according internal procedures.

#### **V. MATERIALS/SUPPLIES**

##### **V.I- Materials**

- disposable (or wire) microbiological loops
- glass slides (approx. 10 x 15 cm)
- micropipettes and tips
- pasteur pipettes and pipette bulbs
- Khan tubes
- timer
- electrical Burner (if wire loops are utilized)
- lamp
- wax pencils

##### **V.II- Supplies**

###### **Media**

- tryptic soy agar (TSA)
- tryptic soy broth (TSB)

- blood agar

**Note:** See Appendix F for the formula of individual media.

### Reagents

- physiological saline solution (NaCl 0.85% w/v)
- 0.5 % formalinized physiological saline solution (0.85 w/v NaCl – 0.5 % formalin v/v)
- *Shigella* O polyvalent antisera
- *Shigella* O monovalent antisera

**Note:** See Appendix F for preparation of solutions.

### Reference strains

According to the antisera to be tested.

## VI. EQUIPMENT

- incubator 36°C (+/- 1°C)
- refrigerator 4 (+/- 1°C)

**Note:** For information related to maintenance, troubleshooting and calibration of applicable equipment, refer to internal manuals, SOPs and/or operating manuals.

## VII. QUALITY ASSURANCE / QUALITY CONTROL (QA/QC)

**Note:** *The following are general QA/QC guidelines. QA/QC requirements may vary based on: institutional policy, national regulations, or accreditation agency (example: ISO or CAP). It is important to consult your institutional QA/QC manual and appropriate regulatory guidelines prior to utilizing this protocol.*

- Each new lot and/or shipment of antisera is tested for positive and negative reactivity using known control strains. Antisera are tested when first used (or first diluted). Routinely used antisera should be quality control tested every six months. Antisera which are less commonly used may be quality control tested prior to use. The quality control test results are recorded on a data sheet (see Appendix C).
- Most of the media required and listed above are commercially available and should be prepared, controlled and sterilized according to the manufacturer's instructions.
  - If QC of media or sera does not meet expected results, technicians must report the anomaly to their supervisor and the lot should be rejected.
  - Laboratory technician(s) performing the tests should be properly trained in this SOP, in QA and H&S (health and security) aspects and in microbiological techniques. This training should be recorded.

Antisera should be checked for reactivity and should produce 3+ or 4+ agglutination when tested against its homologous antigen. No reactivity should be observed with tested with heterologous antigens.

## VIII. PROCEDURE

### VIII.1- Initial culture

Inoculate a Tryptic Soy Agar (TSA) slant (or other non-selective agar) with a single, well isolated, colony of *Shigella* spp. Incubate the slant at 36°C (+/- 1°C) for 18-24 hours.

**Note:** is advisable to keep the original culture (selective plate or transport slant) until the characterization is finished.

### **VIII.2- Bacterial suspension**

Procedure note:

Conventional serotyping of *Shigella* spp. is based on an antigen / antibody reaction. The isolate (antigen) is mixed with antisera of known specificities; the pattern of reactivity observed determines subgroup and serotype.

Two methods for preparing the bacterial antigen suspension are given below. Many operators find "Option A" faster and simpler to perform; whereas "Option B" may provide a more standardized antigen suspension.

It is important to note that antisera producers typically will recommend a method for preparing a bacterial antigen suspension. When specific guidance is provided by the manufacturer; it is important to follow those recommendations.

#### **VIII.2 Option (A):**

Draw a circle approximately 2cm in diameter on to a clean glass slide.

Add 20ul of physiological solution to center of the circle.

Collect a small amount of bacteria (1uL loop) from the TSA slant and suspend the bacteria in the saline.

**Note:** for carrying out each agglutination reaction, a new suspension should be prepared.

#### **VIII.2 Option (B):**

Using a sterile loop, thoroughly suspend the totality of the bacterial growth on the TSA slant with 0.5 ml of 0.5% formalinized physiological saline solution, mix to insure that there are no visible clumps or particles and then transfer the suspension into a Khan tube.

**Note:** all the agglutinations tests can be done with this working suspension.

### **VIII.3- Auto-agglutination control (negative control)**

#### **VIII.3.1-**

Draw a circle approximately 2cm in diameter on to a clean glass slide.

Place 20µl of antigen suspension and 20µl of physiological solution into the circle and mix thoroughly with the microbiological loop or stick.

#### **VIII.3.2-**

Gently tilt the slide back and forth for 1 minute.

Observe for agglutination under indirect light.

**VIII.3.3-** If no agglutination is observed proceed to subgroup / serotype determination. If agglutination is observed; the isolate is rough, refer to "Limitations of Procedure" and do not attempt serotyping.

### **VIII.4- Group/serotype determination**

#### **VIII.4.1-**

Draw a circle approximately 2cm in diameter on to a clean glass slide.

Place 20µl of antigen suspension and 20µl of antiserum into the circle and mix thoroughly with the microbiological loop or stick.

**Procedure Note:**

Serologic identification of *Shigella* begins with the use of polyvalent antisera. Polyvalent antisera is used to identify the species (i.e. *S. dysenteriae*, *S. boydii*, *S. flexneri*, or *S. sonnei*). If agglutination is observed with polyvalent antisera, the isolate is then tested with the individual monospecific serum found in the polyvalent antisera.

Isolates should be initially tested with polyvalent antisera that covers the species / serotypes which are most common in the region.

