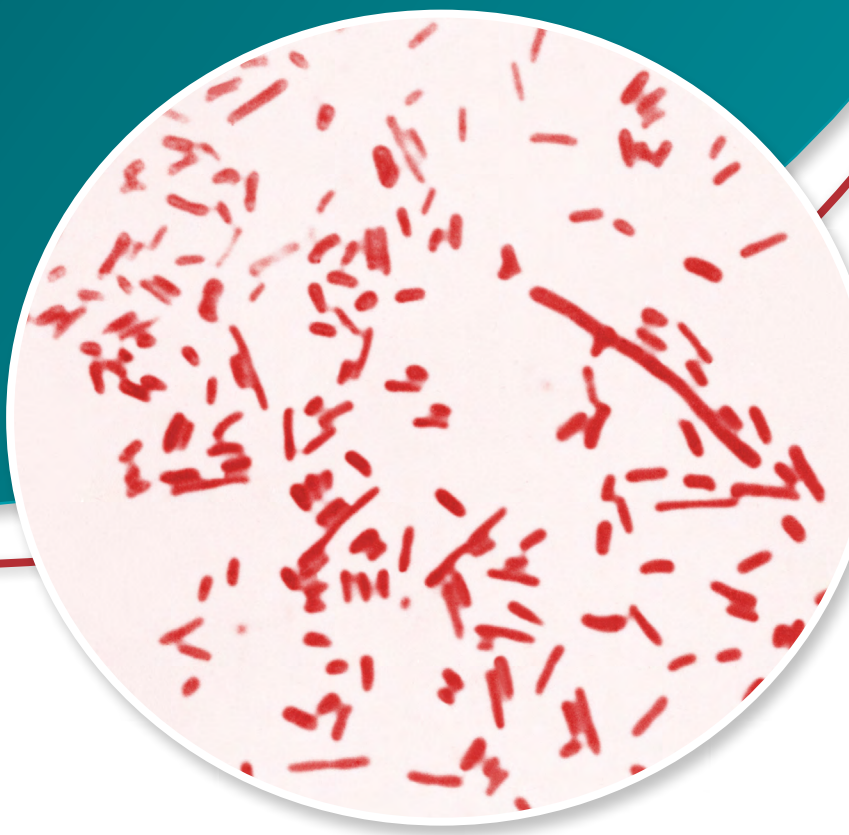
Identification Guide

Isolation and Identification of *Shigella* species and Enteroinvasive *Escherichia coli* from Culture-independent Diagnostic Test Positive Specimens



APHL ASSOCIATION OF PUBLIC HEALTH LABORATORIES®

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Introduction

Purpose

A recommended workflow for the culture and isolation of *Shigella* species from human fecal specimens identified as positive for *Shigella* and enteroinvasive *Escherichia coli* (EIEC) by a culture-independent diagnostic test (CIDT).

Background

The following is a procedure for the culture and isolation of *Shigella* species and EIEC from human feces. *Shigella* is a common etiologic agent of bacterial diarrhea and can cause severe or fatal infections. The genus *Shigella* is composed of four species: *S. dysenteriae* (subgroup A), *S. flexneri* (subgroup B), *S. boydii* (subgroup C) and *S. sonnei* (subgroup D). All species except *S. sonnei* are comprised of multiple serotypes, or subserotypes in the case of *S. flexneri*.[2] If resources permit, determination of the serotype of *Shigella* is useful for epidemiological purposes and can help identify clusters of cases or predict cases associate with foreign travel.

Sensitive and specific laboratory methods for the isolation, identification, serotyping and subtyping of *Shigella* are key to monitoring and control efforts. Advances in clinical diagnostic testing have led to the use of CIDTs, which provide rapid and sensitive testing for a variety of enteric pathogens. To detect *Shigella*, current enteric CIDTs target the Invasion Plasmid Antigen H (*ipaH*) gene, which is present in both *Shigella* and EIEC. Consequently, these assays return a positive result for *Shigella* as "positive for *Shigella*/EIEC." In addition to their shared virulence mechanisms, *Shigella* and EIEC have a high level of genetic and phenotypic similarity which makes their identification by traditional microbiology methods challenging.

