

Hektoen Enteric Agar Protocol

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Information

History

During the 19th century, the connection was made between disease processes and bacteria. Ever since that time, microbiologists have searched for media that allow the rapid recovery of pathogenic organisms from clinical specimens, as well as differentiation between the pathogens and nonpathogens.

At the beginning of the 20th century, microbiologists referred to all non-spore-forming gram-negative bacilli as enteric organisms due to their prevalence in the intestinal tract. However, they recognized that certain species of these enteric organisms were more pathogenic to humans than others (12). The microbiologists also realized that these enteric organisms had distinct patterns of carbohydrate utilization and that those enteric organisms that could not utilize lactose were most likely pathogenic to humans. They began to develop media that could differentiate between lactose-fermenting and non-lactose-fermenting organisms. The first isolation media introduced for the recovery of pathogenic enteric organisms was called fuchsin sulfite infusion agar and was developed by S. Endo in 1903 (2). This media allowed the isolation of these pathogens, but did not inhibit the normal, nonpathogenic enteric organisms also present in feces. Discrimination between the lactose-fermenting enteric organisms and the pathogenic organisms (which are usually non-lactose-fermenting) was difficult because the red color formed by the fermentation of lactose occurred in the media and not in the colonies. When organisms were close together on the agar plate, it was difficult to tell which ones fermented lactose and which ones did not (2). In 1905, MacConkey agar was introduced by Alfred MacConkey (7). MacConkey, utilizing the work done by Fermi in 1898 on the effects of various chemical substances on the inhibition of bacteria (7), added bile in the form of sodium taurocholate to his media. The bile inhibited the gram-positive nonpathogenic enteric organisms allowing only gram-negative rods to grow. The addition of lactose and the dye neutral red allowed differentiation by color of the lactose-fermenting (nonpathogenic) and the non-lactose-fermenting (usually pathogenic) organisms. Eosin methylene blue (EMB) agar followed in 1916, introduced by J. E. Holt-Harris and Oscar Teague (2). EMB media allowed a visual distinction between *Bacillus coli* (now known as *Escherichia coli*), other nonpathogenic lactose-fermenting enteric gram-negative rods, and the *Salmonella* and *Shigella* genera. These media allowed the recovery

and differentiation of suspect organisms belonging to the genera *Salmonella* and *Shigella*. The advantage of both MacConkey and EMB media over Endo agar was that the color change produced by the fermentation of lactose appeared in the colony itself, and not the media, making the pathogens easily distinguishable from the normal enteric organisms in the specimen (2, 7). The color was present in the colonies rather than in the media so even colonies growing in close proximity to one another could be differentiated.

MacConkey and EMB media are only moderately inhibitory and most enteric gram-negative rods grow readily on both, occasionally obscuring the pathogenic organisms. Thus, more selective media was needed to enhance the recovery of *Salmonella* and *Shigella* from contaminated specimens. Microbiologists soon began searching for more selective media (4). They looked for new inhibitory agents and they added higher concentrations of known inhibitory substances to their media to inhibit the growth of nonpathogenic enteric organisms. In 1916, Teague and A. W. Clurman determined that brilliant green dye inhibited most of the nonpathogenic enteric gram-negative rods (11). Their medium, brilliant green agar, enhanced the recovery of *Salmonella* from patients with typhoid fever. Einar Leifson described desoxycholate media in 1935 (5). He used desoxycholic acid and its salts as the inhibitory agent. In 1941, Catherine Mayfield and Maud Gober developed Salmonella Shigella agar (8). Unfortunately, this media was discovered to be overly selective and some strains of *Shigella* were missed (4, 10). In 1965, xylose lysine decarboxylase agar was introduced by Welton I. Taylor for the enhanced recovery of *Shigella* (4). Most recently, Hektoen enteric (HE) agar was introduced in 1968 as another option in the arsenal of selective and differential media utilized by clinical microbiologists trying to recover *Salmonella* and *Shigella* from clinical specimens (4).

Sylvia King and William I. Metzger, working at the Hektoen Institute in Chicago, formulated HE agar (4). Their goal was to increase the recovery of *Shigella* species from mixed cultures. They enriched the media with extra amounts of carbohydrates and peptones to offset the inhibitory effects of the bile salts. The two dyes added to the media, bromthymol blue and acid fuchsin, have lower toxicity than other dyes, thus pathogen recovery was improved (4). HE agar is currently used as both a direct and indirect plating medium for fecal specimens to enhance the recovery of species of *Salmonella* and *Shigella* from mixed normal fecal flora.