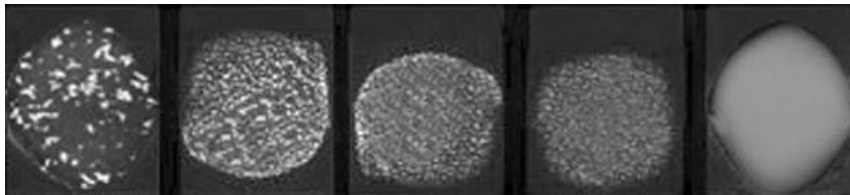
**VIII.4.2-** Holding the slide against a dark background, tilt the slide gently back and forth for up to 1 minute observing for agglutination. **Reactions developing after 1 minute are non-specific.**

**VIII.4.3-** Record the result on the datasheet (see Appendix B).

An homogenous suspension is a negative reaction. Appearance of clumps means a positive reaction.

Score and record the results as follows:

4+	All organisms are clumped and the supernatant, or suspending fluid is clear
3+	About 75% agglutination, the supernatant fluid is slightly cloudy
2+	About 50% agglutination, the supernatant fluid is moderately cloudy
1+	About 25% agglutination, the supernatant fluid is cloudy
Tr	Trace amount of agglutination present
Negative	No agglutination apparent and suspension remains homogenous.



**4+                      3+                      2+                      1+                      Negative**

**VIII.4.4-** If agglutination is observed with one of the polyvalent antisera, the serotype of the isolate is determined by testing the isolate with monovalent antisera specific to the serotypes / sub-factors found in the polyvalent sera.

**VIII.5- Presence of capsular antigens**

In the presence of capsular antigens, heat the bacterial suspension at 100°C in water bath during 45 min. and repeat the procedure (see also point X.2).

**VIII.6- Considerations**

**VIII.6.1-** All the antisera must be used according to the manufacturer's instructions.

**VIII.6.2-** For future studies it is important to preserve the initial culture for short periods (i.e. by inoculating semisolid agar) and /or long periods (i.e. by cryopreservation at -70°C). (For details see Appendix E).

## IX. INTERPRETATION OF RESULTS

**IX.1-** Identification of *Shigella* is based on serologic and phenotypic criteria. As such, serotyping results must be interpreted in conjunction with biochemical test results (Appendix A: Tables 1, 2 & 3).

**IX.2-** All isolates are initially tested with polyvalent antisera, if agglutination with polyvalent antisera is observed, serotype is identified by testing with monovalent antisera. If monovalent antisera is unavailable, agglutination with polyvalent antisera and biochemical profile may be used to obtain a species level identification.

**IX.3-** Agglutination with polyvalent and monovalent antisera is required for serotype level identification.

**IX.4-** Serotype level identification of *S. flexneri* requires the detection of two antigens: “type” antigens and “group” antigens. These antigens are identified using monovalent antisera and identified as follows:

Serotype	Type antigen	Group antigen
1a	I	4
1b	I	4; 6
2a	II	3,4
2b	II	7,8
3a	III	(3,4); 6; 7,8
3b	III	(3,4); 6
4a	IV	3,4
4b	IV	6
4c*	IV	7,8
5a	V	3,4
5b	V	7,8
6	VI	(4)
Variant X	--	7,8
Variant Y	--	3,4

Symbols in parentheses indicate variably expressed antigens.

\*The subserotype 4c was proposed to the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Enterobacteriaceae, for its incorporation in the *Shigella flexneri* scheme (Pryamukhina NS, Khomenko NA -1988); however this subserotype is not been officially recognized.

## X. LIMITATIONS OF PROCEDURE

### X.1- Rough strains:

Serotyping should not be attempted on strains which autoagglutinate saline. Rough isolates seldom revert to smooth forms. However, if autoagglutination in saline is observed, an attempt to recover smooth colonies may be made by performing 2-4 serial sub-cultures on enriched media, such as blood agar or Mueller Hinton agar.



## **X.2- Capsular antigens:**

Occasionally, the presence of capsular antigens may prevent some isolates of *Shigella* spp. from reacting with polyvalent antisera. The presence of capsular antigens should be considered when isolates which are biochemically typical of *Shigella* spp., fail to agglutinate (or agglutinate poorly) with *Shigella* polyvalent antisera. In these situations, an attempt should be made to remove the capsular antigen (by boiling); as discussed in step VIII.5.

**Note:** the capsular antigens are thermolabile.

**X.3-** When conflicting results are observed (example: biochemical profile disagrees with serological profile or epidemiology); three (3) to five (5) additional, well isolated, colonies should be taken from the original plate and tested.

**X.4.-** Due to the presence of conserved antigens, *S. sonnei* polyvalent antisera can produce cross-reactions with some cultures of *S. boydii* type 6. In these situations, biochemical profile and the use of *Shigella boydii* type 6 monovalent antiserum will be necessary to confirm the identification.

## **XI. REPORTING**

A final, confirmatory, identification of *Shigella* spp. is based on immunologic and biochemical profile. Serotyping results must be interpreted in the context of biochemical data and the serologic and biochemical identification must be consistent prior to reporting. Inconsistent / discordant results must be confirmed by a reference centre.

If biochemical and serological results are in agreement, the isolate may be reported to the extent it was characterized. For example:

If an isolate was serotyped as *S. dysenteriae* Type 1 and is biochemically consistent with *S. dysenteriae* Type 1, it may be reported as: *Shigella dysenteriae* Type 1.

If an isolate was reactive with *S. boydii* polyvalent antiserum and is biochemically consistent with *S. boydii*, but not further serotyping was performed, the isolate may be reported as: *Shigella boydii*.

