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:

"Biochemical Identification of *Salmonella* and *Shigella* Using an Abbreviated Panel of Tests"

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LABORATORY SOP

Title: Biochemical Identification of *Salmonella* and *Shigella* Using an Abbreviated Panel of Tests	
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REVISION HISTORY

HISTORY OF CHANGES				
Rev. Level	**Sections Changed**	**Description of Change (From—To)**	**Date**	**Approval**
0	New Document	Formatted and simplified historical GFN/GSS protocols into CLSI / ISO template	01 Jan 2010	GFN Laboratory Subcommittee
1	Appendix III	Changed error. Original version listed all *Shigella* as anaerogenic. Current version corrects Appendix III to indicate gas production by *Shigella* varies by strain / serotype.	13 Feb 2014	M. Mikoleit
	Appendix IV Page 12 and 14	Deleted KCN from "Supplementary Identification Panels" and "*Salmonella* Subspecies Differentiation Panel: Interpretative Criteria". KCN is extremely toxic and not critical for the differentiation of *Salmonella* species/subspecies.		
	Various	Minor grammatical edits		
2	Various	References checked and updated. Serological characteristics of *Shigella flexneri* serotypes added (Appendix IV) Overview over appendices added under 'XIII. APPENDICES' Grammatical edits.	16 Oct 2015	Malika Gouali, Institute Pasteur, France and Elena Campos, INCIENSA, Costa Rica

I. PURPOSE

Standardized process for the biochemical identification of *Salmonella* and *Shigella.*

II. TEST PRINCIPLES

Isolation and identification remains the gold-standard for the diagnosis of infections due to *Salmonella* and *Shigella.* Theoretically, culture is 100% specific and unlike rapid tests, yields an isolate which may be subjected to further characterisation (e.g. antimicrobial susceptibility testing, serotyping, or pulsed-field gel electrophoresis).

Virtually all isolation protocols for *Salmonella spp.* and *Shigella spp*. include the use of selective and differential media to enhance recovery of the targeted organisms. Selective media are formulated to suppress background flora. These media also provide preliminary, macroscopic, differentiation of enteric organisms on the basis of colony color and morphology.

When interpreting *Salmonella* and *Shigella* cultures, it is important to remember that colony morphology on selective agar is not diagnostic. Colony morphology is used simply as a means to identify colonies for additional testing. Colonies that produce *Salmonella*-like or *Shigella*-like morphology ("suspect colonies") on selective agar must be subjected to additional biochemical (and serological) testing to confirm the identification (see Appendix I). As other *Enterobacteriaceae* may look similar to *Salmonella* or *Shigella* on selective and media, the presence of suspect colonies alone cannot be considered diagnostic. A final genus / species level identification requires additional testing for confirmation. Similarly, isolates presumptively identified as suspect-*Salmonella spp*. or suspect-*Shigella spp*. on the basis of agglutination with polyvalent antisera must be subjected to biochemical confirmation. Similarly, polyvalent antisera can be a useful screening tool; however, some O and H antigenic types are found in multiple genera among the *Enterobacteriaceae*, so reaction with any given antiserum without adequate biochemical testing is not diagnostic.

Identification of *Enterobacteriaceae*

Conventional phenotypic testing is typically used to identify and characterize microorganisms. Phenotypic testing includes tests that identify the ability of an organism to perform specific biochemical reactions (e.g., ability to ferment glucose) or exhibit certain growth characteristics (e.g., motility, ability to grow in the presence of potassium cyanide). Conventional identification is a two-step process: i). The organism is phenotypically characterized using biochemical and growth tests. Phenotypic characterization assays commonly utilized to characterize *Enterobacteriaceae* typically include carbohydrate fermentation assays, amino acid utilization assays, and sole carbon source utilization assays. ii) The results of individual tests are compiled to form the biochemical profile of the unknown organism. The profile is then compared, often with the aid of statistical analysis programs, to profiles of known organisms and identification is obtained.

Selection of tests for Identification of *Enterobacteriaceae*

Many biochemical tests have been described in peer-reviewed literature. However, only a subset of substrates has been shown to be useful for the identification of *Enterobacteriaceae*. Additionally, some substrates, while potentially useful for identification of *Enterobacteriaceae* are too toxic, unstable, or costly for routine use. As such, the selection of substrates must be based on available resources, extent of characterization required, and previous laboratory findings.

Phenotypic testing has historically been performed using tube or plate media. Over time miniaturized and automated systems have been developed which allow multiple substrates to be tested on a single strip or card. Automated systems can be easier to inoculate and often come with automated interpretation software; however they can also be more expensive and typically are not customizable.

Conventional tube and plate media allow the user to select an appropriate panel of substrates necessary to identify the test organism, to extend incubation time if needed. Finally, many reference centers receive isolates which have previously been tested on automated systems, typically in clinic or hospital laboratories, in these situations conventional testing allows verification of results using an alternate method.

In resource-limited settings, biochemical testing is typically limited to key substrates necessary to rule-in or rule-out pathogens of interest. These simplified algorithms rely on key phenotypes that identify a particular

genus/species of interest and phenotypes necessary to differentiate that genus/species from other related organisms.

This protocol presents a simplified algorithm for the identification of *Salmonella* and *Shigella* (**The *Salmonella/Shigella* Panel,** Appendix I). In this algorithm, ten phenotypic characteristics are captured using five conventional biochemical tests (described in Appendices VI-X). This algorithm is not sufficient to identify all *Enterobacteriaceae*; but will identify *Salmonella* spp. and *Shigella* sp.; and provides serovar level identifications of *Salmonella* serovars Typhi and Paratyphi A; and species level identification of *Shigella sonnei* (see Appendix III for interpretative criteria).

Additional algorithms are available to further characterize *Salmonella* and *Shigella,* and to identify additional members of the *Enterobacteriaceae* (see Appendix IV):

> **Basic Enteric Panel:** Twenty five tests that identify *Salmonella, Shigella,* and several other commonly encountered *Enterobacteriaceae*.
>
> **Full Enteric Panel:** Fifty tests that is sufficient to identify most enteric organisms. This panel is recommended for isolates which produce ambiguous results with the above panels or when a biochemically atypical organism is encountered.
>
> **Salmonella Subspecies Panel:** Ten tests intended for use with known isolates of *Salmonella* and provides differentiation between the *Salmonella* species and subspecies.
>
> **Biochemically-Unique Serovar Panel:** Tests that can be used rule-in or rule-out suspected isolates of biochemically unique *Salmonella* serovars, such as Choleraesuis, Paratyphi C, and Sendai.

