U.S. FOOD & DRUG ADMINISTRATION

Bacteriological Analytical Manual

Chapter 6: *Shigella*

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Revision History

- March 2023: Section D, corrected a minor typo and added a new point of contact
- February 2013: Section D1: Enrichment of *Shigella sonnei* modified; use of waterbaths for incubation modified to use of forced air incubators
- June 2012: Table 1 restored
- May 2000 and October 2000: chapter revised

Introduction

Shigellosis, although commonly regarded as waterborne, is also a foodborne disease restricted primarily to higher primates, including humans. It is usually spread among humans by food handlers with poor personal hygiene. Foods most often incriminated in the transmission have been potato salad, shellfish, raw vegetables, and Mexican dishes.

The genus *Shigella* consists of four species: *S. dysenteriae* (subgroup A), *S. flexneri* (subgroup B), *S. boydii* (subgroup C), and *S. sonnei* (subgroup D). *Shigella* organisms may be very difficult to distinguish biochemically from *Escherichia coli.* Brenner (1) considers *Shigella* organisms and *E. coli* to be a single species, based on DNA homology. Nonetheless, *Shigella* species are Gram-negative, facultatively anaerobic, nonsporulating, nonmotile rods in the family *Enterobacteriaceae*. They do not decarboxylate lysine or ferment lactose within 2 days. They utilize glucose and other carbohydrates, producing acid but not gas. However, because of their affinity to *E. coli*, frequent exceptions may be encountered, e.g., some biotypes produce gas from glucose and mannitol. Neither citrate nor malonate is used as the sole carbon source for growth, and the organisms are inhibited by potassium cyanide.

A. Equipment and Materials

1. Same as for *Salmonella,* Chapter 5
2. Forced air incubators, maintained at 42.0 ± 0.3°C and 44.0 ± 0.3°C
3. Anaerobic jar
4. Anaerobic gas generating pouch/sachet
5. Anaerobic indicator

B. Media

1. *Shigella* broth with novobiocin (M136)
2. Trypticase soy-yeast extract (TSYE) broth (M157)
3. MacConkey agar (M91)
4. Triple sugar iron (TSI) agar (M149)
5. Urea broth (M171)
6. Motility test medium (semisolid) (M103)
7. Potassium cyanide (KCN) broth (M126)
8. Malonate broth (M92)
9. Tryptone (tryptophane) broth, 1% (M164)
10. MR-VP broth (M104)
11. Christensen citrate agar (M39)
12. Veal infusion agar (M173)
13. Bromcresol purple broth (M26) supplemented with the following carbohydrates, each at a level of 0.5%: adonitol, salicin, rhamnose, glucose, inositol, lactose, mannitol, raffinose, sucrose, xylose, dulcitol, and glycerol.
14. Acetate agar (M3)
15. Mucate broth (M105)
16. Mucate control broth (M106)
17. Decarboxylase basal medium (lysine, Falkow) (M44)
18. Decarboxylase basal medium (ornithine)(M44)

C. Reagents and stains

1. Kovacs' reagent (R38)
2. Voges-Proskauer test reagents (R89)
3. 1 N Sodium hydroxide solution (R73)
4. 1 N Hydrochloric acid (R36)
5. Methyl red indicator (R44)
6. Physiological saline solution, 0.85% (sterile) (R63)
7. Novobiocin
8. Polyvalent *Shigella* antisera for groups A, A_1, B, C, C_1, C_2, D and Alkalescens-Dispar biotypes 1-4
9. Gram stain reagents(R32)

D. Enrichment

Two approaches are provided for the recovery of *Shigella*. The first approach is a conventional culture method that involves the use of a specially formulated medium, *Shigella* broth. Novobiocin is added to provide a selective environment. Sample enrichments are incubated as described below, and streaked to MacConkey agar. Typical colonies are biochemically and serologically confirmed as *Shigella* spp.

The second approach uses DNA hybridization. The enzyme DNA gyrase induces negative supercoiling into closed circular DNA. It has been reported, however, that novobiocin inhibits

DNA gyrase (3). Thus, the use of novobiocin in *Shigella* broth may cause this medium to be incompatible with DNA hybridization for detecting *Shigella*. Because DNA hybridization can detect *Shigella* in the presence of overwhelming numbers of competitors, a selective agent such as novobiocin is not needed in the enrichment medium and may actually be counterproductive. Thus, the use of tryptic soy broth with yeast extract added (TSYE) to a final concentration of 0.6% is the recommended enrichment, if DNA hybridization is being used.

1. **Conventional culture method**

    a. **Enrichment of *Shigella sonnei***. Aseptically weigh 25 g sample into 225 ml *Shigella* broth to which novobiocin (0.5 µg/ml) has been added. Hold suspension 10 min at room temperature and shake periodically. Pour supernatant into sterile 500 ml Erlenmeyer flask. Adjust pH, if necessary, to 7.0 ± 0.2 with sterile 1 N NaOH or 1 N HCl. Place flask in anaerobic jar, insert anaerobic gas generating pouch/sachet (use number recommended by the anaerobic jar manufacturer, according to the volume of the jar), insert an anaerobic indicator, and tighten the lid. Incubate jars at 44.0°C in a forced air incubator for 20 h. Agitate enrichment culture suspension and streak on a MacConkey agar plate. Incubate 20 h at 35°C.

    b. Enrichment of other *Shigella* species. Proceed as above, but use novobiocin at 3.0 µg/ml and incubate anaerobically at 42.0°C in a forced air incubator.