If smooth colonies cannot be recovered from a rough culture, the organism cannot be serotyped. The isolate should be biochemically identified and reported as:

“Biochemically consistent with *Shigella* (species)

Comment: This isolate was rough, as such serotyping could not be performed.”

Each institution must establish its own policy regarding how, where, and to whom results are reported.

Policies should also be established to facilitate the expedited reporting of critical results. Critical results may include:

Identification of *Shigella dysenteriae* Type 1 (etiologic agent of epidemic dysentery).

Unusual or sudden increase in the number of isolates identified.

Unusual or sudden increase in a particular subgroup (or serotype).

## XII. REFERENCES

**Allison, G., Verma, N.** -2000-. Serotype-converting bacteriophages and O-antigen modification in *Shigella flexneri*; Trends in Microbiology, 8, (17-23).

**Ansaruzzaman, M., Kibriya, A., Rahman, A., Neogi, P., Faruque, A., Rowe, B., Albert. M.** - 1995 - Detection of provisional serovars of *Shigella dysenteriae* and designation as *S. dysenteriae* serotypes 14 and 15. J. Clin. Microbiol., 33 (1423-1425)

**Bopp, Ch., Brenner, F., Wells, J., Strockbine N.** -1999- Manual of Clinical Microbiology. Chapter 28.

**Brenner, D.J.** -1984- Recommendation on recent proposals for the classification of shigellae. Int. J. Syst. Bacteriol, 34, (87-88).

**Brenner, D.J., Krieg, N., Staley, J.** Bergey's Manual of Systematic Bacteriology 2<sup>nd</sup> Edition, Vol. 2, Part B. 2005,

**Ewing, W., Edwards, P..** -1986-. Identification of *Enterobacteriaceae*. 4<sup>th</sup> edition. Elsevier Science Publishers.

**Andrews, W.H., Jacobson, A.** -1995- FDA Bacteriological Analytical Manual. 8<sup>th</sup> edition, Chapter 6.

**Food Directorate's (Health Canada's) website at [www.hc-sc.gc.ca](http://www.hc-sc.gc.ca)**

[www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp25-eng.php](http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp25-eng.php)

[www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp26-eng.php](http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp26-eng.php)

**Gemski, P., Koeltzow, D. E., Formal, S. B.** -1975-. Phage conversion of *Shigella flexneri* group antigens. Infection and Immunity, 11, (685-691).

**Gray, L. D.** -1995- Manual of Clinical Microbiology. Chapter 33.

**Houng, H., Sethabutr, O. and Echeverría, P.** -1997- A simple polymerase chain reaction technique to detect and differentiate *Shigella* and enteroinvasive *Escherichia coli* in human feces. Diagn. Microbiol. Infect. Dis., 28, (19-25).

**Kaisar A., et al.** -2002- Phenotypic and Genotypic Characterization of serologically atypical strains of *Shigella flexneri* type 4 isolated in Dhaka, Bangladesh. J. Clin. Microbiol, 40, (2490-2497).

**Le Minor, L., Richard, C.** -1993- Méthodes de laboratoire pour l'identification des Entérobactéries. Institut Pasteur.

**Liu, B., Knirel, Y. and als.** -2008- Structure and genetics of *Shigella* O antigens. FEMS Microbiol. Rev., 32, (627-653).

**Mikoleit, M. L.** -2009- **Laboratory Protocol: "Biochemical identification of *Salmonella* spp. and *Shigella* spp."** Global Salm Surv.

**Parsot, C. and Sansonetti, P.** Pathogenicity islands and the other mobile virulence elements Chapter 8 (ed. J Kaper, J Hacker).

**Popoff, M.** -2001- Guidelines for the preparation of *Salmonella* Antisera, 6<sup>th</sup> Ed., WHO Collaborating Centre for Reference and Research on *Salmonella* Pasteur Institute.

**Post, D.E.** -1998- Food-Borne Pathogens. Oxoid Monograph Number 5: *Escherichia coli*, *Shigella* species.

**Pryamukhina NS, Khomenko NA.** -1988- Suggestion to supplement *Shigella flexneri* classification scheme with the subserovar *Shigella flexneri* 4c: phenotypic characteristics of strains. J. Clin. Microbiol., 26 (1147-1149).

**Radnedge, L. et al.** -1997- Plasmid maintenance functions of the large virulence plasmid of *Shigella flexneri*. The Journal of Bacteriology, 179, (3670-3675).

**Simmons, D. A. R.** -1971- Immunochemistry of *Shigella flexneri* O-Antigens: a study of structural and genetic aspects of the biosynthesis of cell-surface antigens. Bacteriological Reviews, 35, (117-148).

**Simmons, D. A. R., Romanowska, E.** -1987- Structure and biology of *Shigella flexneri* O-antigens. J. Med. Microbiol., 23, (289-302).

**Woodward, D., Clark, C., Caldeira, R., Ahmed, R, Soule, G., Bryden, L., Tabor, H., Melito, P., Foster, R., Walsh, J., Ng, L., Malcolm, G., Strockbine, N., Rodgers, F.** -2005- Identification and characterization of *Shigella boydii* 20 serovar nov., a new and emerging *Shigella* serotype. J. Clin. Microbiol., 54 (741-748).