Detailed instructions for the inoculation and interpretation of media described in these supplementary panels may be found in standard texts or obtained from WHO Collaborating Centres (http://www.who.int/collaboratingcentres/database/en/).

Salmonella and *Shigella*

Salmonella and *Shigella* are two genera within the family *Enterobacteriaceae*. Like other *Enterobacteriaceae*, they are Gram-negative, non-spore forming rods. The *Enterobacteriaceae* are oxidase negative, catalase positive (with the exception of *S. dysenteriae* Type 1), facultative anaerobes that grow on MacConkey agar and reduce nitrate to nitrite.

The genus *Salmonella* is comprised of two species, *S. enterica* and *S. bongori*. *S. enterica* is subdivided into six subspecies which are identified by name or Roman numeral:

***Salmonella enterica* subspecies**	
I	*Salmonella enterica* subsp. *enterica*
II	*Salmonella enterica* subsp. *salamae*
IIIa	*Salmonella enterica* subsp. *arizonae*
IIIb	*Salmonella enterica* subsp. *diarizonae*
IV	*Salmonella enterica* subsp. *houtenae*
VI	*Salmonella enterica* subsp. *indica*

Based in the immuno-reactivity of “O” (LPS), “H” (flagellin protein) antigens, the genus is further sub-divided into serovars. As of 2007, a total of 2,557 serovars of *S. enterica* and 22 serovars of *S. bongori* have been recognized. The majority, but not all, of human clinical isolates, including *Salmonella* serovars Enteritidis, Typhimurium, and Typhi (etiologic agent of typhoid fever) are found within *S. enterica* subspecies *enterica*.

Conventional biochemical testing is typically used to differentiate the genus *Salmonella* from other *Enterobacteriaceae*, also between the six subspecies of *S. enterica* and to differentiate *S. enterica* from *S. bongori*. With limited exceptions, *Salmonella* serovars cannot be differentiated from each other on the basis of biochemical profile. In the case of *Salmonella*, a serovar level identification typically can only be the

characterization of O and H antigens using specific antisera, a technique known as serotyping according to the Kauffman-White scheme.

Some serovars within *Salmonella enterica* subspecies *enterica* have unique biochemical profiles (e.g., *Salmonella* serovars Typhi, Paratyphi A, Choleraesuis, Pullorum, and Gallinarum). These biochemical profiles can be used to differentiate these serovars from other serovars of *Salmonella* and other *Enterobacteriaceae*. Ideally all isolates should be serotyped after being biochemically characterized; however if it is not possible to perform serology, a serovar level identification of these biochemically unique serovars may be made on the basis of biochemical profile as described in the present protocol.

Biochemical identification becomes an essential supplement to serotype data when multiple subspecies share an identical antigenic formula, or when all antigenic factors are not expressed, such as with non-motile, mucoid, or rough isolates.

Shigella spp. are by definition non-motile and lysine decarboxylase negative. Recent phylogenetic studies indicate that *Shigella* and *Eschericha coli* comprise a single species. However, to facilitate disease surveillance the shigellae have not been merged with *E. coli*.

The genus *Shigella* is comprised of four species: *Shigella dysenteriae* (also referred to subgroup A), including *S. dysenteriae* serotype 1, the etiologic agent of epidemic dysentery; *Shigella flexneri* (also referred to subgroup B), *Shigella boydii* (also referred to subgroup C), & *Shigella sonnei* (also referred to subgroup D). With the exception of *S. sonnei*, each species may be further divided into serogroups on the basis of reactivity with hyperimmune serum (for serological characteristics of *Shigella flexneri t*ypes, see Appendix IV).

III. RESPONSIBILITIES

A. Staff Responsibilities

This section summarizes responsibilities as specific to execution of this SOP, please refer to applicable manuals within the facility/locality for complete set of responsibilities to properly conduct this procedure.

B. Specific Safety Requirements and Responsibilities

Universal precautions to prevent the transmission of blood-borne viruses must always be employed when processing any human, clinical sample.

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 with clinical isolates of unknown *Enterobacteriaceae*, *Salmonella* spp., and *Shigella* spp.

Biosafety level 3 (BSL-3/RG-3) practices and procedures should be considered working with production quantities of *S. dysenteriae* 1 and invasive serovars of *Salmonella* spp. (e.g. *Salmonella* serovars Typhi, Paratyphi A, Paratyphi B, Paratyphi C, and Choleraesuis). BSL-3/RG-3 practices and procedures should also be utilized when performing procedures likely to generate aerosols of *S. dysenteriae* 1 or invasive serovars of *Salmonella* spp.

Organism specific information may be obtained at:
http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/index-eng.php

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

IV. SAMPLE COLLECTION/TRANSPORT/STORAGE

Only pure cultures shall be used for biochemical testing. Isolates for testing may be referred cultures submitted by other laboratories or “suspect”, *Salmonella*-like or *Shigella*-like, colonies recovered from selective media.

When selecting colonies from selective media for biochemical testing, it is critical to pick well isolated colonies; if several suspicious colonies are present, three to five separate colonies should be selected for biochemical testing. The growth from a single colony is used to inoculate all biochemical test media. If there is insufficient growth present to inoculate the biochemical test media, the colony should be subcultured to fresh media and incubated overnight.

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"). 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 5% Sheep Blood Agar (for biochemical testing) AND to a selective agar (e.g. MAC) to verify purity.

V. MATERIALS/SUPPLIES

Reagents

Biochemical test media and reagents specified in Appendices VI-X.

Supplies

General laboratory supplies (inoculating loops, gloves, etc.)

VI. EQUIPMENT

36°C Incubator (non-CO_2)

VII. QUALITY ASSURANCE

Media should be quality control (QC) tested to insure appropriate reactivity and growth properties. Specific QC requirements are included in individual protocols.

VIII. PROCEDURE

Selection of Colonies:

Selective and differential plating media (Hektoen Enteric Agar, MacConkey Agar, & XLD Agar), are incubated overnight (18-24 hours) at 36°C (+/- 1°C). The plates are then examined for *Salmonella*-like or *Shigella*-like colonies. See Appendix II for description of typical colony morphology for these media.

Suspect colonies are picked using an inoculating needle or 1uL inoculating loop and transferred to a non-selective agar, such as 5% Sheep Blood Agar (SBA). It is critical to pick well isolated colonies; if several suspicious colonies are present, three separate colonies should be selected for biochemical testing.