Modification and refinement by later microbiologists of their predecessors' formulas has been ongoing. As a result, much of the media discussed in this history is still available and is still used in microbiology labs around the world. In addition, new selective and differential media for *Salmonella* and *Shigella* continues to be developed and made available for use by clinical microbiologists (12).

Purpose

Hektoen enteric agar is used to recover gastrointestinal pathogens, such as *Salmonella* and *Shigella*, from food, water, and fecal samples

suspected of containing these organisms. Because of its selective nature, it inhibits most nonpathogenic enteric organisms and thus is used in clinical microbiology to recover *Salmonella* and *Shigella* from feces. It is also a differential medium that allows microbiologists to note visual differences in colony morphology and quickly eliminate nonpathogenic gram-negative rods from pathogenic gram-negative rods with minimal additional testing.

HE agar can be used for the primary plating of fecal specimens. It may also be used to subculture the overnight growth from enrichment broths (such as gram-negative broth or selenite broth) inoculated with fecal specimens suspected of containing low numbers of *Salmonella*. Direct inoculation of colonies from agar plates may produce sufficient growth of organisms that would otherwise be inhibited in a more dilute inoculum from diarrheal feces or broth culture.

Theory

Hektoen enteric agar is a selective and differential media for the isolation and differentiation of enteric pathogens from clinical specimens. Animal peptones and yeast extract provide the nutritive base (Hektoen enteric agar instructions for use package insert; Remel, Lenexa, KS).

The presence of the bile salts and dyes inhibit most gram-positive organisms allowing only gram-negative rods to grow on HE agar (4, 5, 7, 9, 11). The high concentration of bile salts partially or fully inhibits most of the nonpathogenic coliform flora of the intestinal tract (4, 5, 7, 9, 11). Since the enteric pathogens *Salmonella* and *Shigella* can tolerate these inhibitory substances they generally grow faster and larger than the coliforms.

The fermentation of carbohydrates such as lactose, sucrose, and salicin, is one of the differentiating characteristics used to identify the coliforms. *Salmonella* and *Shigella* are unable to utilize these three specific carbohydrates, whereas most nonpathogenic coliforms can use at least one of them. Thus, the nonpathogenic coliforms, if they are able to grow in the presence of the bile salts, will produce orange-yellow colonies due to the production of acid from at least one of the carbohydrates. This acid causes the bromthymol blue indicator to change from its neutral green color to an orange-yellow color. The bile salts may precipitate out of the media and appear as a hazy zone around the colonies. This is due to the acid produced by the utilization of the lactose, sucrose, or salicin interacting with the bile salts present in the media (6). If a lactose- and sucrose-negative organism utilizes salicin, salmon-pink to orange-yellow colonies will be present. The inability of *Salmonella* and *Shigella* to produce acid from the utilization of lactose, sucrose, or salicin results in colonies that are translucent, light green, or greenish blue and allows them to be quickly differentiated from nonpathogenic organisms. Additional testing must then be performed on these colonies to confirm or rule out the presence of *Salmonella* or *Shigella*.

The production of H₂S by certain enteric gram-negative rods, such

as *Salmonella*, can be detected on HE agar due to the addition of thiosulfate and ferric ammonium citrate to the formula. *Salmonella* produces bacterial enzymes that cause a sulfide molecule to be released from the thiosulfate present in the media. This sulfide molecule then couples with a hydrogen ion to form H₂S gas. The H₂S gas reacts with the ferric ammonium citrate, forming a precipitate, resulting in colonies that are black or have a black center (12). Other nonpathogenic enteric organisms, such as *Proteus* sp. and *Citrobacter freundii*, also produce H₂S, but these organisms are usually inhibited by the bile salts in the HE agar. If these organisms can overcome the inhibitory effects of the bile salts and grow, they usually can be differentiated from the pathogens because *Proteus* and *Citrobacter freundii* can utilize at least one of the carbohydrates present in the HE agar. An orange-yellow colony with a black center is most likely not an intestinal pathogen, although rare strains of *Salmonella* are capable of lactose fermentation and would appear this way.

Since HE agar is primarily a screening agar, additional testing is required to confirm or rule out *Salmonella* or *Shigella*. Several options are available for confirmatory testing ranging from commercial identification kits (e.g., API 20E, MicroID, Enterotube, Microscan panels) to tubed biochemicals (e.g., TSI agar, KIA agar, LIA agar, urea agar, lysine decarboxylase) to serological typing of somatic and capsular antigens.