## **XIII ATTACHMENTS**

**XIII.1-** Appendix A: Background information

**XIII.2-** Appendix B: *Shigella* serotyping worksheet

**XIII.3-** Appendix C: *Shigella* antisera quality control record data sheet.

**XIII.4-** Appendix D: Flowchart for *Shigella* spp. serotyping.

**XIII.5-** Appendix E: Storage of *Shigella* spp. cultures.

**XIII.6-** Appendix F: Preparation of culture media and solutions.

## APPENDIX A

### BACKGROUND INFORMATION

- **Introduction**

The genus *Shigella* belongs to the family *Enterobacteriaceae*. The *Shigella* are Gram-negative rods, 0.3 to 1 µm in diameter and 1 to 6 µm in length, appearing singly, in pairs and in chains, are non-sporeforming, facultatively anaerobic, oxidase negative, and ferment glucose and other carbohydrates without producing gas. By definition, all *Shigella spp.* are nonmotile and lysine decarboxylase negative. Additionally, the *Shigella* are Voges-Proskauer negative and methyl-red positive, do not utilize Simmons citrate, nor produce H<sub>2</sub>S and are arginine dihydrolase and urease negative. The shigellae, with the exception of *S. dysenteriae* Type 1, are catalase positive. The optimum temperature of growth is 37°C.

The genus *Shigella* is divided into four species: *Shigella dysenteriae* (Group A), *Shigella flexneri* (Group B), *Shigella boydii* (Group C), and *Shigella sonnei* (Group D). Each of these species, with the exception of *S. sonnei*, has several serotypes based on the reactivity with hyperimmune serum. The proportion of each species varies from country to country and from region to region. The hallmark of infection with *Shigella* is diarrhea with blood, often termed “dysentery.” However, in most cases, *Shigella spp.* causes acute non-bloody diarrhea that cannot be distinguished clinically from diarrhea caused by other enteric pathogens. *S. dysenteriae* 1, the etiologic agent of epidemic dysentery, differs from other *Shigella* in four important characteristics: produces a potent cytotoxin (Shiga toxin); causes illness that is more severe, more prolonged, and more frequently fatal than illness caused by other *Shigella*; resistance to antimicrobials occurs more frequently than in other *Shigella*; and it causes large, often regional, epidemics, frequently with high attack rates and high fatality rates.

The genetic information for *Shigella* pathogenesis is codified at both the chromosomal and plasmid level. These microorganisms have a megaplasmid of invasion (220kb) and synthesizes Type-III secretion system that allows the insertion of a pore through which the effector proteins are distributed in the gastrointestinal eucariotic cell.

*Shigella* invades the colonic epithelium causing patchy destruction, which leads to the formation of micro-ulcers and inflammatory exudates, with the subsequent appearance of inflammatory cells (polymorphonuclear leucocytes) and blood in the stool.

The only reservoirs for *Shigella* are humans and primates. Transmission occurs primarily through direct or indirect fecal-oral contact with patients or carriers. Fecally contaminated water and foods also transmit the disease; contamination of foods can also occur by infected food handlers.

*Shigella* has a low infectious dose which facilitates person to-person spread, the ingestion of 10 to 100 cells typically produces disease. The severity of the illness depends on the age and the nutritional condition of the person and it is also dose-related.

A definitive diagnosis of *Shigella* infection can only be made by isolating and identifying the organism from a stool culture. The isolate is also required to determine the antimicrobial sensitivity and additional subtyping. Sensitive molecular techniques to detect *Shigella*, such as PCR, have been developed and they are practical and rapid screening techniques for monitoring food samples (“Isolation and identification of *Shigella spp.* from foods”, “Detection of *Shigella spp.* in foods by the polymerase chain reaction”, published on the Food Directorate’s (Health Canada’s) website at [www.hc-sc.gc.ca](http://www.hc-sc.gc.ca)). There are others PCR protocols that have been developed for the analysis of human samples for identification of *Shigella* genus and species. It must be considered that the *ipaH* gene used as target for *Shigella* in these PCR protocols is also present in enteroinvasive *Escherichia coli* (EIEC), therefore it is necessary to perform differential testing to complete the characterization (Houng, H, Sethabutr, O. and Echeverria; P. – 1997).

- **Biochemical identification of *Shigella* spp.**

The biochemical properties common to the genera *Shigella* are indicated in Table 1.

TABLE 1. Biochemical reactions of *Shigella* spp.

Test or Substrate	<i>Shigella</i> spp. (% positive)
Hydrogen sulfide	- (0)
Glucose (gas)	- (2)
Glucose (acid)	+ (100)
Motility	- (0)
Simmons' citrate	- (0)
Adonitol fermentation	- (0)
Malonate utilization	- (0)
Methyl-red	+ (100)
Voges-Proskauer	- (0)
Arginine dihydrolase	- (5)
Lysine decarboxylase	- (0)
Ornithine decarboxylase	<i>Sh. sonnei</i> + (100) ; other <i>Shigella</i> – (0)
Urea hydrolysis	-
Phenylalanine deaminase	-
Salicin fermentation	-
Lactose fermentation	-*
Sucrose fermentation	-
Xylose fermentation	-
Inositol fermentation	-
Catalase	+ (all species and serotypes except <i>S. dysenteriae</i> 1)

\**Shigella* spp. will typically be lactose negative following overnight incubation (18-24 hours).

However, it is not uncommon for *Shigella* spp. to ferment lactose following prolonged incubation (5-7 days).