SBA plates are incubated at 36°C (+/- 1°C) for 18-24 hours. The growth from a single colony is used to inoculate all biochemical test media. If there is insufficient growth present to inoculate the biochemical test media, the colony should be subcultured to fresh media and incubated overnight.

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"). 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 5% Sheep Blood Agar (for biochemical testing) AND to a selective agar (e.g. MAC) to verify purity.

Selection of Test Panel:

All *Salmonella*-like or *Shigella*-like colonies should be screened with the biochemical test media described in Appendix I. The "*Salmonella* and *Shigella* Panel" consists of: triple sugar iron agar (TSI), lysine iron agar (LIA), motility-indol-ornithine agar (MIO), Simmons citrate agar, and urea agar. This panel is sufficient to both rule-in / rule-out *Salmonella* or *Shigella* and to biochemically differentiate *Salmonella* serovars Typhi and Paratyphi A from other serovars of *Salmonella*. This panel is particularly suitable in a resource-limited situation or when screening large numbers of isolates.

Tests used to characterize specific specimens may be modified on a case-by-case basis. This protocol describes the inoculation and interpretation of the biochemical tests used in the *Salmonella* and *Shigella* Panel. Additional testing algorithms are described in Appendix IV.

Inoculation of Biochemical Test Media:

Following incubation, examine plates for purity. Do not proceed unless the growth appears pure.

Procedures for the inoculation of biochemical test media vary by substrate. Stepwise procedures for the inoculation and interpretation of the biochemical test *Salmonella* and *Shigella* Panel is described in Appendices VI-X.

IX. INTERPRETATION OF RESULTS

Individual biochemical results are interpreted and recorded according to specific test procedures (Appendices VI-X).

Result profiles are compared to standard biochemical tables (e.g. appendices or reference texts). Interpretation of a large number of biochemical results from a single sample may be facilitated by the use of an automated ID program (e.g. PibWin: http://www.som.soton.ac.uk/staff/tnb/pib.htm).

X. LIMITATIONS OF PROCEDURE

Biochemically atypical isolates may be encountered. Atypical results should be repeated, ideally by an alternate method. Additional phenotypic testing as described in Appendix IV may help clarify unexpected results, or aberrant results may warrant verification at a reference centre.

XI. REPORTING

Isolates that are biochemically consistent with *Salmonella* species based on the *Salmonella* and *Shigella* Panel may be reported with a genus level identification: "*Salmonella* species*".*

Isolates that are biochemically consistent with *Salmonella spp.* and that have been biochemically identified as serovar Typhi or Paratyphi A may be reported as: "*Salmonella* serovar Typhi or *Salmonella* serovar Paratyphi A, respectively, with a comment: Identification based on biochemical profile*".*

All isolates of *Salmonella* should be serotyped or referred to a reference laboratory for serotyping.

Confirmatory identification of *Shigella* species requires biochemical and serological characterization. If serotyping cannot be performed, isolates that are biochemically consistent with *Shigella* species may be reported with a genus level identification of: "*Shigella* species*"*. These reports should include the comment: "Identification based on biochemical profile*.*"

XII. REFERENCES

Centers for Disease Control and Prevention. Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera. Atlanta, GA. CDC. 2002.

Kauffman-White. Antigenic Formulae of the Salmonella Serovars, 9th edition. 2007.

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WHO Global Salm-Surv. Laboratory Protocol: Isolation of *Salmonella*. 5th Edition. 2007.

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XIII. APPENDICES

Appendix I BIOCHEMICAL TESTING ALGORITHM

Appendix II Morphology of *Salmonella* spp., *Salmonella* serovar Typhi, and *Shigella* sp. on Various Selective and Differential Media After Overnight (18-24 hour) Incubation at 36°C (+/- 1°C)

Appendix III Comparative Phenotypic Profiles of *Salmonella* species, *Salmonella* ser. Typhi, *Salmonella* ser. Paratyphi A, and *Shigella* sp.

Appendix IV Supplementary Identification Panels

Appendix V GROWTH OF VARIOUS *ENTEROBACTERIACEAE* IN:
Triple Sugar Iron Agar (TSI), Urea Agar (Urea), Lysine Iron Agar (LIA), Simmons Citrate Agar (Citrate), & Motility-Indol-Ornithine Agar (MIO)