EIEC infections cause similar symptoms to *Shigella* but tend to be milder. EIEC transmission is primarily via the fecal-oral route. It is difficult to determine the burden of EIEC due to the challenges of detecting and distinguishing EIEC from *Shigella*, but it appears to be much less common compared to *Shigella*.[1] The infective dose of EIEC is 1,000–10,000 times that of *Shigella*, which decreases the transmissibility of EIEC.[2]

While CIDTs can be a benefit to patient care by providing faster results, they do not yield isolates, which are necessary for public health testing and surveillance. Isolates are critical for subtyping, monitoring disease trends and identifying antibiotic resistance in public health laboratories (PHLs). In recent years, PHLs have seen a drastic increase in the volume of primary specimens that have tested positive for a reportable enteric pathogen when tested at a clinical laboratory using a CIDT platform for enteric pathogens. In many cases, the stool has tested positive for *Shigella*/EIEC at a clinical laboratory using a CIDT before submitting the stool specimen to a PHL for culture and further characterization. The goal of this document is to provide PHLs with methods to isolate and identify *Shigella* from stool specimens that have tested positive for *Shigella*/enteroinvasive *E. coli* as efficiently and cost-effectively as possible.

Fecal specimens are the preferred laboratory sample for diagnosis of infectious diarrhea. However, the recovery of enteric pathogens from feces is often complicated by multiple factors, including prior antibiotic treatment, transport stress, intermittent shedding of pathogens in the feces and a low inoculum of *Shigella* bacteria in relation to other enteric flora. These factors necessitate the use of culture algorithms that employ selective enrichment and the use of selective and differential media.

Selective enrichment suppresses fecal flora while allowing the target pathogen to grow. Selective media can also utilize phenotypic characteristics to preliminarily differentiate potential pathogens from fecal flora.

The number of media and reagents that can be used to identify *Shigella* continues to expand. It is important to routinely review literature and manufacturer product announcements.

Specimen Collection and Transport

The preferred specimen for *Shigella* culture is fresh stool collected in a nonnutritive transport media, such as Cary-Blair (CB).[7] Stool in Gram Negative (GN) broth or buffered glycerol saline is also acceptable.[1] Unpreserved stool and fecal or rectal swabs submitted in non-transport media may also be acceptable, however these specimens should be considered for rejection if transit time exceeds limits stated by the receiving laboratory (often two hours after specimen collection). If the unpreserved sample cannot reach the laboratory within the specified time, it is recommended that the raw stool cultures be transferred into a non-nutritive buffered transport medium (such as CB) and stored at 4°C to preserve pathogen viability. Rectal swabs are not a preferred sample and should only be utilized when the patient cannot produce a fecal sample. The rectal swab should be examined after collection; fecal matter should be visible on the swab. If fecal matter is not visible on the swab, the swab should not be submitted for culture.

Suspect *Shigella* specimens collected in CB transport media should be shipped at 2-8°C.[3] Ideally, specimens should be received as quickly as possible, not exceeding four days since collection as isolate recovery may decline. At the PHL, testing for *Shigella* should begin as soon as reasonably possible. Specimens should be held at 2–8°C until culture is completed.

Materials and Supplies

Media

- Selective/Differential Media (use at least one): Hektoen Enteric agar (HE), Xylose-Lysine-Deoxychocolate agar (XLD), *Salmonella-Shigella* agar (SS)
- Less-selective Media (use at least one): Blood agar (BAP), MacConkey Agar (MAC), Trypticase Soy Agar plate (TSA)
- Enrichment broths (optional): Gram-negative broth (GN), Selenite (SEL)
- Biochemicals for identification: Lysine decarboxylase (LDC), Motility, Glucose Gas, Acetate, Mucate, Lactose, Indole (Note: Some PHLs use API-20E in place of individual biochemicals, as well as Triple Sugar Iron (TSI), Ornithine, Mannitol and ONPG)

Before using media, review the manufacturer's instructions to determine appropriate incubation times and organisms for media quality control. All testing and media used for identification and isolation of *Shigella* should be performed according to the manufacturer's recommendations. See **Additional Comments (page 6)** for information about why specific media were included in the workflow.