TABLE 2. Biochemical properties of *S. dysenteriae* & *S. flexneri*

Group, serotype	Indol Production	Ornithine Decarboxylase	Mannitol fermentation	Xylose fermentation	ONPG
<b><i>S. dysenteriae</i> (Group A)</b>					
1	0%	0%	0%	0%	100%
2	100%	0%	0%	0%	2%
3	0%	0%	0%	0%	25%
4	0%	0%	0%	0%	70%
5	0%	0%	0%	0%	0%
6	0%	0%	0%	0%	71%
7	100%	0%	0%	0%	85%
8	100%	0%	0%	76%	0%
9	0%	0%	0%	0%	50%
10	0%	0%	0%	100%	0%
11	0%	0%	0%	0%	63%
12	0%	0%	0%	0%	54%
13	0%	0%	0%	0%	0%
14	0%	0%	0%	0%	0%
15	0%	0%	0%	0%	0%
<b><i>S. flexneri</i> (Group B)</b>					
1a [I:4]	83%	0%	100%	0%	0%
1b [I:4,6]	33%	0%	100%	0%	0%
2a [II:3,4]	73%	0%	100%	0%	0%
2b [II:7,8]	78%	0%	0%	0%	0%
3a [III:(3,4),6,7,8]	83%	0%	91%	0%	0%
3b [III:(3,4),6]	64%	0%	100%	0%	0%
4a [IV:3,4]	74%	0%	76%	10%	n/a
4b [IV:6]	70%	0%	99%	0%	n/a
5a [V:(3,4)]	31%	0%	96%	4%	0%
5b [V:7,8]	95%	0%	98%	0%	n/a
6 [VI:4], bioserotype Boyd 88	0%	0%	100%	0%	n/a
6 [VI:4], bioserotype Manchester	0%	0%	100%	2%	n/a
6 [VI:4], bioserotype Newcastle	0%	0%	0%	0%	n/a
variant X [-:7,8]	75%	0%	100%	0%	0%
variant Y [-:3,4]	30%	0%	96%	0%	5%

TABLE 3. Biochemical properties of *S. boydii* & *S. sonnei*

Group, serotype	Indol Production	Ornithine Decarboxylase	Mannitol fermentation	Xylose fermentation	ONPG
<b><i>S. boydii</i> (Group C)</b>					
1	0%	0%	100%	13%	8%
2	0%	0%	100%	0%	3%
3	0%	0%	100%	18%	22%
4	0%	0%	99%	0%	0%
5	100%	0%	100%	4%	0%
6	0%	0%	71%	0%	0%
7	100%	0%	100%	98%	0%
8	0%	0%	100%	29%	50%
9	100%	0%	93%	0%	75%
10	0%	0%	93%	4%	24%
11	100%	0%	100%	61%	0%
12	0%	0%	100%	0%	0%
13	100%	100%	100%	0%	0%
14	0%	1%	45%	7%	0%
15	100%	0%	91%	0%	33%
16	100%	0%	100%	100%	0%
17	100%	0%	100%	100%	20%
18	0%	0%	100%	18%	18%
19	0%	0%	100%	33%	6%
<b><i>S. sonnei</i> (Group D)</b>	0%	99%	99%	1%	95%

**Differentiation between *Shigella* spp and *E. coli***

Differentiation of *Shigella* strains from *E. coli* is one of the problems faced by a microbiological laboratory and may reflect the fact that *E. coli* and all four *Shigella* species are very closely on the basis of the DNA-DNA relationship.

The tests indicated in Table 5 are suggested for the differentiation (Edwards P.R., Ewing, W. H. – 1986).

TABLE 4. Differentiation of *Shigella* spp and *E. coli*.

Test	<i>Shigella</i> spp	<i>E. coli</i>
Mucate (fermentation)	-	+
Acetate (utilization)	-	+ or (+)
Christensen's Citrate	-	V
Indole production	V	+
Lysine decarboxylase	-	V
Ornithine decarboxylase	V	V
Glucose (gas)	-	+
Salicine (fermentation)	-	V
Motility	-	+ or -

+ 90% or more positive

- 90% or more negative

The biochemical reactions: Christensen's citrate, sodium acetate and sodium mucate are of considerable value in the differentiation of members of the genus *Shigella* from *Escherichia*.

The following guidelines may help for identifying *Shigella* spp.

- a) Isolates are always nonmotile and lysine negative.
- b) Gas production from Glucose is rare although it may be seen occasionally on strains of *Sh. flexneri*, *Sh. boydii* and *Sh. dysenteriae*.



- **Serotyping of *Shigella* spp**

Serotyping is subtyping method based on the immuno-reactivity of various antigens. Microorganisms produce a variety of antigens: structural components of the cells (cell wall constituents, capsules or envelopes, flagellae, fimbriae); secretion products of the cells (toxins, extracellular enzymes) or antigens contained in the interior of the cells. Chemically the antigens used for such purposes are of two main kinds: proteins and carbohydrates (including mixtures of both components).

The *Shigellae* are by definition non-motile, as such, only the somatic (O) antigens are utilized for the determination of serotype. Flagellar (H) antigens are not expressed. The O antigen consists of repeat units of oligosaccharide, and is part of the lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria and contributes to the main antigenic variability on the cell surface.

On the basis of O antigen structure and biochemical profile, *Shigella* spp can be classified into four species: *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*. Each species (with the exception of *S. sonnei*) can be further divided into multiple serotypes).

- ✓ Group A or *Shigella dysenteriae*: includes 15 serotypes (*S. dysenteriae* 1 to the 15).
- ✓ Group B or *Shigella flexneri*: includes 8 serotypes (*S. flexneri* 1 to the 6, *S. flexneri* X and *S. flexneri* Y) that can be divided into subserotypes according to its antigenic group factors (designated 3,4 – 6 and 7,8). Therefore with all the possible different antigenic combinations, the serotypes 1 to 5 of *S. flexneri* are subdivided into 11 subserotypes (see table in section IX - Interpretation of Results) (Annual Summary 2005 CDC <http://www.cdc.gov/ncidod/dbmd/phlisddata/shigella.htm>)
- ✓ Group C or *Shigella boydii*: includes 20 serotypes (*S. boydii* 1 to the 20).
- ✓ Group D or *Shigella sonnei*: contains only one serotype that may occur in two forms, form I (smooth) and form II (rough).



