Indole Test Protocol

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Information

History

As far back as 1889, the indole test was used as a means to distinguish between *Escherichia coli* and*Enterobacter aerogenes* (4). The numerous variations of the indole test alone and in combination with other biochemical tests attest to the central role this test has played in the characterization of coliforms (gram-negative nonsporulating bacilli that ferment lactose, producing acid and gas) (5). The indole test is still used as a classic test to distinguish indole-positive *E. coli* from indole-negative*Enterobacter* and *Klebsiella*. (8)

Purpose

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. It is used as part of the IMViC procedures, a battery of tests designed to distinguish among members of the family Enterobacteriaceae.

Theory (3, 5)

Tryptophan is an amino acid that can undergo deamination and hydrolysis by bacteria that express tryptophanase enzyme.

tryptophan + water = indole + pyruvic acid + ammonia

The chief requirement for culturing an organism prior to performing the indole test is that the medium contains a sufficient quantity of tryptophan (5). The presence of indole when a microbe is grown in a medium rich in tryptophan demonstrates that an organism has the capacity to degrade tryptophan. Detection of indole, a by-product of tryptophan metabolism, relies upon the chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB) under acidic conditions to produce the red dye rosindole (5, 8).

RECIPES

The main requirement for a suitable indole test medium is that it contain a sufficient amount of tryptophan. Although many media meet this criterion, tryptone broth is commonly used.

Tryptone broth (2)

Ingredient	Amount
Tryptone	10.0 g
Sodium chloride	5.0 g

Dissolve the ingredients in 1 liter of sterile water. Dispense 4 ml per tube. Cap tube and autoclave at 121°C under 15 psi pressure for 15 minutes. Store the tubes in the refrigerator at 4 to 10°C.

Kovács reagent (3, 5)

Ingredient	Amount
Amyl or isoamyl alcohol, reagent grade (butyl alcohol may be substituted)	150.0 ml
p-dimethylaminobenzaldehyde (DMAB)	10.0 g
HCl (concentrated)	50.0 ml

Dissolve DMAB in the alcohol. Gentle heating might be required to get the aldehyde into solution.

Slowly add the acid to the aldehyde-alcohol mixture. The solution should be a pale yellow color and is only stable for a short time. Store the mixture in a brown glass bottle in the refrigerator. Kovács reagent also is commercially available.

PROTOCOL

Inoculate the tube of tryptone broth with a small amount of a pure culture. Incubate at 35°C (+/- 2°C) for 24 to 48 hours.

To test for indole production, add 5 drops of Kovács reagent directly to the tube (3, 5).

A positive indole test is indicated by the formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent (Fig. 1b).

If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy (Fig. 1c).