For the optimal isolation of *Shigella*, two different selective media should be used: a general-purpose plating media with low selectivity (e.g., MAC) and a more selective agar medium (e.g., XLD). SS agar should be used with caution because it inhibits the growth of some strains of *Shigella,* including *S. sonnei* and *S. dysenteriae*.[1]

Other Supplies

- Inoculating loops
- Applicator swabs
- Supplies for MALDI-TOF (if performed)
- Serotyping regents (depending on method)
- PCR reagents (if *ipaH* PCR is performed)

Culture and Identification Workflows

The following describes specific workflows that could be utilized at PHLs. The Standard Workflow (Appendix A, page 8) is the most cost-effective, but it has reduced sensitivity for detection of *Shigella,* as it does not utilize an enrichment broth. The Optional Enrichment Broth workflow (Appendix B, page 9) uses more reagents and takes longer but it will increase the chances to recover *Shigella* spp. One study showed that using an enrichment broth nearly doubled the amount of *Shigella* recovered.[6] If using the Enrichment Broth workflow, it is recommended to incubate enrichment broth for six to eight hours prior to plating on solid media to reduce overgrowth of normal microbiota that may prevent recovery of potential pathogens.[1,4,5] Studies show that GN broth better supports *Shigella* growth compared to SEL.[5,6] PCR for the *ipaH* gene can be used to identify EIEC and *Shigella*, however additional methods will be needed to distinguish *Shigella* from EIEC. PHLs should choose the workflow(s) that best fits their surveillance needs and resources.

Standard Workflow

See "Appendix A: Standard Flow Diagram for Isolation and Identification of Shigella" (page 8).

Day 0

Stool specimen collected in transport media is received at the public health laboratory.

Inoculate at least one of the following selective media: XLD, HE, SS (**Step 1** in Appendix A) and one less selective media (BAP, TSA, MAC).

Day 1

1. Pick two or more suspicious colonies (or one of each colony type) to BAP or MAC plates and incubate overnight. (**Step 2** in Appendix A). Alternatively, some PHLs pick suspicious colonies directly to biochemicals including TSI, MIL, MIO, ONPG and TSA slant for serology (results are in Appendix E on page 11).
2. If there are no suspicious colonies, consider adding stool to an enrichment broth (see Optional Enrichment Broth Workflow on page 6 and Appendix B).
3. Some PHLs perform *ipaH* PCR on suspicious colonies. The *ipaH* gene is present in both *Shigella* and EIEC. One strategy for utilizing *ipaH* PCR is:
    - Pick two or more suspicious colonies (or one of each colony type) and perform *ipaH* PCR. In addition, perform PCR on a sweep of colonies from several quadrants from the plate.
    - If any of the picks are positive, subculture to a BAP or MAC for identification.
    - If only the sweep is positive, perform PCR on additional suspicious colonies to identify an *ipaH* positive colony.
    - If a colony tests positive for *ipaH* but is not identified as *Shigella* based on biochemical tests, it is a presumptive EIEC.

Day 2

Subculture growth from BAP or MAC to recommended biochemicals listed in Appendix E for identification and a TSA slant for serology to determine the species (**Step 3** in Appendix A). See Appendix E for a full list of biochemicals and expected reactions for *Shigella* and *E. coli*.

Note: Using biochemicals is the most common identification method; a list of other methods is in Appendix F (page 12).

Day 3

Observe results of biochemical reactions to identify *Shigella*. If *Shigella* is identified, begin species identification. Species identification using serology is the most common method, but a list of other species identification methods is in Appendix G (page 13).

Optional Enrichment Broth Workflow

See "Appendix B: Optional Enrichment Broth Flow Diagram for Isolation and Identification of Shigella*" (page 9).*

Day 0

Stool specimen collected in transport media is received at the public health laboratory.

Inoculate one of the following enrichment broths: GN or SEL (**Step 1** in Appendix B). It is recommended to incubate the broth for six to eight hours prior to plating on solid media to reduce overgrowth of normal microbiota that may prevent recovery of potential pathogens.

Day 1

Inoculate at least one of the following selective media: XLD, HE, SS and one less selective media (MAC, BAP, TSA) (**Step 2** in Appendix B). Some PHLs perform *ipaH* PCR on the inoculated broth.