### ***Shigella flexneri***

*Shigella flexneri* serotypes are subdivided into subserotypes (see table in section IX - Interpretation of Results) (Edwards P.R., Ewing, W. H. – 1986; Brenner, D.J., 1984). The O antigen is an important virulence factor, and their diversity within Group B of *Shigella* is due to the presence of temperate bacteriophages that generates the serotype conversion. Several phages serotype-converting have been isolated and the genes involved in the O antigen modification were identified and characterized. (Allison, G.E., Verma, N.K. - 2000).

The serotypes of *S. flexneri* (with the exception of serotype 6) have some degree of antigenic relatedness between them attributable to a common repeating tetrasaccharide unit, to which  $\alpha$ -D-glucopyranosyl and O-acetyl residues are added, providing the basis for their type (i.e., I to VI) and group (i.e., 3,4, 6, and 7,8) antigenic factors. The addition of these residues is promoted by the insertion of temperate-bacteriophages. The figure 1 shows the chemical composition of the different serotypes of *Shigella flexneri*.

FIGURE 1 – Chemical composition of different serotypes of *S. flexneri*

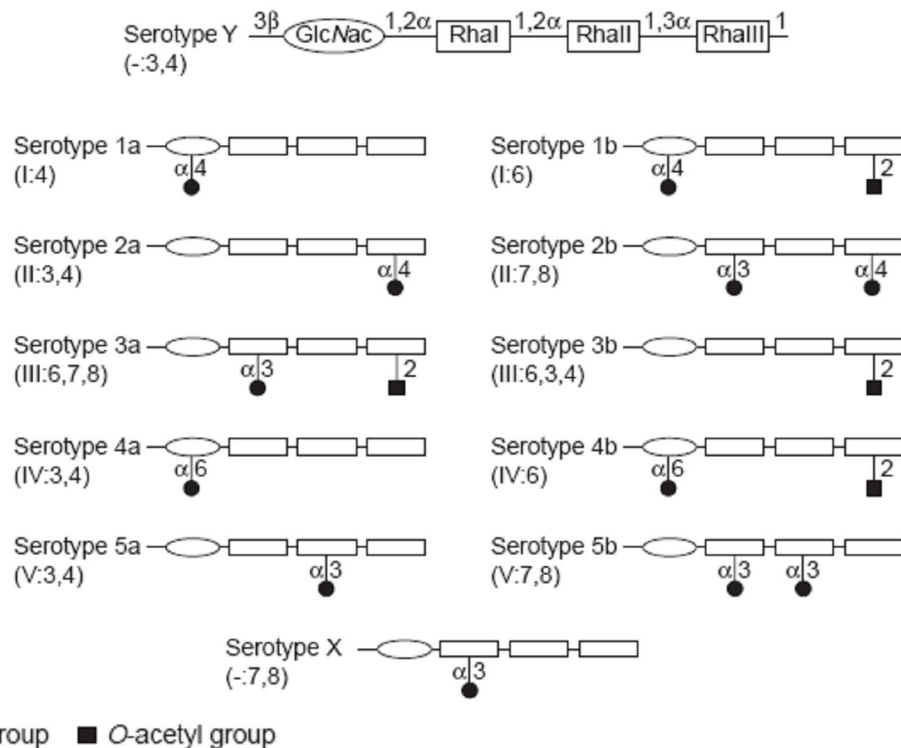


Fig. 1. Chemical composition of the different serotypes of *Shigella flexneri*. The basic O-antigen (serotype Y) consists of repeating units of the N-acetylglucosamine-rhamnose-rhamnose-rhamnose tetrasaccharide. The linkages between each sugar residue are indicated for serotype Y. Serotypes differ by the addition of either glucosyl or O-acetyl groups to different sugars within the tetrasaccharide repeat unit via the linkages indicated. Each serotype has one type-specific (Roman numeral) and one or more group-specific (Arabic numeral) antigenic determinants. Abbreviations: GlcNac, N-acetylglucosamine; Rha, rhamnose. (Allison, G.E., Verma, N.K. - 2000).



The *Shigella* serogroup and serotype are identified by agglutination tests with polyclonal antibodies, where a positive interaction indicates that the bacterium in study contains antigens that react specifically with the serum antibodies

These polyclonal antisera are obtained by hyperimmunization of healthy rabbits with whole cells of *Shigella* spp. heat inactivated. The antisera are tested for specificity and if it is necessary they are absorbed to remove cross-reacting agglutinins.

There are polyvalent and monovalent *Shigella* antisera. The polyvalent antisera, are those that their antibodies recognize antigens present in the different serotypes of *Shigella* (i.e. polyvalent antisera for *Sh. flexneri*, recognizes all the serotypes of this group). The monovalent antisera, only recognize specific epitopes of a serotype (i.e. monovalent antisera for *S. flexneri* 2) or of a group factor (i.e. monovalent antisera for *S. flexneri* group factor 7,8).

These reagents are used on slide agglutination test, to serotype well defined biochemical isolates. The final identification of the microorganism must be based on the results of biochemical and serological approaches.

The commercially available antisera may not be able to cover all possible epitopes of the O antigen of *Shigella* spp. There are probably a multitude of epitopes not covered by the typing scheme currently in use. New serotypes or subserotypes are being isolated from different parts of the world (Kaiser A., et al; 2002 – Ansaruzzaman, M. et al; 1995 – Woodward, D. et al; 2005).