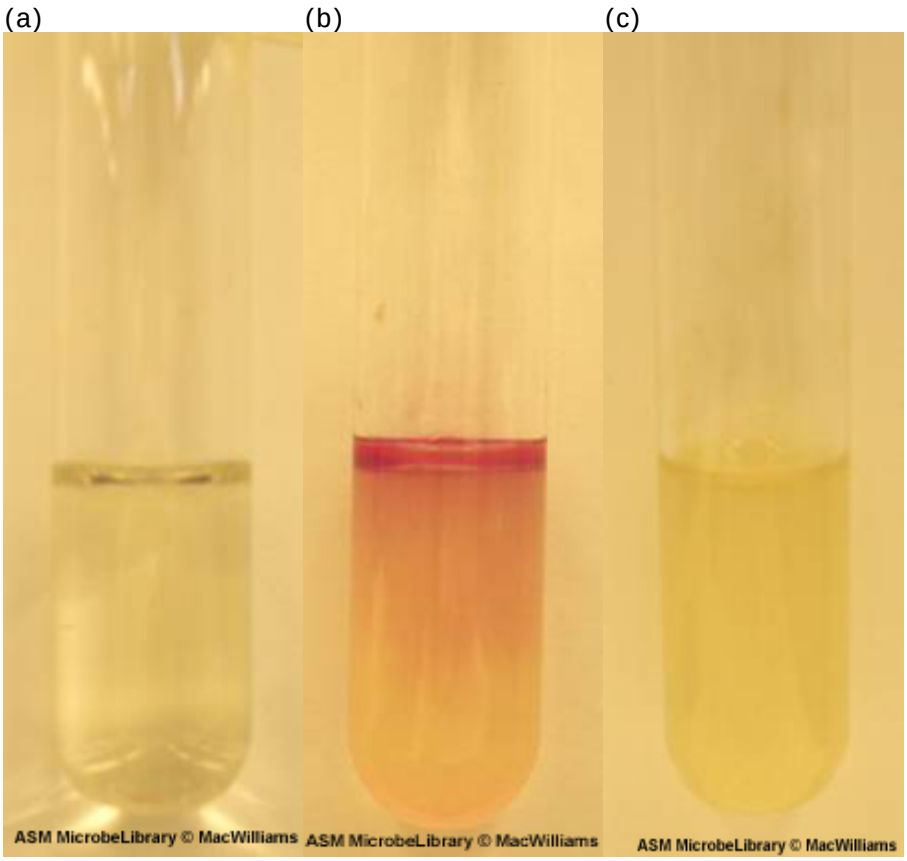

FIG 1. The indole test. (a) An uninoculated tube of tryptone broth. (b) A positive indole test. The 48-hour *Escherichia coli* culture grown at 37°C tests positive for the presence of indole as indicated by the red reagent layer after the addition of Kovács reagent. (c) A negative indole test. The 48-hour *Enterobacter aerogenes* culture incubated at 37°C has not broken down the tryptophan in the medium and thus no color change occurs upon addition of the Kovács reagent. The reagent appears as a thin yellow layer on top of the culture medium.

See Indole Test Atlas images of indole test using tryptone broth.

Alternate Methods of Detecting Indole Production

Tryptophan peptone broth

Tryptophan peptone broth (1, 5)

Ingredients	Amount
Casein peptone	10.0 g
Sodium chloride	5.0 g
Tryptophan[a]	10.0 g

[a]The tryptophan concentration can be varied; the amount used here will result in a 1% final concentration.

Dissolve ingredients in 1 liter of distilled water. Dispense 4 ml per tube. Cap tube and autoclave at 121°C under 15 psi pressure for 15 minutes.

Store tubes in the refrigerator at 4 to 10°C.

Inoculate, incubate, and perform test as described above for tryptone broth.

Sulfide-indole-motility (SIM) medium (1, 3, 5)

The SIM medium is a multitest agar used to test for indole production while simultaneously determining other characteristics of the bacterium (see Comments and Tips section).

SIM medium

Ingredients	Amount
Peptone	30.0 g
Beef extract	3.0 g
Ferrous ammonium sulfate	0.2 g
Sodium thiosulfate	0.025 g
Agar	3.0 g

Dissolve ingredients, except agar, in 1 liter of distilled water. Adjust pH to 7.3. Add agar and heat mixture to boiling to dissolve agar. Cool to 50°C. Dispense in 4.0 to 5.0 ml aliquots in 16-mm test tubes. Cap tubes and autoclave at 121°C under 15 psi pressure for 15 minutes. After autoclaving, allow tubes to cool in an upright position to form the agar deep. Tubes can be stored at 4 to 8°C for several months. SIM medium is commercially available both as a premixed powder and as premade deep tubes.

To inoculate SIM medium, pick an isolated colony with a needle. Stab the needle approximately two-thirds of the way into the deep and then remove it following the same path as the entry.

Incubate at 35°C (+/-2°C) for 24 to 48 hours or until growth is evident.

To test for the presence of indole, a by-product of tryptophan metabolism, 5 drops of Kovács reagent should be added to the top of the deep.

A positive indole test is indicated by the formation of a red color in the reagent layer on top of the agar deep within seconds of adding the reagent.

If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy.

See Indole Atlas images—Alternative Methods for Determining Indole Production for SIM result images.

Motility-indole-ornithine (MIO) medium (1, 2)

The MIO medium is a multitest agar used to test for indole production while simultaneously determining other characteristics of the bacterium (see Comments and Tips section).