RECIPE

Hektoen enteric agar may be purchased as prepared agar plates from various suppliers of microbiological media. Follow the manufacturer's recommendation for quality control and storage of prepared plates.

Hektoen enteric agar can also be prepared from dehydrated powder available from various suppliers of dehydrated media. Be sure to prepare the medium according to the manufacturer's directions.

Ingredients for Hektoen enteric agar per liter of purified water (1)

Proteose, peptone	12 g	Sodium chloride	5 g
Yeast extract	3 g	Sodium thiosulfate	5 g
Bile salts no.3	9 g	Ferric ammonium citrate	1.5 g
Lactose	12 g	Agar	14 g
Saccharose	12 g	Bromthymol blue	0.06 g
Salicin	2 g	Acid fuchsin	0.1 g

Suspend the components listed above in 1 liter of purified water. Mix thoroughly. Heat with frequent agitation to boiling to completely dissolve the components. Do not overheat. Do not autoclave. Dispense into 100-mm diameter sterile petri dishes, allowing approximately 20 to 25 ml of liquid per plate. Allow to solidify at room temperature, then store at 4 to 8°C in plastic to minimize dehydration during storage. Minimize exposure of the plates to light. Hektoen enteric agar is stable for approximately 70 days from the date of preparation (Remel Technical Services, personal communication). Each lab should verify the quality and functionality of each batch of prepared media by testing known strains of organisms periodically as the 70-day expiration date approaches.

Quality assurance procedures (1)

Appearance:

- The dehydrated powder should appear light purplish beige, homogeneous, and free flowing.
- The prepared medium should appear brown with a greenish cast and slightly opalescent prior to pouring the plates.
- The prepared plates should appear green with a yellowish cast and slightly opalescent. The agar surface should be smooth and moist, but without excessive moisture. Do not use plates if drying or cracking of the agar is apparent, or if there is evidence of microbial contamination.
- The pH must be 7.5 ± 0.2 at 25°C for optimum results.

Performance characteristics:

Once the HE agar plates have solidified, several plates should be removed from each batch and tested against organisms with known characteristics. Recommended organisms and their growth characteristics are shown in Table 1. Using a sterilized inoculating loop streak each organism onto a plate so that the growth of isolated colonies is achieved. Incubate 18 to 24 hours at 35°C and examine the plates for growth. If the expected results are not seen, do not use the media.

TABLE 1. Recommended quality control organisms and expected reactions (1) (Hektoen enteric agar instructions for use package insert; Remel, Lenexa, KS)

Organism	Growth	Color of colony
<i>Enterobacter aerogenes</i> ATCC ^a 13048	Fair to good	Orange-yellow
<i>Escherichia coli</i> ATCC 25922	None to fair	Orange-yellow (may have bile precip
<i>Salmonella enterica</i> ATCC 13076	Excellent	Greenish-blue ^b
<i>Salmonella typhimurium</i> ATCC 14028	Excellent	Greenish-blue ^b
<i>Shigella flexneri</i> ATCC 12022	Excellent	Greenish-blue
<i>Streptococcus faecalis</i> ATCC 29212	None	

^aAmerican Type Culture Collection, Manassas, VA

^bMay have a black center due to H_2S production.

PROTOCOL

1. Streak a plate of HE agar using the quadrant streak plate method (3) to obtain isolated colonies. Well-isolated colonies will provide the best results in the biochemical differentiation of bacteria using HE agar. The inoculum may be obtained from several different sources. Follow the instructions below for the source that meets the requirements of your laboratory activity.

a. Source: a previously inoculated and incubated culture plate of the organism to be tested grown on blood agar, MacConkey agar, EMB agar, tryptic soy agar, etc. (see Comments and Tips)

i. Using a sterile inoculating loop touch one isolated colony from the source plate and transfer this to the HE agar plate. Use the quadrant

streak plate method to obtain isolated colonies of the organism. (see Comments and Tips)

b. Source: feces from human or animal sources.

i. Working under a biological safety hood, insert a sterile swab into the fecal specimen to be tested.

ii. Roll the swab across one-third of the HE agar plate. Discard the swab into an appropriate container.

iii. With a sterile inoculating loop, use the quadrant streak plate method to obtain isolated colonies of the organism.

c. Source: an enrichment broth such as selenite broth or gram-negative broth.

i. Insert a sterile swab into the enrichment broth.

ii. Roll the swab across one-third of the HE agar plate. Discard the swab into an appropriate container.

iii. With a sterile inoculating loop, use the quadrant streak plate method to obtain isolated colonies of the organism.

d. Source: an environmental sample or food source.

i. The preculturing of foods for the recovery of pathogenic enteric organisms is dependent on the type of food being cultured. Consult a reputable source such as the FDA's Bacteriological Analytical Manual (13) for instructions on handling food items.