Appendix VI Inoculation and Interpretation of Simmons Citrate Agar

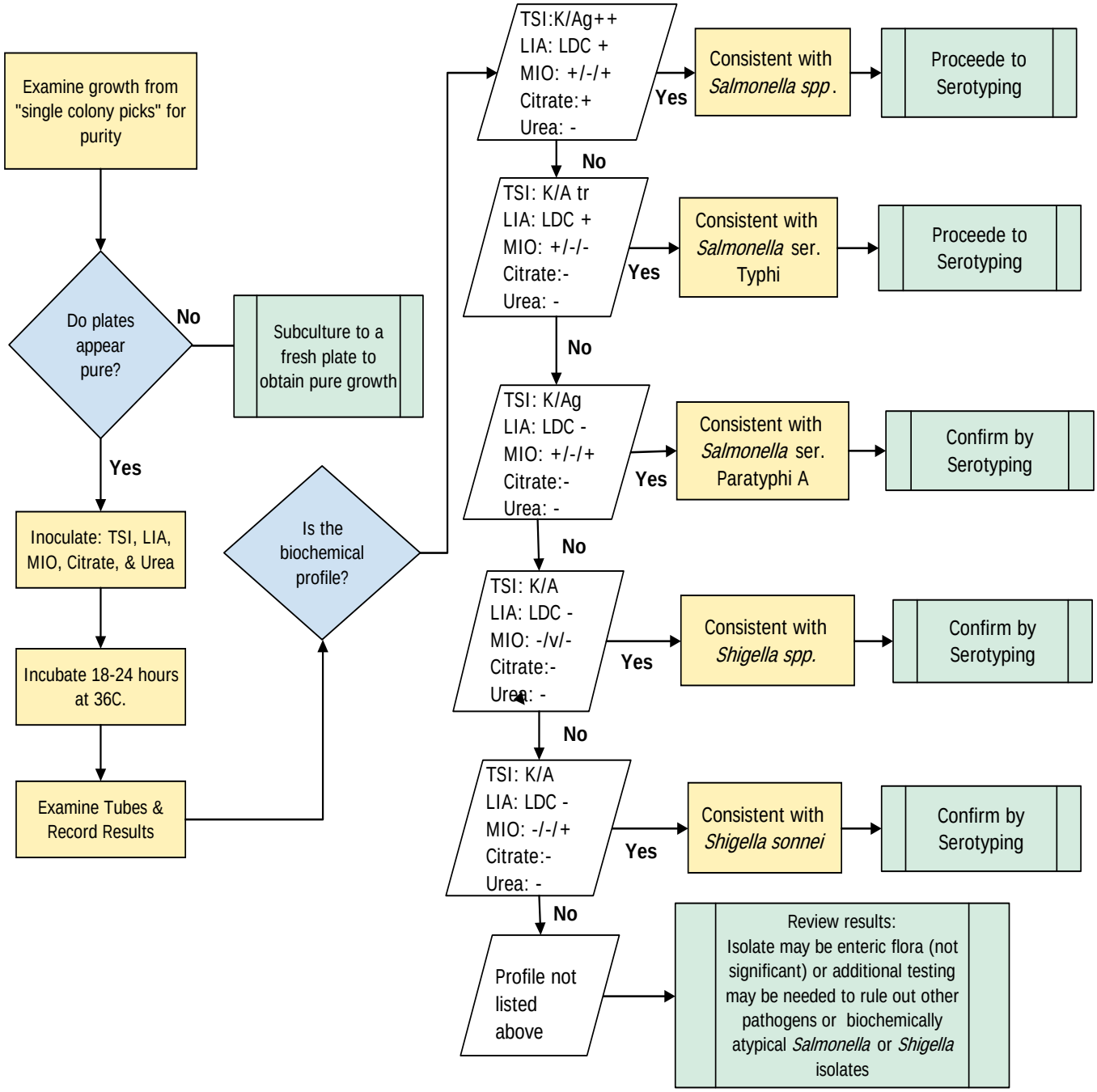
Appendix VII Inoculation and Interpretation of Lysine Iron (LIA) Agar

Appendix VIII Inoculation and Interpretation of Motility-Indol-Ornithine Agar (MIO Agar)

Appendix IX Inoculation and Interpretation of Triple Sugar Iron (TSI) Agar

Appendix X Inoculation and Interpretation of Christensen's Urea Test Medium (Urea Agar)

APPENDIX I
BIOCHEMICAL TESTING ALGORITHM



APPENDIX II

Morphology of *Salmonella spp*., *Salmonella* serovar Typhi, and *Shigella* sp. on Various Selective and Differential Media After Overnight (18-24 hour) Incubation at 36°C (+/- 1°C)

Media	***Salmonella*** **(majority)**	***Salmonella*** **Typhi**	***Shigella*** **sp.**
MacConkey Agar (MAC)	Smooth, colourless colonies. 2-4 mm	Smooth, colourless colonies. 1-3 mm	Smooth, colourless colonies. 2-3 mm
Hektoen Enteric Agar (HE)	Clear colonies with black centres. 2-4 mm	Clear colonies. Some may produce pinpoint black centres. 1-3 mm	Clear / green colonies 2-3 mm
Xylose Lysine Desoxycholate Agar (XLD)	Colonies may range in colour from clear to pink /red. Most colonies 2-4 mm with black centres.	May be inhibited. 1-3 mm clear colonies. Some with pinpoint black centres.	Red colonies 1-2 mm

APPENDIX III

Comparative Phenotypic Profiles of *Salmonella* species, *Salmonella* ser. Typhi, *Salmonella* ser. Paratyphi A, and *Shigella* sp.

	Salmonella **(majority)**	***Salmonella*** **serovar Typhi**	***Salmonella*** **serovar Paratyphi A**	***Shigella spp*****.**
TSI (slant)[1]	K	K	K	K
TSI (butt)[1]	A	A	A	A
TSI (H_2S)[2]	+	Trace amount	Negative	Negative
TSI (gas)[3]	+	No gas	+	- (most)
LIA[4]	+	+	-	-
MIO (Motility)[5]	+	+	+	-
MIO (Ornithine)[6]	+	+	+	*S. dysenteriae, S. flexneri, & S. boydii*: - *S. sonnei*: +
MIO (Indol)[7]	-	-	-	Varies by species / serotype
Urea[8]	-	-	-	-
Citrate (Simmons)	+	-	-	-

Phenotypes recorded after 18-24 hours at 36ºC.

TSI: Triple Sugar Iron Agar
LIA: Lysine Iron Agar
MIO: Motility-Indol-Ornithine Agar
Urea: Urea Agar
Citrate (Simmons): Simmons Citrate Agar

1) A: Acid (yellow colour); K: Alkaline (red colour)
2) H_2S: Production of hydrogen sulphide evidenced by blackening of agar.
3) Gas: Gas production evidenced by splitting of agar or presence of bubbles.
4) LIA: Lysine decarboxylation (positive) evidenced by purple colour in butt of tube.
5) MIO (Motility): Motility (positive result) evidenced by diffuse growth through agar
6) MIO (Ornithine): Ornithine decarboxylation (positive) evidenced by purple colour in bottom 3/4 of tube
7) MIO (Indol): Indol production (positive reaction) evidenced by red colour development after addition of Kovacs' reagent.
8) Urea: Urease activity (positive reaction) detected by red colour development on slant.
9) Citrate: Citrate utilisation (positive reaction) evidenced by blue colour development on slant

APPENDIX IV
Supplementary Identification Panels

Biochemical Substrates By Panel	Indole production	Methyl red	Voges-Proskauer	Citrate (Simmons')	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility (36°C)	Gelatin hydrolysis (37°C)	Malonate utilization	D-Glucose	Lactose	Sucrose	D-Mannitol	Dulcitol	Salicin	Adonitol	*myo* -Inositol	D-Sorbitol	L-Arabinose	Raffinose	L- Rhamnose	Maltose	D-Xylose	Trehalose	Cellobiose	α-methyl-D-glucoside	Erythritol	Esculin hydrolysis	Melibiose	D-Arabitol	Glycerol	Mucate	Tartrate, Jordan's	Acetate	Lipase (corn oil)	DNase at 25°C	Nitrate to nitrite	Oxidase, Kovacs	ONPG*	Citrate (Christensen)	Peptone Iron Agar	D-Mannose	Tyrosine Utilization	MUG (Beta-glucuronidase)	Galacturonate	
Salmonella / Shigella Panel	X			X	X	X		X		X	X																																						
Basic Enteric Panel	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X		X	X													X	X						X					X	X	
Complete Enteric Panel	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Salmonella Subspecies Panel													X		X			X	X			X														X	X						X					X	X	
Biochemically Unique Serovar Panel				X	X			X	X	X	X			X				X				X	X		X		X	X							X	X						X					X	X	
Andrade's sugar solutions have historically been used for the determination of carbohydrate fermentation patterns by *Enterobacteriaceae* . MIO may be used to simultaneously detect motility, indol production, and ornithine decarboxylation																																																	