MIO medium

Ingredients	Amount
Yeast extract	3.0 g
Peptone	10.0 g
Tryptone	10.0 g
L-ornithine HCl	5.0 g
Dextrose	1.0 g
Agar	2.0 g
Bromcresol purple	0.02 g

Bring to 1 liter with distilled water. Heat mixture to boiling to dissolve agar. Cool to 50°C. Dispense in 4.0 to 5.0 ml aliquots in 16-mm test tubes. Cap tubes and autoclave at 121°C under 15 psi pressure for 15 minutes. After autoclaving, allow tubes to cool in an upright position to form the agar deep. Tubes can be stored at 4 to 8°C for several months. MIO medium is commercially available both as a premixed powder and as premade deep tubes.

To inoculate MIO medium, pick an isolated colony with a needle. Stab the needle approximately two-thirds of the way into the deep and then remove it following the same path as the entry. Incubate at 35°C (+/- 2°C) for 24 to 48 hours or until growth is evident.

To test for the presence of indole, a by-product of tryptophan metabolism, 5 drops of Kovács reagent should be added to the top of the deep.

A positive indole test is indicated by the formation of a red color in the reagent layer on top of the agar deep within seconds of adding the reagent. If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy.

See Indole Atlas images—Alternative Methods for Determining Indole Production for MIO result images.

Ehrlich's reagent (5)

Ehrlich's reagent, an alternative to Kovács reagent, also contains DMAB, which reacts with indole to produce a red product. The Ehrlich formulation is more sensitive but contains additional toxic or flammable solvents; it is recommended when testing bacterial groups that produce

little indole such as nonfermentative bacilli or anaerobes. Kovács reagent is apparently more stable and the absence of the additional organic extraction (required with Ehrlich's) makes Kovács formulation more suitable for undergraduate laboratories.

Ehrlich's reagent

Ingredients	Amount
Ethyl alcohol (absolute)	95.0 ml
p-dimethylaminobenzaldehyde (DMAB)	1.0 g
HCl (concentrated)	20.0 ml

Dissolve components and store solution at room temperature in a brown glass bottle.

To test for indole production, inoculate a 4-ml tryptone broth tube with one loopful of culture. After 24 to 48 hours of incubation, add to the culture 1.0 ml of either ether or xylene. Mix well and then allow organic solvent to rise to the top of the medium. Add 0.5 ml of Ehrlich's reagent so that it runs down the side of the tube into the medium. Development of a red color in the reagent layer indicates a positive test. If the reagent stays yellow, the test is negative.

Indole spot test (5, 7)

Inoculate the bacterium to be tested on an agar medium that contains tryptophan. Trypticase soy agar or sheep blood agar can be used. Incubate for 18 to 24 hours at the appropriate temperature to allow for growth.

To conduct the test, place a small piece of Whatman filter paper in a petri dish cover. Saturate paper with Kovács reagent (1 to 1.5 ml). Smear the paper with cell paste from an 18- to 24-hour culture.

If indole is present, a red pink color will develop within 1 to 3 minutes.

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

1. The indole test results can vary with different isolates of the same species.

2. SIM medium. In addition to using SIM medium to test for indole production, SIM agar is used to determine the status of two other characteristics: motility and the ability to produce hydrogen sulfide as a by-product of metabolism. These additional characteristics can be helpful when attempting to distinguish between *Salmonella* and *Shigella* (2).

If hydrogen sulfide is produced as a by-product of metabolism, it will react with the ferrous iron in the medium to form ferrous sulfide, a black insoluble compound. With organisms that are incapable of forming H_2S in sufficient quantities, no precipitate will form (2, 5).

Motility is evidenced by the spread of bacterial growth radiating out from the stab line. Nonmotile bacteria will only grow along the stab line (2, 5).

3. MIO medium. In addition to testing for indole production, MIO agar is used to test for motility and ornithine decarboxylase. Ornithine decarboxylation causes an increase in pH in the tube. This is indicated by the purple-grey color throughout the tube (2). A negative result for ornithine decarboxylation is indicated by yellow in the tube due to glucose fermentation and no overriding pH increase (2).

Motility is evidenced by the spread of bacterial growth radiating out from the stab line. Nonmotile bacteria will only grow along the stab line (2, 5).

See Indole Atlas images—Alternative Methods for Determining Indole Production for MIO result images.

4. Other multitest media that can be used to perform the indole test in addition to other tests include trypticase nitrate broth, motility-indole-lysine (MIL), and motility-indole-sulfide (MILS) medium (1, 5).

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