2. Incubate the HE agar plate at 35 to 37°C in an aerobic incubator for 18 to 24 hours. Do not incubate in a CO₂ atmosphere as this produces acid and will alter the pH of the medium.

3. Examine the isolated colonies for color reaction and whether or not a black precipitate is present in the colonies. Do not examine areas of confluent growth as false negative fermentation reactions may occur (see Limitations). Areas of confluent growth may also contain mixed organisms. Refer to the interpretative guidelines in Table 2 and the images later in this protocol.

4. Perform follow-up testing as needed for your laboratory activity.

5. Discard plates into an appropriate waste container.

TABLE 2. Interpreting HE agar reactions (1, 6) (Hektoen enteric agar instructions for use package insert; Remel, Lenexa, KS)

Observation	Interpretation
Growth on the HE agar plate	The organism is not inhibited by bile
Yellow or orange precipitate around the colonies	Bile salts have been precipitated by organism. Typical of some nonpath
Yellow	Fermentation of lactose, sucrose, or salicin; not likely to be an enteric pathogen
Salmon to orange	Fermentation of salicin, not likely to enteric pathogen
Yellow, salmon to orange with black centers	Fermentation of one of the carbohydrates plus the production of H ₂ S; not likely

Greenish-blue, light green, or transparent

Greenish-blue, light green, or transparent with black centers

be an enteric pathogen (other than the rare lactose-fermenting *Salmonella*)
No fermentation present, suspect *Shigella*; confirm with additional tests

No fermentation present, H₂S production present, suspect *Salmonella*, confirm with additional tests



FIG. 1. *Salmonella enterica*. Note the black center of the transparent colonies indicating H₂S production in the absence of carbohydrate utilization. All serotypes of *Salmonella* have this appearance on HE agar except for serotype Typhi, which is a weak H₂S producer, and rare strains of lactose-fermenting *Salmonella*.

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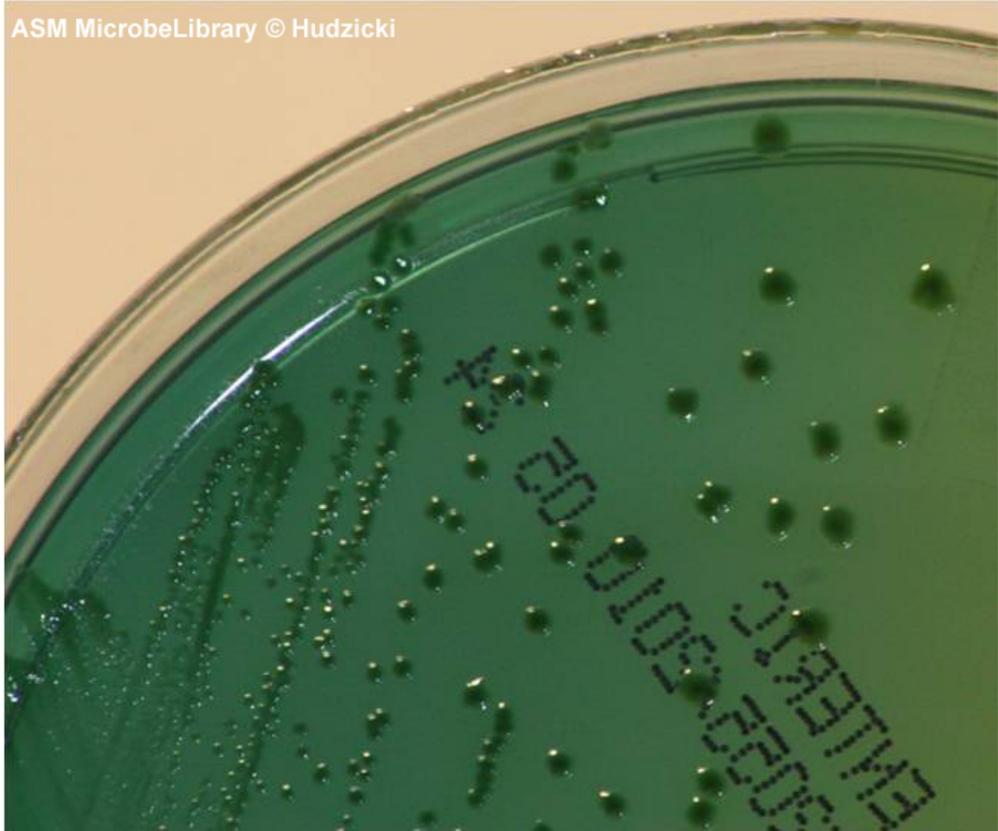