Serological characteristics of *Shigella flexneri* serotypes											
	TYPING							GROUPING			
Serotype	**I**	**II**	**III**	**IV**	**V**	**VI**	**1c**	**Y3(4)**	**6**	**X7(8)**	**MASF IV-1**
1a	+	-	-	-	-	-	-	+	-	-	-
1b	+	-	-	-	-	-	-	+	+	-	-
1c	-	-	-	-	-	-	+	-	-	-	-
1d	+	-	-	-	-	-	-	-	-	+	-
2a	-	+	-	-	-	-	-	+	-	-	-
2b	-	+	-	-	-	-	-	-	-	+	-
3a	-	-	+	-	-	-	-	+/-	+	+	-
3b	-	-	+	-	-	-	-	+/-	+	-	-
3c	-	-	+	-	-	-	-	-	+	-	-
4	-	-	-	+	-	-	-	-	-	-	-
4a	-	-	-	+	-	-	-	+	-	-	-
4 av	-	-	-	+	-	-	-	+	-	-	+
4b	-	-	-	+	-	-	-	-	+	-	-
4c	-	-	-	+	-	-	-	-	-	+	-
5a	-	-	-	-	+	-	-	+	-	-	-
5b	-	-	-	-	+	-	-	-	-	+	-
6	-	-	-	-	-	+	-	+/-	-	-	-
6a	-	-	-	-	-	+	-	+	-	-	-
X	-	-	-	-	-	-	-	-	-	+	-
Xv	-	-	-	-	-	-	-	-	-	+	-
4X	-	-	-	-	-	-	-	-	-	-	+
Y	-	-	-	-	-	-	-	+	-	-	-
Yv	-	-	-	+	-	-	-	+/-	-	-	+
7b	-	-	-	-	-	-	+	-	+	-	-

Salmonella **Subspecies Differentiation Panel: Interpretative Criteria[5]**

Substrate	***S. enterica***						***S. bongori*** (formerly ssp. V)
	ssp. I	**ssp. II**	**ssp. IIIa**	**ssp. IIIb**	**ssp. IV**	**ssp. VI**	
Dulcitol	+	+	-	-	-	V	+
Galacturonate	-	+	-	+	+	+	+
Lactose	-	-	- (75%)	+ (75%)	-	V	-
Malonate	-	+	+	+	-	-	-
Mucate	+	+	+	- (70%)	-	+	+
MUG	V	V	-	+	-	V	-
ONPG	-	-	+	+	-	V	+
Salicin	-	-	-	-	V *	-	-
Sorbitol	+	+	+	+	+	-	+
Tartrate (Jordan's)	+	-	-	-	-	-	-

(+) = >90% positive
(-) = <10% positive
V = Variable

* Varies by serovar (Kauffman White table, 2007, 9th edition)

Biochemically Unique Serovars Panel: Interpretative Criteria [2&5]

Substrate	Paratyphi A	Typhi	Choleraesuis var. Kunzendorf	Choleraesuis en *sensu stricto*	Paratyphi C	Typhisuis	Sendai	Miami		Pullorum*	Gallinarum*	*S. enterica* ssp. I (most serovars)
ADH	-	-	Variable	Variable	Variable	Variable				-	-	+
Arabinose	+	-	-	-	+	+	+	+	-	+	+	+
Citrate (Simmons)	-	-	Variable	Variable	+	-	-	+	+	-	-	+
Dulcitol	+	-	-	-	+	-		+	-	-	+	+
Glucose (gas)	+	-	+	+	+	+				+	-	+
LDC	-	+	+	+	+	-	Variable	+	+	+	+	+
Motility	+	+	+	+	+	+				-	-	+
Mucate	-	-	-	-	-	-		+	-	-	+	+
Rhamnose	+	-								+	-	+
Sorbitol	+	+	Variable	Variable	+	-				-	-	+
Tartrate (Jordan's)	-	+	+	+	+	-	-	+	+	-	+	+
Trehalose	+	+	-	-	Variable	+				+	Variable	+
TSI (H2S)	- / Trace	Trace	+	-	+	-				Trace	Trace	+
Xylose	-	+	+	+	+	+				+	Variable (70%)	+

* *Salmonella* Pullorum has been merged with *Salmonella* Gallinarum and is no longer recognized as a unique serovar in the Kauffmann-White Scheme. To conform with its current classification within the Kauffmann-White Scheme, this organism would most appropriately be described as *Salmonella* Gallinarum biovar Pullorum. However, these organisms have distinct clinical presentations and the World Organization for Animal Health (OIE and many national ministries of agriculture still report and track these organisms separately. As accurate reporting is crucial for disease surveillance, we have listed these serovars individually.

APPENDIX V
GROWTH OF VARIOUS *ENTEROBACTERIACEAE* IN: Triple Sugar Iron Agar (TSI), Urea Agar (Urea), Lysine Iron Agar (LIA), Simmons Citrate Agar (Citrate), & Motility-Indol-Ornithine Agar (MIO)

SALMONELLA serovar NEWPORT
(Representative of most non-typhoidal serovars of *S. enterica*)

A B C D E F

A) TSI: Alkaline slant / Acid Butt / H_2S Positive / Gas (K / A_g $^{+++}$)

B) Urea: Negative

C) LIA: Lysine Decarboxylase Positive

D) Citrate: Positive

E) MIO: Motile / Ornithine Positive

F) MIO w/ indol reagent: Indol negative

SALMONELLA serovar TYPHI

A B C D E F

A) TSI: Alkaline slant / Acid Butt / Trace H_2S / No Gas (K / A ^{TR})

B) Urea: Negative

C) LIA: Lysine Decarboxylase Positive

D) Citrate: Negative

E) MIO: Motile / Ornithine Negative

F) MIO w/ indol reagent: Indol negative

Salmonella serovar Paratyphi A

A B C D E F

A) TSI: Alkaline slant / Acid Butt / No H_2S / Gas (K / A_g)