Day 2

1. Pick two or more suspicious colonies (or one of each colony type) to BAP or MAC and incubate overnight. (**Step 3** in Appendix B). Alternatively, some PHLs pick suspicious colonies directly to biochemicals, including TTSI, MIL, MIO, ONPG and TSA slant for serology.
2. Some PHLs perform *ipaH* PCR on suspicious colonies. The *ipaH* gene is in both *Shigella* and EIEC.
    - Pick two or more suspicious colonies (or one of each colony type) and perform PCR that targets the *ipaH* gene. Perform PCR on a sweep of colonies from several quadrants from the plate.
    - If any of the picks are positive, subculture to a BAP or MAC for identification.
    - If only the sweep is positive, perform PCR on additional suspicious picks to identify an *ipaH* positive colony.
    - If an *ipaH* positive colony is identified as *E. coli*, it is EIEC.

Day 3

Subculture growth from BAP or MAC to recommended biochemicals in Appendix E for identification as a TSA for serology to determine the species (**Step 4** in Appendix B). See Appendix E for a full list of biochemicals and expected reactions for *Shigella*.

Day 4

Observe results of biochemicals to identify *Shigella*. If *Shigella* is identified, begin species identification. Species identification using serology is the most common method, but a list of other species identification methods is in Appendix F.

Additional Comments

One study where parallel plating was performed found that XLD was most efficient at detecting *Shigella* in stool specimens (90%), followed by HE (80%) and SS (68%).[6] The authors also observed that HE and SS agar produced twice the amount of false-positive colonies (non-*Shigella* colonies) as XLD.