**APPENDIX B**

**SHIGELLA SEROTYPING WORKSHEET**

**Patient:** \_\_\_\_\_ **Accession Number:** \_\_\_\_\_

**Date Received:** \_\_\_\_\_ **Date Inoculated:** \_\_\_\_\_ **Inoculated By:** \_\_\_\_\_

**Serotyping Performed By:** \_\_\_\_\_ **Date:** \_\_\_\_\_

**Lots of the media and solutions employed:** \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Antiserum	Lot	Colony 1	Colony 2 (if tested)	Boiled Suspension (if tested)
POLY A				
POLY A1				
POLY B				
POLY C				
POLY C1				
POLY C2				
POLY C3				
POLY D				
Single factor: _____				
Single factor: _____				
Single factor: _____				
Single factor: _____				
Single factor: _____				
Single factor: _____				
Single factor: _____				
Single factor: _____				
Single factor: _____				
Single factor: _____				
Single factor: _____				
Single factor: _____				
Single factor: _____				

**Final Result:** \_\_\_\_\_

**Reported By:** \_\_\_\_\_ **Approved By:** \_\_\_\_\_ **Date:** \_\_\_\_\_

APPENDIX C

**SHIGELLA ANTISERA QC RECORD DATASHEET**

Serotyping Performed By: \_\_\_\_\_ Date: \_\_\_\_\_

Lots of the media and solutions employed: \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Antisera: \_\_\_\_\_

Manufacturer / Lot #	Expiration:	Positive Control Strain:	Result:	Control of the reference strain:*	Result of QC Testing:	Comments:
					Pass / Fail	
					Pass / Fail	
					Pass / Fail	

\* Refers to the autoagglutination test (positive or negative)

Interpretation:

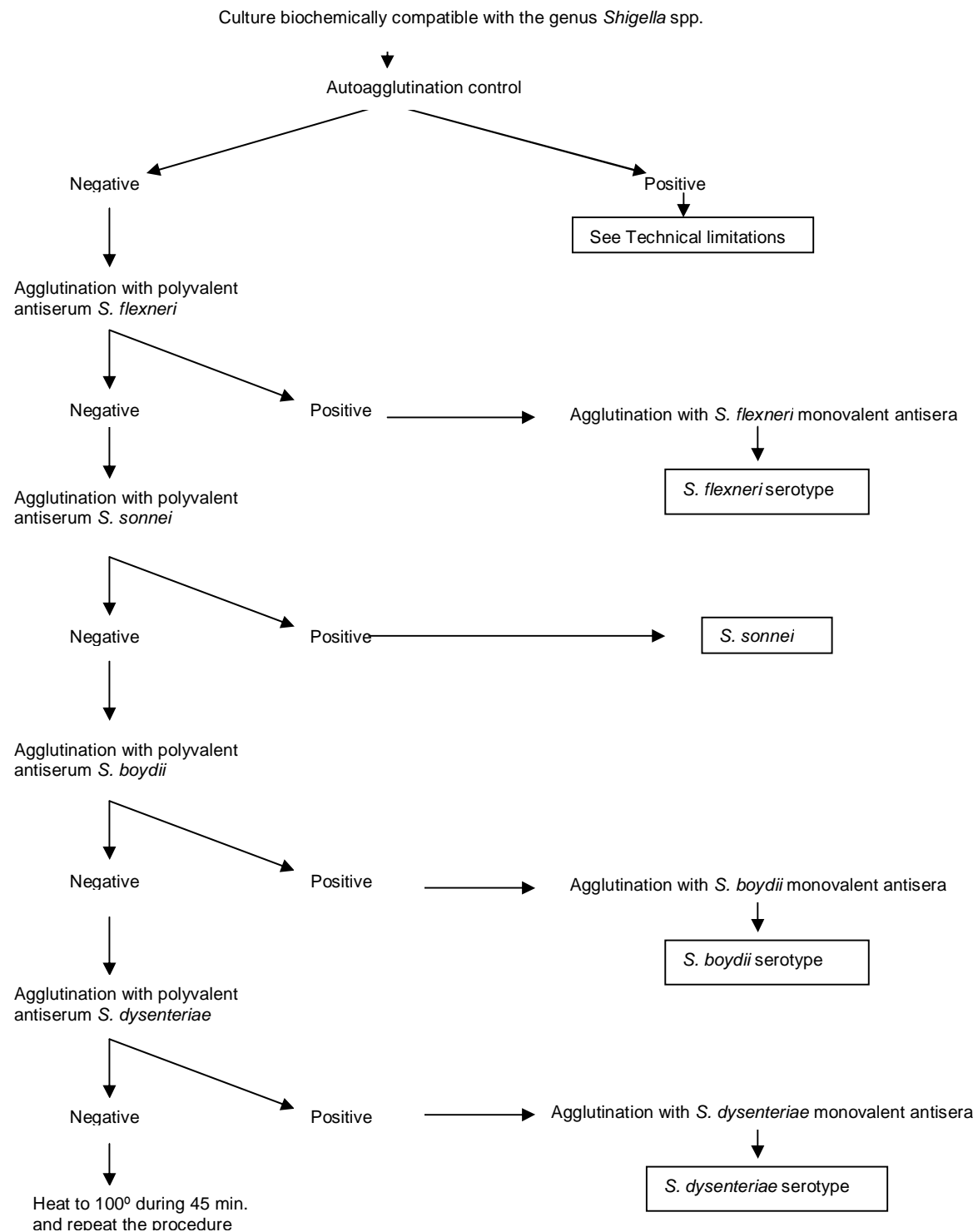
Pass: Antiserum produces 3-4+ agglutination with positive control (< 60 seconds) & the reference strain not autoagglutinate

Fail: No reaction / weak reaction with positive control and / or autoagglutination of the reference strain

## APENDIX D FLOWCHART FOR *Shigella* spp. SEROTYPING

Note: This is an example of a testing flow utilised by South American laboratories and was developed based on regional trends in *Shigella* epidemiology. In this algorithm, polyvalent sera against *S. flexneri* is used first because *S. flexneri* is the most common species in the region. If a different species is more common in a particular region, the test plan may be adjusted accordingly.