B) Urea: Negative

C) LIA: Lysine Decarboxylase Negative

D) Citrate: Negative

E) MIO: Motile / Ornithine Positive

F) MIO w/ indol reagent: Indol negative

Shigella sonnei

A B C D E F

A) TSI: Alkaline slant / Acid Butt / No H_2S / No Gas (K / A)

B) Urea: Negative

C) LIA: Lysine Decarboxylase Negative

D) Citrate: Negative

E) MIO: Nonmotile / Ornithine Positive

F) MIO w/ indol reagent: Indol negative

Shigella flexneri
(Also representative of most serotypes of *S. dysenteriae* & *S. boydii*)

A B C D E F

A) TSI: Alkaline slant / Acid Butt / No H_2S / No Gas (K / A)

B) Urea: Negative

C) LIA: Lysine Decarboxylase Negative

D) Citrate: Negative

E) MIO: Nonmotile / Ornithine Negative

F) MIO w/ indol reagent: Indol negative (most)

Proteus mirabilis

A B C D E F

A) TSI: Alkaline slant / Alkaline Butt / H_2S positive / No Gas (K / A $^{+++}$)

B) Urea: Positive (often in < 6 hours)

C) LIA: Lysine Deaminase Positive

D) Citrate: Negative (some strains positive)

E) MIO: Motile / Ornithine Positive

F) MIO w/ indol reagent: Indol negative

Citrobacter freundii

A B C D E F

A) TSI: Alkaline slant / Acid Butt / H_2S positive / Gas (K / A_g^{+++})

B) Urea: Negative (Some strains positive)

C) LIA: Lysine Decarboxylase Negative

D) Citrate: Positive

E) MIO: Motile / Ornithine Negative

F) MIO w/ indol reagent: Indol negative

Escherichia coli

A B C D E F

A) TSI: Acid slant / Acid Butt / No H_2S / Copious Gas (A / A_g)

B) Urea: Negative

C) LIA: Lysine Decarboxylase Positive

D) Citrate: Negative

E) MIO: Motile / Ornithine Positive

F) MIO w/ indol reagent: Indol positive

Pseudomonas aeruginosa

A B C D E F

A) TSI: No fermentation / No H_2S / No Gas

B) Urea: Negative

C) LIA: Lysine Decarboxylase / Deaminase Negative

D) Citrate: Positive

E) MIO: Nonmotile / Ornithine Positive

F) MIO w/ indol reagent: Indol negative

APPENDIX VI

Inoculation and Interpretation of Simmons Citrate Agar

Purpose

Simmons citrate agar is a synthetic medium containing inorganic ammonium salts as a nitrogen source and sodium citrate as a carbon source. It is used to distinguish members of the *Enterobacteriaceae* and other gram-negative rods on the basis of citrate utilization.

Policy

This assay is utilized for phenotypic characterization of bacteria.

Background

- Citrate utilization is one of several phenotypic assays (biochemical tests) utilized in the identification / characterisation of bacteria.
- Identifications are based on the interpretation of multiple phenotypic tests.

Reagents

A. Source:
Simmons citrate agar

B. Preparation procedure for reagent: follow instructions from the manufacturer.
None

C. Storage conditions:
Store at 4°C, not to exceed the expiration date on the label.

Equipment & Supplies

Inoculating loop / needle

Specimen

Fresh 18 - 24 h culture of organism to be identified.

Quality Control

Quality control testing is performed with each new lot and shipment of media.
Prior to use, the media is tested for sterility: uninoculated media should have no growth following 48 hours of incubation at 36°C (+/- 1°C). Each new lot / shipment of media must also produce expected reactions with QC organisms. The following reactions must be observed:

Positive: *Enterobacter aerogenes*
Negative: *Escherichia coli*

Note: Phenotypically equivalent strains may be substituted based on local availability.

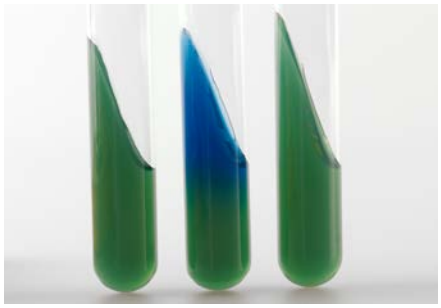
Procedure

A small amount of growth is harvested with a sterile (1µL) loop.
Lightly inoculate the surface of the agar slant.
Do not use a heavy inoculum.
Tubes are incubated under aerobic conditions at 36°C (+/- 1°C) with caps loosened.
Tubes should be examined and results recorded at 24 hours, 48 hours, and 3-5 days.

Interpretation/Results/Reporting

Positive - intense blue color (initially the color change may only occur on the agar slant)

Negative - agar remains green



A B C

A: Uninoculated agar
B: Citrate Positive (*Salmonella* serovar Newport)
C: Citrate Negative (*E. coli*)

Calculations

N/A

Expected Values

N/A

Method limitation

N/A

Procedure Notes

Utilization of cellular debris or residual media may produce false positive results. As such, it is critical to avoid over inoculating the agar and to use a fresh loop or needle.

References

Edwards, P. R. and W. H. Ewing. 1962. Identification of *Enterobacteriaceae*, 2nd ed. Burgess Publishing Co., Minneapolis.

MacFaddin, J. 1976. Biochemical Tests for the Identification of Medical Bacteria. P. 35-40.

Simmons Citrate Agar: There are many media providers. An example is BD BBL™ Simmons Citrate Agar (211620)

Appendices

N/A

APPENDIX VII

Inoculation and Interpretation of Lysine Iron (LIA) Agar

Purpose

LIA is used for the differentiation of *Enterobacteriaceae* and other gram negative rods.

Policy

This assay is utilized for phenotypic characterization of bacteria.

Background

- Bacterial identification are based on the interpretation of multiple phenotypic tests.
- LIA is one of several phenotypic assays (biochemical tests) utilized in the identification / characterisation of bacteria.
- LIA agar is utilized to detect hydrogen sulphide production, lysine decarboxylation and lysine deamination by enteric organisms.

Reagents

A. Source:
LIA agar slants

B. Preparation procedure for reagent: follow instructions from the manufacturer.

C. Storage conditions:
Store at 4°C, not to exceed the expiration date on the label.

Equipment & Supplies

Inoculating needle

Specimen

Fresh 18-24 h culture of organism to be identified.