2. **DNA hybridization method**

    Aseptically weigh 25 g sample into 225 ml TSYE. Hold suspension 10 min at room temperature and shake periodically. Pour supernatant into sterile 500 ml Erlenmeyer flask and adjust pH, if necessary, to 7.0 ± 0.2 with sterile 1 N NaOH or 1 N HCl. Incubate sample enrichment 20-24 h at 35-37°C.

E. Isolation of Shigella species

1. **Conventional culture method**

Examine MacConkey agar plates. *Shigella* colonies are slightly pink and translucent, with or without rough edges. Inoculate suspicious colonies into the following media: glucose broth, TSI agar slant, lysine decarboxylase broth , motility agar, and tryptone. Incubate at 35°C for 48 h, but examine at 20 h. Discard all cultures showing motility, H_2S, gas formation, lysine decarboxylation, and fermentation of sucrose or lactose. With respect to indole formation, discard positive cultures from 44.0°C enrichment. All suspicious isolates from 42°C enrichment may be either positive or negative and consequently should be retained.

2. **DNA hybridization method.**

Proceed as described in Chapter 24.

F. Physiological Characterization

Perform Gram stain and inoculate cultures giving satisfactory screening reactions to the other recommended biochemicals. The characteristics of *Shigella* are summarized as follows: Gram-negative rods; negative for H_2S, urease, glucose (gas), motility, lysine decarboxylase, sucrose, adonitol, inositol, lactose (2 days), KCN, malonate, citrate, and salicin; positive for methyl red. Pick isolates having positive reactions for *Shigella* to veal infusion agar slants. Use antisera for identification of serotype or compare with physiological behavior of the 32 serotypes presented in Table 1. If serotype cannot be identified by these tests, two explanations are possible: 1) Several provisional serotypes have not been accepted by an international commission on the taxonomy of *Shigella* species. Resolve by referral to the U.S. Centers for Disease Control and Prevention (CDC), Atlanta, GA, or to the World Health Organization (WHO), *Shigella* spp. Reference Laboratories. 2) The cultures may be *E. coli*. Proper interpretation of the mucate and acetate reactions should help. *Shigella* species tend to be negative in all these reactions, whereas anaerogenic *E. coli* tend to be positive in at least one of the reactions (Table 2) (2).

Table 1. Biochemical reactions of serotypes of Shigella[a]

Subgroup and serotype	Mannitol	%+	Dulcitol	%+	Xylose	%+	Rhamnose	%+	Raffinose	%+	Glycerol	%+	Indole	%+	Ornithine decarboxylase	%+
Subgroup A - *S. dysenteriae*																
1	–	0	–	0	–	0	–	0	–	0	+or(+)	100	–	0	–	0
2	–	0	–	0	–	0	+	98	–	0	(+)or+	98	+	100	–	0
3	–	0	–	0	–	0	–	0	–	0	(+)or+	100	–	0	–	0
4	–	0	–	0	–	0	–	0	–	0	(+)or+	100	–	0	–	0
5	–	0	+or(+)	100	–	0	–	0	–	0	+or(+)	100	–	0	–	0
6	–	0	–	0	–	0	–	0	–	0	–or(+)	38	–	0	–	0
7	–	0	–	0	–	0	(+)or+	90	–	0	–	0	+	100	–	0
8	–	0	–	0	+or(+)	96	–	8	–	0	+or(+)	100	+	100	–	0
9	–	0	–	0	–	0	–	0	–	0	+or(+)	100	–	0	–	0
10	–	0	–	0	+	100	–	0	–	0	–	0	–	0	–	0
Subgroup B - *S. flexneri*																
1	+	95	–	0	–	0	–	0	D	89	–	0	–or+	35	–	0
2	+	99	–	0	–	0	–	0	D	77	–	0	–or+	44	–	0
3	+	98	–	0	–	0	D	12	D	88	–	0	+or–	88	–	0
4	+	99	–	0	–	0	D	23	D	82	–	0	+or–	55	–	0
4	–	0	–	0	D	71	–or+	48	–	3	–	0	+	98	–	0
5	+	99	–	0	–	0	–	S	D	72	–	0	+	95	–	0
6	+	>99	D	80	–	4	–	6	–	0	D	88	–	0	–	0
6[b]	+	100	D	86	D	75	–	0	–	0	+or(+)	100	–	0	–	0
6[b]	–	0	+or(+)	100	–	0	–	0	–	0	(+)	100	–	0	–	0
Subgroup C - *S. boydii*																
1	+	100	–	1	+or(+)	97	–	0	–	0	(+)or+	96	–	0	–	0
2	+	100	–	1	–	0	–	0	–	0	+or(+)	100	–	0	–	0
3	+	100	D	75	D	86	–	0	–	0	+or(+)	91	–	0	–	0
4	+	99	–or(+)	28	–	0	–	0	–	0	+or(+)	100	–	0	–	0
5	+	100	–	0	(+)	94	–	0	–	0	D	61	+	100	–	0
6	+or(+)	100	(+)or+	100	+	100	–	0	–	0	(+)or+	100	–	0	–	0
7	+	100	–	0	+	98	–	0	–	0	(+)or+	98	+	100	–	0
8	+	100	–	0	+	94	–	0	–	0	(+)or+	100	–	0	–	0
9	+	95	–	0	–	0	D	80	–	0	(+)or –	82	+	100	–	0
10	+	94	+	100	D	84	–	0	–	0	(+)or+	100	–	0	–	0
11	+	100	–or(+)	34	+or(+)	100	–	0	–	0	(+)or+	100	+	100	–	0
12	+	100	–or(+)	14	–	0	–	0	–	0	–or+	14	–	0	–	0
13	+	100	–	0	–	0	–	0	–	0	(+)or–	63	+	100	+	100
14	–or+	29	–	0	(+)or+	100	–	0	–	0	+or(+)	100	–	0	–	0
15	+	90	–	0	–	0	–	0	–	0	(+)or–	64	+	100	–	0
Subgroup D - *S. sonnei*	+	99	–	1	–	1	+or(+)	98	D	84	D	46	–	0	+	*>99*