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Acknowledgments

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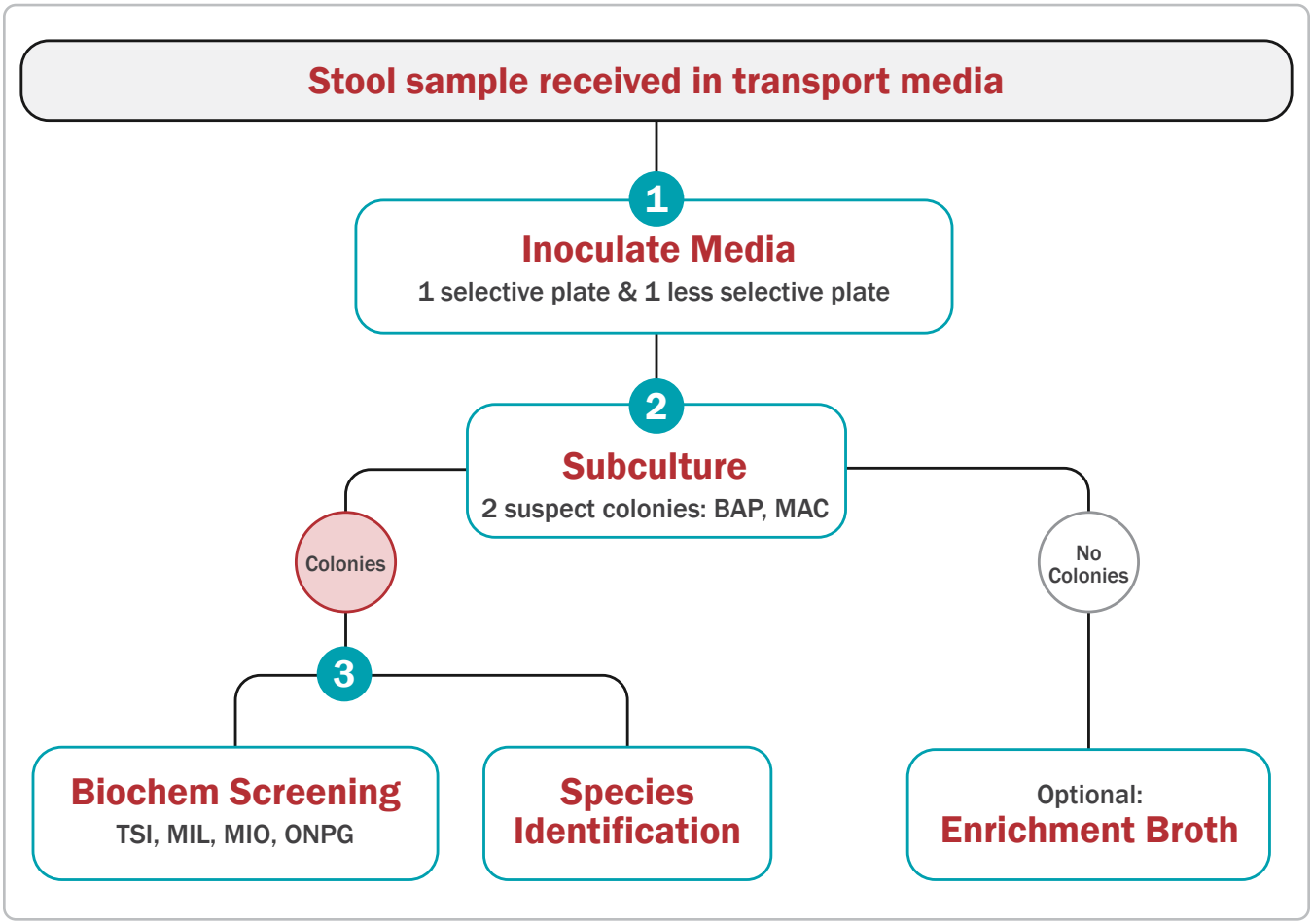
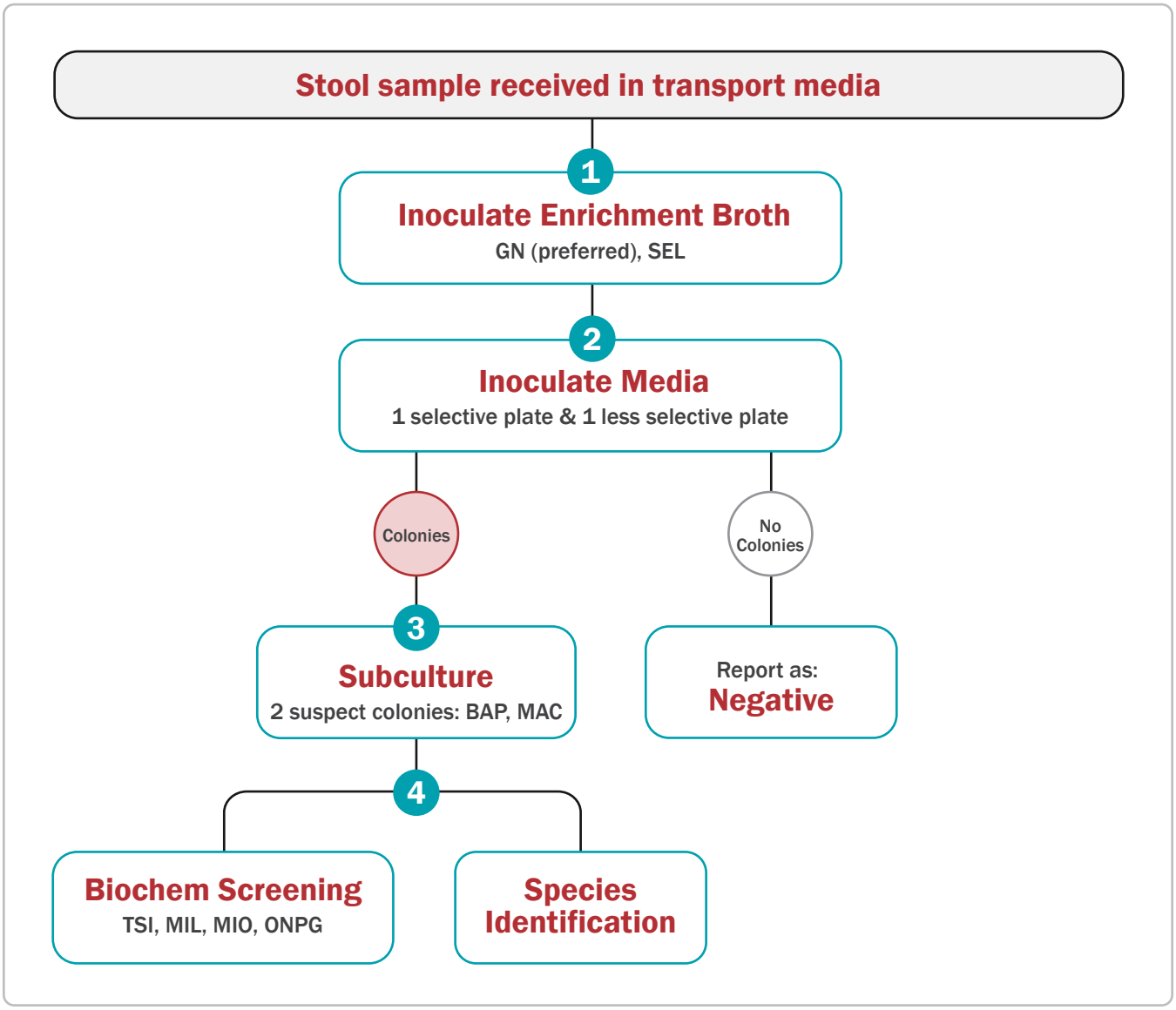
Appendix