Quality Control

Quality control testing is performed with each new lot and shipment of media.
Prior to use, the media is tested for sterility: uninoculate media should show no growth following 48 hours of incubation at 36°C (+/- 1°C). Each new lot / shipment of media must also produce expected reactions with QC organisms. The following reactions must be observed:

Bacteria	**Slant**	**Butt**	**H_2S**
Proteus mirabilis	Red	Yellow	-
Salmonella Typhimurium	Purple	Purple	+
Shigella flexneri	Purple	Yellow	-

Note: Phenotypically equivalent strains may be substituted based on local availability.

Procedure

A small amount of growth is harvested with a sterile inoculating needle.

Lightly inoculate the surface of the agar slant.

Make a single stab into the butt of the tube.

Tubes are incubated under aerobic conditions at 36°C (+/- 1°C) with caps loosened.

Tubes should be examined and results recorded at 24 hours, 48 hours, and 5-7 days (unless H_2S production occurs sooner).

Interpretation/Results/Reporting

A. H_2S production:
Positive - black color along the streak or throughout the medium
Negative - no black color

B. Lysine Decarboxylase (LDC):
Decarboxylation of lysine is detected in the butt of the tube.
LDC positive organisms will turn the agar in the butt of the tube purple.
LDC negative organisms will turn the agar in the butt of the tube yellow.

C. Lysine Deamination:
Lysine Deamination is detected on the agar slant.
Lysine deaminase positive organisms will turn the agar slant red.
Lysine deaminase negative organisms will turn the agar slant purple.

A B C D E

A: Uninoculated LIA
B: LDC negative /lysine deaminase negative/ H_2S negative (*Citrobacter freundii*)
C: LDC positive /lysine deaminase negative/ H_2S negative (*Salmonella* ser. Typhi)
D: LDC negative /lysine deaminase positive/ H_2S negative (*Proteus mirabilis*)
E: LDC positive /lysine deaminase negative/ H_2S positive (*Salmonella* ser. Newport)

Calculations

N/A

Expected Values

N/A

Method Limitation

LIA can detect H_2S production, however this media is less sensitive than triple sugar iron agar (TSI) or peptone iron agar (PIA). Many lysine decarboxylase negative organisms (e.g. *Citrobacter* or *Proteus*) fail to produce H_2S on LIA.

Procedure Notes

Do not use a loop to stab the agar as it will split the agar and give false-positive indications of gas production.

References

Faulkner, W. R. and J. W. King. 1970. Manual of Clinical Laboratory Practices, p. 291. Chemical Rubber Co., Cleveland.

220953 - BD BBL™ Lysine Iron Agar (LIA), Slants (100/sp)

Appendices

N/A

APPENDIX VIII

Inoculation and Interpretation of Motility-Indol-Ornithine Agar (MIO Agar)

Purpose

MIO agar is utilised to demonstrate motility, ornithine decarboxylase activity, and indol production.

Policy

This assay is utilized for phenotypic characterization of bacteria.

Background

- The demonstration of motility, ornithine decarboxylase activity, and indol production are performed in a single tube.
- Following overnight (18-24 hour) incubation, motility and ornithine decarboxylase activity are determined by visual examination.
- After the motility and ornithine decarboxylase results are interpreted, indol results are interpreted following the addition of Kovacs' reagent.

Reagents

A. Source
MIO agar
Kovacs' Indol Reagent

B. Preparation procedure for reagent: follow instructions from the manufacturer.

C. Storage conditions
Store at 4°C, not to exceed the expiration date on the label.

Equipment & Supplies

Inoculating needle

Specimen

Fresh 18 - 24 h culture of organism to be identified.

Quality Control

Quality control testing is performed with each new lot and shipment of media.
Prior to use, the media is tested for sterility: Uninoculated media should show no growth following 48 hours of incubation at 36°C (+/- 1°C). Each new lot / shipment of media must also produce expected reactions with QC organisms. The following reactions must be observed:

Motile / Ornithine Positive / Indol Positive: *E. coli*
Non-motile / Ornithine Negative / Indol Negative: *Shigella flexneri*

Note: Phenotypically equivalent strains may be substituted based on local availability.

Procedure

A small amount of growth is harvested with an inoculating needle.

Make a single stab into the tube of MIO agar. The stab should be made straight into the agar and stop approximately 1 cm from the bottom of the tube.

Do not make multiple stabs into the agar and do not twist the needle into the media.

Tubes are incubated under aerobic conditions at 36°C (+/- 1°C) with caps loosened.

Tubes should be examined and results recorded following overnight (18-24 hours) incubation.

Interpretation/Results/Reporting

Motility

Positive: Visible growth extending away from the stab line. Typically the agar will become visibly turbid.

Negative: Growth only along the stab line. The agar remains clear. Isolates which only produce small tufts of growth along stab line (similar to bristles on a brush) are considered non-motile.

Ornithine Decarboxylase

Positive: The agar in the middle of the tube turns a light, purple colour. These tubes are distinctly purple; however they will be a lighter shade of purple than their uninoculated counterparts.

Negative: The agar in the middle of the tube turns yellow. Only the colour of the agar in the middle of the tube should be noted. Oxidation may cause the agar on the surface of the tube to turn purple this is not significant.

Indol

IMPORTANT: Kovacs' reagent will cause the agar to turn yellow. Record motility and ornithine prior to adding indol reagent.
Add 3-4 drops of Kovacs' reagent to the surface of the tube.

Positive: Kovacs' reagent turns pink-red.

Negative: No colour change is observed. Kovacs' reagent remains orange-yellow.

A B C D E F

A: Uninoculated MIO tube
B: Indol Negative (*Shigella flexneri*)
C: Indol Positive (*E. coli*)
D: Motile / Ornithine Positive (*Salmonella* serovar Newport)
E: Nonmotile / Ornithine Negative (*Shigella flexneri*)
F: Nonmotile / Ornithine Positive (*Shigella sonnei*)

Calculations

N/A

Expected Values

N/A

Method Limitation

N/A

Procedure Notes

Kovacs' indol reagent will cause the agar to turn yellow. Record motility and ornithine prior to adding indol reagent.

References

Edwards, P. R. and W. H. Ewing. 1962. Identification of *Enterobacteriaceae*, 2nd ed. Burgess Publishing Co., Minneapolis.