[a] +, 90% or more positive in 1 or 2 days; –, 90% or more negative; + or –, majority positive; – or +, majority negative; (+) delayed positive; D, different reactions [+, (+), –].

[b] Some *S. flexneri* 6 cultures (the Newcastle and Manchester biotypes) produce gas from fermentable substrates; other shigellae are anaerogenic.

NOTE: In this table percentages of + and (+) reactions are combined.

Ref: Ewing, W.H. 1986. Edwards and Ewing's Identification of Enterobacteriaceae, 4th ed., pp.146-147. Elsevier, New York. Reproduced with permission.

Table 2. Reactions of *Shigella* and *Escherichia coli* in acetate, citrate, and mucate media[a,b]

Genera and species	Sodium acetate	%+	(%+)	Christensen's citrate	%+	(%+)	Sodium mucate	%+	(%+)
S. dysenteriae	−	0	0	−	0	0	−	0	0
S. flexneri	−	0	0	−	0	0	−	0	0
S. boydii	−	0	0	−	0	0	−	0	0
S. sonnei	−	0	0	−	0	0	D	6.4	(30.3)
E. coli	+ or (+)	83.8	(9.7)	D	15.8	(18.4)	+	91.6	(1.4)
Alkalescens-Dispar biotypes	+ or (+)	89.6	(4.7)	D	75	(12.5)	D	29.5	(27.

[a]+, 90% or more positive in 1 or 2 days; −, 90% or more negative; + or −, majority positive; − or +, majority negative; (+) delayed positive; D, different reactions [+,(+), −].
[b]From ref. 2. Reproduced with permission.

G. Serological characterization

Suspend growth from 24 h veal infusion agar slant in 3 ml 0.85% saline to McFarland Turbidity Standard No. 5. Mark nine 3 × 1 cm rectangles on clear glass petri dish with wax pencil. Add drops of suspension, antisera, and saline in accordance with the following protocol.

Polyvalent antiserum: A, A_1, B, C, C_1, C_2, D, A-D

Rectangle	Suspension	A	A_1	B	C	C_1	C_2	D	A - D	Saline
1	+	+								
2	+		+							
3	+			+						
4	+				+					
5	+					+				
6	+						+			
7	+							+		
8	+								+	
9	+									+

Mix contents of each rectangle with a needle, taking care that no mixing between rectangles occurs. Rock petri dish 3-4 min to accelerate agglutination. Read extent of agglutination as follows: 0 = no agglutination; 1+ = barely detectable agglutination; 2+ = agglutination with 50% clearing; 3+ = agglutination with 75% clearing; 4+ = visible floc with suspending fluid totally cleared. Re-examine suspension in monovalent sera belonging to each polyvalent in which a distinct positive reaction (2+, 3+, 4+) has occurred. In the event of a negative reaction, heat suspension in steamer 30 min to hydrolyze interfering capsular antigen. Re-examine in polyvalent, and, if positive, in corresponding monovalent sera. Because of tentative serotypes, a negative reaction may occur with the available sera. Consequently, it is advised that cultures retrieved from an outbreak and suspect foods giving *Shigella*-like reactions in physiological tests be referred to the CDC or to a WHO *Shigella* laboratory for confirmation.

References

1. Brenner, D.J. 1984. Family I. *Enterobacteriaceae,* pp.408-420. *In*: Bergey's Manual of Systematic Bacteriology, Vol. 1. N.R. Krieg (ed). Williams & Wilkins, Baltimore.

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3. Mehlman, I.J., Romero, A., and Wentz, B.A. 1985. Improved Enrichment for Recovery of Shigella sonnei from Foods. *J. Assoc. Off. Anal. Chem*. **68**(3):552-555.

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