Appendix A: Standard Flow Diagram for Isolation and Identification of *Shigella*



Appendix B: Optional Enrichment Broth Flow Diagram for Isolation and Identification of *Shigella*



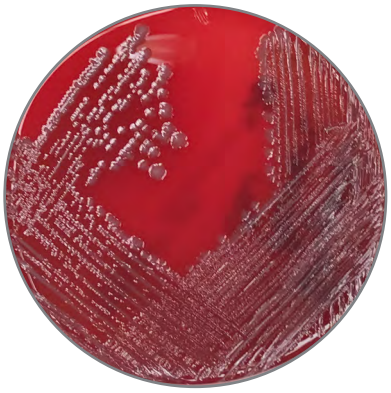
Appendix C: Typical Morphology Description of *Shigella* on Selective Media

Media	*Shigella**	*E. coli*
XLD	Red colonies	Small, yellowish colonies that may have bile precipitate
HE	Greenish blue colonies	Small, salmon-orange colonies that may have bile precipitate
SS	Colorless colonies	Small, pink to red colonies
MAC	Colorless colonies	Pink colonies

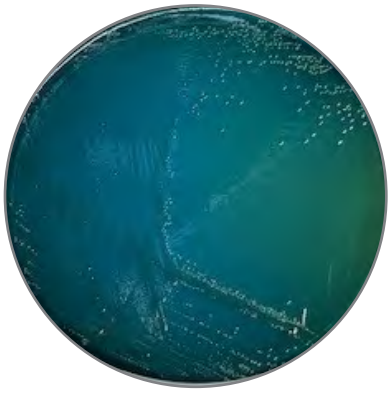
* Note that *S. sonnei* frequently shows two different colony morphologies on BAP and MAC media, a smooth morphology and a rough morphology.[7,8] This dual colony morphology is less frequently seen with non-*sonnei Shigella* spp. The difference of the colony morphologies is due to the presence or absence of the large and unstable pINV plasmid. The smooth colonies have retained the plasmid while the larger irregular colonies have lost the plasmid.[8]

Appendix D: Morphology of *Shigella* spp. on Non-selective and Differential Media

1. *S. flexneri* on BAP

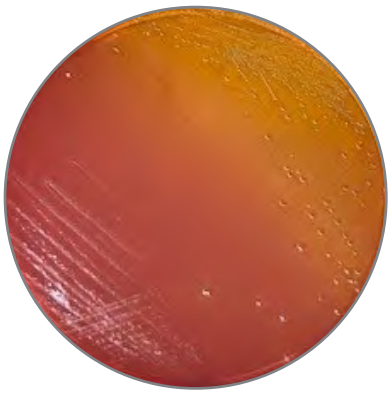


2. *S. sonnei* on HE



3. *S. flexneri* on MAC

4. *S. flexneri* on XLD



Appendix E: Typical Results of Biochemicals for *Shigella* and *E. coli*

Biochemicals	Reaction*		
	Shigella	*E. coli*	Inactive *E. coli*
Lysine decarboxylase	-	+	d†
Motility	-	+	-
Gas from glucose	-	+	-
Acetate	-	+	d
Mucate	-	+	d
Lactose	-	+	d
Indole	-	+	-
TSI (slant)	K	K	K
TSI (butt)	A	A	A
TSI (H2S)	-	-	-
TSI (gas)	-	+	-
Ornithine	+/- †	d	d
Mannitol	+ ‡	+	+
ONPG	+/- §	+	+

* For TSI: K = alkaline (red), A = acid (yellow)
d = different reactions [+, (+)(variable), -]

† 98% of *S. sonnei* are ornithine +, all other *Shigella* species are ornithine –