Appendices

N/A

APPENDIX IX

Inoculation and Interpretation of Triple Sugar Iron (TSI) Agar

Purpose

TSI is used for the differentiation of *Enterobacteriaceae* and other gram negative rods.

Policy

This assay is utilized for phenotypic characterization of bacteria.

Background

- TSI is one of several phenotypic assays (biochemical tests) utilized in the identification / characterisation of bacteria.
- Identifications are based on the interpretation of multiple phenotypic tests.
- TSI contains three sugars: glucose (0.1%), lactose (1%), and sucrose (1%); pH indicator phenol red and ferrous sulfate to demonstrate H_2S production (by blackening of the medium).
- An alkaline slant (pink) and acid butt (yellow) occur when only glucose is fermented.
- *Salmonella* and *Shigella* are two pathogens which yield an alkaline slant and acid butt.

Reagents

A. Source
TSI agar slant

B. Preparation procedure for reagent
None

C. Storage conditions
Store at 4°C, not to exceed the expiration date on the label.

Equipment & Supplies

Inoculating needle

Specimen

Fresh 18 - 24 h culture of organism to be identified.

Quality Control

Quality control testing is performed with each new lot and shipment of media.
Prior to use, the media is tested for sterility: uninoculated media should show no growth following 48 hours of incubation at 36°C (+/- 1°C). Each new lot / shipment of media must also produce expected reactions with QC organisms. The following reactions must be observed:

Bacteria	Slant	Butt	Gas	H_2S
Enterobacter aerogenes (e.g. strain CDC 659-66)	Acid	Acid	+	-
Citrobacter freundii	Alkaline	Acid	+	+
Pseudomonas aeruginosa (e.g. ATCC 27853)	Alkaline	Alkaline	-	-

Note: Phenotypically equivalent strains may be utilized based on local availability.

Procedure

A small amount of growth is harvested with a sterile inoculating needle.

Lightly inoculate the surface of the agar slant.

Make a single stab into the butt of the tube.

Tubes are incubated under aerobic conditions at 36°C (+/- 1°C) with caps loosened.

Tubes should be examined and results recorded at 24 hours, 48 hours, and 5-7 days (unless H_2S production occurs sooner).

Interpretation:

A. Carbohydrate fermentation:
Alkaline slant/alkaline butt- no sugars fermented
Alkaline slant/acid butt- only glucose fermented
Acid slant/acid butt- glucose fermented along with lactose and/or sucrose

B. Gas production:
Positive- gas bubbles in agar or splitting of agar
Negative- no bubbles or splitting of agar

C. H_2S production:
Positive - black colour along the streak or throughout the medium
Negative - no black colour

Recording Results:

TSI results are recorded using the following notations:

Acidification is indicated with the capital letter “A”
Alkalization is indicated with the capital letter “K”
Gas production is indicated with a lower case letter “g”

Hydrogen sulphide production is indicated as follows:

“Tr”	= Trace amount of hydrogen sulphide
“+”	= Small to moderate amount of hydrogen sulphide
“+++”	= Large amount of hydrogen sulphide

The fermentation reactions on the slant and butt are recorded. The reactions are separated by a diagonal line. The gas production is noted in subscript and H2S production is noted in subscript.

Examples of typical TSI reactions are shown on the following page:

A B C D E F

A: Uninoculated TSI

B: *Salmonella* serovar Typhi K/A^{TR} (Alkaline slant / Acid Butt / Trace H_2S / No Gas)

C: *Salmonella* serovar Newport K/A_g^{+++}(Alkaline slant / Copious H_2S / Gas)

D: *Shigella flexneri* K/A (Alkaline slant / Acid Butt / No H_2S / No Gas)

E: *E. coli* A/A (Acid slant / Acid Butt / No H_2S / Copious Gas)

F: *Pseudomonas aeruginosa* (Non-fermenter / No H_2S / No Gas)

Calculations

N/A

Expected Values

N/A

Method Limitation

N/A

Procedure Notes

Use a needle to stab the agar. Do not use a loop, as it will split the agar and give false-positive indications of gas production.

References

Faulkner, W. R. and J. W. King. 1970. Manual of Clinical Laboratory Practices, p. 291. Chemical Rubber Co., Cleveland.

TSI : Biokar, reference : BK059HA, 500 g

Appendices

N/A

APPENDIX X

Inoculation and Interpretation of Christensen's Urea Test Medium (Urea Agar)

Purpose

Urea agar is used to differentiate organisms based on urease activity.

Policy

This assay is utilized for phenotypic characterization of bacteria.

Background

- Urease activity is one of several phenotypic assays (biochemical tests) utilized in the identification / characterization of bacteria.
- Identifications are based on the interpretation of multiple phenotypic tests.
- Organisms which produce urease split urea into carbon dioxide and ammonia.
- The ammonia combines with water to form ammonium carbonate which raises the pH of the medium. This pH shift is detected by the phenol red indicator (changes from salmon to pink)

Reagents

A. Source
Urea agar slant

B. Preparation procedure for reagent
None

C. Storage conditions
Store at 4C, not to exceed the expiration date on the label.

Equipment & Supplies

Inoculating needle

Specimen

Fresh 18 - 24 h culture of organism to be identified.

Quality Control

Quality control testing is performed with each new lot and shipment of media.
Prior to use, the media is tested for sterility: uninoculated media should show no growth following 48 hours of incubation at 36°C (+/- 1°C). Each new lot / shipment of media must also produce expected reactions with QC organisms. The following reactions must be observed:

Positive: *Providencia rettgeri*
Negative: *Enterobacter aerogenes*

Note: Phenotypically equivalent strains may be substituted based on local availability.

Procedure

A small amount of growth is harvested with a sterile (1uL) loop or needle.

Lightly inoculate the surface of the agar slant.

Tubes are incubated under aerobic conditions at 36°C (+/- 1°C) with caps loosened.

Tubes should be examined and results recorded at 24 hours, 48 hours, and 5-7 days.

Interpretation/ Results/Reporting

Positive - intense pink colour on the slant
Negative - no colour change

A B C

A: Uninoculated Urea Agar
B: Positive (*Proteus mirabilis*)
C: Negative (*E. coli*)

Calculations

N/A

Expected Values

N/A

Method Limitation

N/A

Procedure Notes

Many *Proteus* spp. will exhibit a positive reaction within 6 h.

References

Edwards, P. R. and W. H. Ewing. 1962. Identification of *Enterobacteriaceae*, 2nd ed. Burgess Publishing Co., Minneapolis.

Appendices

N/A