FIG. 2. *Shigella flexneri*. The colonies are transparent, indicating the absence of carbohydrate utilization and no H₂S production. All species of *Shigella* have this appearance on HE agar.

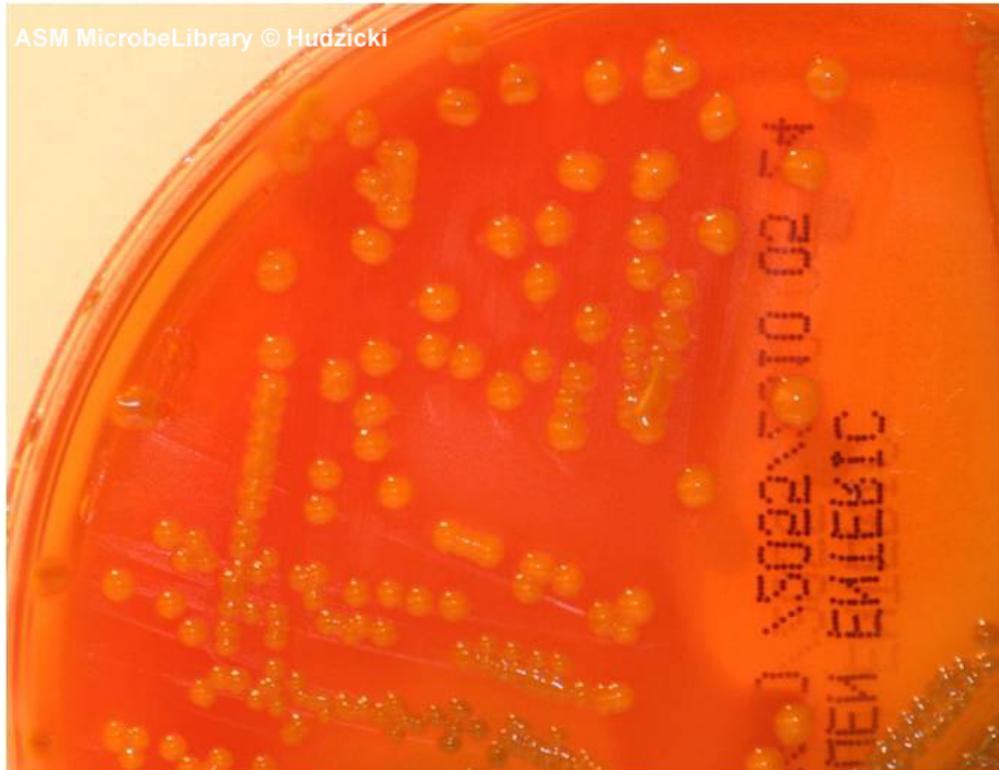


FIG. 3. *Enterobacter aerogenes*. The colonies are yellow orange, indicating the utilization of at least one of the carbohydrates present in the media. No H₂S is produced. The orange haze around the colonies is due to the precipitation of the bile salts by the organism. The appearance of *E. aerogenes* on HE agar is typical of most nonpathogenic enteric gram-negative rods.