‡ All *S. dysenteriae* are mannitol –

§ *S. sonnei* is mostly ONPG +. *S. dysenteriae* is ONPG variable and *S. boydii* and *S. flexneri* are mostly ONPG -

Appendix F: Methods for *Shigella* Identification

Techniques	Pros	Cons
Biochemicals	• Common in PHLs to ID pathogens	• Time consuming and can be costly
MALDI-TOF	• Quick and cost effective • Already performed in many PHLs, so would add minimal changes	• Does not differentiate *E. coli* and *Shigella*, so additional methods are needed for identification.
PCR	• Quick and cost effective • Already performed in many PHLs, so would add minimal changes	• No current FDA-approved assays • Depending on the target, may not distinguish *Shigella* from EIEC.
WGS	• Routinely performed in PHLs • Can provide an indication of *Shigella* at the pathotype and serotype level • WGS is the established subtyping method for PulseNet	• Takes several days, which can delay reporting results • Current methods used by PulseNet, cannot reliably differentiate *E. coli* and *Shigella* • *Shigella* can be high volume during outbreaks; in those instances serotyping by WGS may be cost prohibitive
Wellcolex Slide Agglutination	• Quick and easy to perform • Can confirm and speciate *Shigella*	• Not commonly used in PHLs • Cannot identify very rare serotypes

Biochemicals

The traditional method for identifying *Shigella* is through biochemicals. Additional biochemicals may be needed to identify *Shigella*, see Appendix E for an expanded list of biochemicals that may be used for *Shigella* identification. Note that some PHLs use API-20E as opposed to individual biochemicals for identification. API-20E is easy to use but does carry an increased cost.

MALDI-TOF

MALDI-TOF is not routinely able to distinguish *Shigella* and *E. coli*. The ability to identify *Shigella* can differ between manufacturers and libraries. Some PHLs use MALDI-TOF as a screen and purity check for *Shigella*/*E. coli*.

PCR

There is not a widely used *Shigella*-specific PCR. Most PHLs that perform PCR target the invasion plasmid antigen H (*ipaH*) gene. *ipaH* is carried by all four *Shigella* species as well as EIEC. Some PHLs use *ipaH* as a screening method to identify potential *Shigella* colonies then use another method to complete identification.

WGS

Currently, PulseNet 2.0 predicts some species of *Shigella* based on correlation with other genotyping information output by the genotyping tool. New WGS analysis tools such as ECTyper,[10] ShigaPass, ShigaTyper and ShigEiFinder are also able to distinguish *E. coli* and *Shigella* spp. as well as predict their serotype. These tools could be evaluated for use in PHLs to identify *Shigella* spp. and predict their serotype. There are plans for PulseNet to add a module to identify *Shigella* species and their serotypes in the future.

Wellcolex

WellcolexTM Colour *Shigella* is a qualitative latex slide agglutination test for the detection and species identification of *Shigella* isolates present on solid culture media.[11] This assay cannot detect rare serotypes of *S. boydii*.

Appendix G: Common Methods to Identify the *Shigella* species

Determination of the species of *Shigella* is important for epidemiological purposes and can help to identify clusters of cases or travel.

Techniques	Pros	Cons
Antisera	• Routinely performed in PHLs • Relatively quick and easy to perform	• Requires stock of multiple antisera, including those rarely seen in the US
WGS	• Routinely performed in PHLs • Can provide an indication of *Shigella* at the pathotype and serotype level • WGS is the established subtyping method for PulseNet	• Takes several days, which can delay reporting results • Current methods used by PulseNet cannot reliably differentiate *E. coli* and *Shigella* • *Shigella* can be high volume during outbreaks; in those instances serotyping by WGS may be cost prohibitive
Wellcolex Slide Agglutination	• Quick and easy to perform • Can confirm and speciate *Shigella* • May be less expensive to use than antisera, depending on laboratory *Shigella* volume.	• Not commonly used in PHLs • Cannot identify very rare serotypes

Association of Public Health Laboratories

The Association of Public Health Laboratories (APHL) works to strengthen laboratory systems serving the public's health in the US and globally. APHL's member laboratories protect the public's health by monitoring and detecting infectious and foodborne diseases, environmental contaminants, terrorist agents, genetic disorders in newborns and other health threats.

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