Alternatively: If complimentary biochemical data which suggests a particular species / type (example: ornithine decarboxylase positive: *S. sonnei* or catalase negative: *S. dysenteriae* Type 1) the initial algorithm may be modified



## APPENDIX E

### STORAGE OF *Shigella* spp. CULTURES

#### (A) Preservation in semisolid agar

The semisolid agar is dispensed in small tubes, sterilized 15 min at 121°C and allow to cool. Tubes are stab-inoculated. After overnight incubation, the tubes are stored in the dark at room temperature. It is not necessary –at least in countries with temperate climate- to store in the refrigerator. When the stored strains need to be used again, a portion of the culture is transferred to a sterile broth with an inoculating loop. After overnight incubation at 35-37°C, a loopful of broth cultures is streaked on a nutrient agar, as TSA. (Popoff, 2001, Guidelines for the preparation of *Salmonella* Antisera, 6<sup>th</sup> Ed., WHO Collaborating Centre for Reference and Research on *Salmonella* Pasteur Institute)

#### (B) Freezing at -70°C

Freezing can conveniently be done as follows:

Add tryptic soy broth (TSB) containing 15% (v/v) glycerol and approximately 30, sterile glass beads (2mm in diameter) to a sterile, freezer-safe (10 x 30 mm) screw-capped vial. Prepare as many vials as needed.

Using a fresh, pure, culture, make a heavy suspension of bacteria in the vial. Carefully remove as much of the excess suspension as possible. Label the vial and place the vial in a tray. The tray is then immediately stored at -70°C. To recover a frozen strain, a vial is removed from the freezer and one bead is picked up using a platinum loop or sterile forceps. The removed bead is transferred to nutrient broth and incubated overnight at 35-37°C and the vial is immediately returned to to freezer. (Popoff, 2001, Guidelines for the preparation of *Salmonella* Antisera, 6<sup>th</sup> Ed., WHO Collaborating Centre for Reference and Research on *Salmonella* Pasteur Institute)

## APPENDIX F

### PREPARATION OF CULTURE MEDIA AND SOLUTIONS

#### **\*\*TRYPTIC SOY BROTH (TSB)**

##### **Formula (grams per liter)**

Pancreatic Digest of Casein	17.0
Soy peptone	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Glucose	2.5
<b>pH 7.3 ± 0.2</b>	

##### **Instructions**

To rehydrate the medium, suspend 30 g of the powder in 1 liter of distilled water. Mix and heat until it's dissolved. Distribute and sterilize in the autoclave for 15 minutes at 121°C.

#### **\*\*TRYPTIC SOY AGAR (TSA)**

##### **Formula (grams per liter)**

Pancreatic Digest of Casein	15.0
Soy peptone	5.0
Sodium chloride	5.0
Agar	15.0
<b>pH 7.3 ± 0.2</b>	

##### **Instructions**

Suspend 40 g of the powder in 1 liter of distilled water. Mix and let stand for 5 minutes. Heat until dissolution. Distribute and sterilize in the autoclave for 15 minutes at 121°C.

#### **\*\*BLOOD AGAR**

##### **Instructions**

To a sterile blood agar base or TSA cooled to 45-50°C, and while still liquid, aseptically add 5% sterile, defibrinated blood (horse or sheep blood is recommended). Mix thoroughly while avoiding the incorporation of air bubbles. Distribute in sterile plates. It is strongly recommended to incubate plates before use to insure sterility.

**\*\*SEMI-SOLID AGAR (FOR STORAGE)**

**Formula (grams per liter)**

Beef extract	5.0
Peptone	10.0
NaCl	3.0
Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O	2.0
Agar	10
<b>pH 7.4 ± 0.1</b>	

**Instructions**

Suspend the components in 1 liter of distilled water. Let stand for 10 to 15 minutes. Heat to boil. Adjust to pH 7,4 ±0,1. Sterilize in autoclave for 15 minutes at 121°C.

**\*\*PHYSIOLOGICAL SALINE SOLUTION (NaCl 0,85% w/v)**

**Formula (grams per liter)**

NaCl	8,5
------	-----

**Instructions**

Suspend 8,5 g of the NaCl in 800 ml of distilled water. Complete volume up 1000 ml with distilled water. Distribute and sterilize in the autoclave for 15 minutes at 121°C.

**\*\*0,5 % FORMALINIZED PHYSIOLOGICAL SALINE SOLUTION (NaCl 0,85 w/v – 0,5 % formalin v/v)**

**Formula (grams per liter)**

NaCl	8,5
Formalin	5.0 ml

**Instructions**

Suspend 8,5 g of the NaCl in 995 ml of distilled water. Autoclave for 15 minutes at 121°C. Allow to cool to room temperature. Add 5 ml of formalin. DO NOT AUTOCLAVE AFTER ADDING FORMALIN.