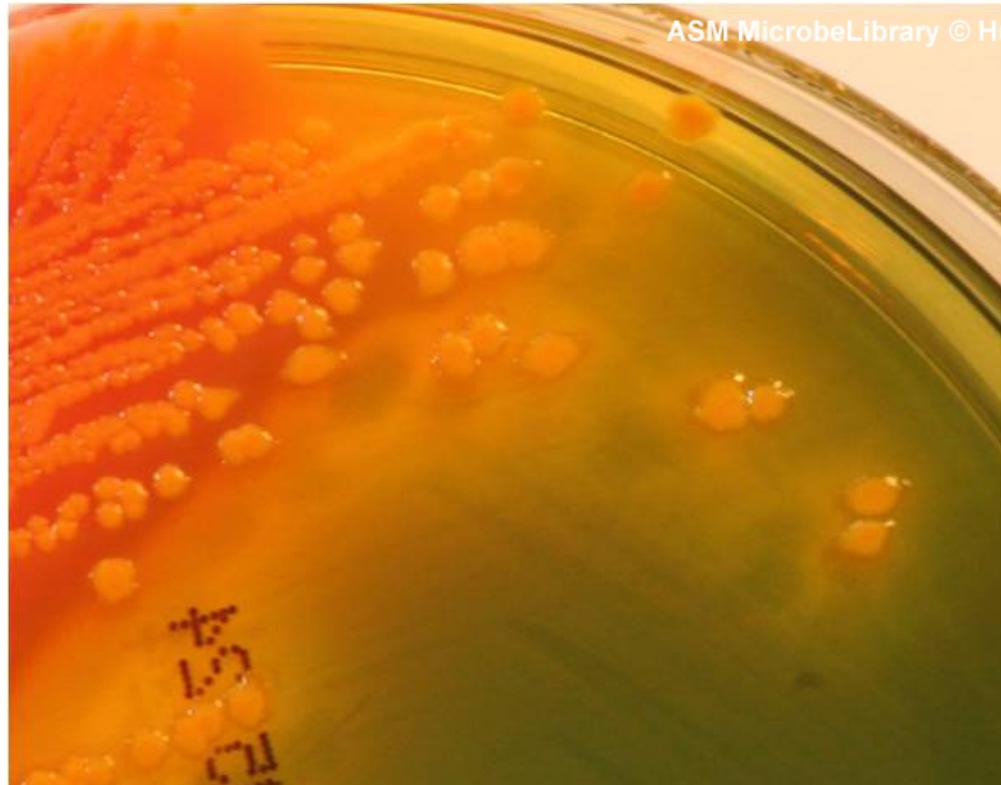


FIG. 4. Salicin-fermenting strain of *Proteus vulgaris* (50% of strains are positive). The colonies are more yellow than orange and flatter than *Enterobacter aerogenes*. There is no precipitation of the bile salts by this organism.

Limitations:

- Interpretation of carbohydrate utilization must be determined within 18 to 24 hours of the start of the incubation period. If the HE agar plates are allowed to incubate longer than 24 hours, the carbohydrates present in the medium may be exhausted by the continuing metabolic fermentation of these substrates by the organism. At this point, acid production ceases. The organism then begins to utilize the peptones and proteins present in the medium. This utilization produces alkaline end products that might overcome the acid production that occurred during the first 24-hour period, causing a loss of the yellow or orange colony color. Thus, the organism would be interpreted as not utilizing any of the carbohydrates and would appear as a suspect pathogen.
- This media should only be used as a screening media. Additional testing is required to confirm the presence of *Salmonella* or *Shigella* from HE agar.
- This medium should not be used alone for the recovery of intestinal pathogens. It should be used as part of a battery of selective and nonselective media when attempting to recover intestinal pathogens from human and animal fecal specimens.

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the [ASM Curriculum Recommendations: Introductory Course in Microbiology](#) and the [Guidelines for Biosafety in Teaching Laboratories](#).

COMMENTS AND TIPS

- During storage of the HE agar plates, the bile salts may crystallize and precipitate out into the media. This will not affect the performance of the HE agar (Hektoen enteric agar instructions for use package insert; Remel, Lenexa, KS).
- Liquid medium should be hunter green prior to pouring. Be sure to allow the liquid medium to cool prior to pouring.
- Although the author has had no problems with deterioration of plates prepared from dehydrated powder during the suggested stability time frame of 70 days, some reviewers indicated that their HE agar, prepared from dehydrated powder, is not that stable. Some recommended an expiration date of 1 week.
- Inhibition of growth does not necessarily equal prevention of all growth. Growth may take longer to appear and the colonies may be smaller than those seen on other media for enteric gram-negative rods.
- When transferring growth from a previously inoculated plate for subculture (see Protocol, section 1a), lab instructors may want to have the students make a light suspension of the organism in sterile saline or sterile water prior to inoculation of the HE agar. If too much organism is transferred to the HE agar from another plate, the organism may be able to overcome the inhibitory properties of the HE agar.

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