

Methyl Red and Voges-Proskauer Test Protocols

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Information

History

In 1898 Voges and Proskauer (16) characterized the fermentation of sugars by various bacterial isolates. They showed that the gas produced during fermentation was a mix of CO₂ and H₂ and that by adding KOH to cultures that had grown in glucose peptone media for prolonged incubation in the presence of oxygen, some organisms produced a red fluorescent color (16). Although the nature of the coloration was not clear at the time, this method was suggested for differentiation between bacterial isolates that produced the color and those that did not (16).

In 1906, Arthur Harden analyzed the fermentation products of *Enterobacter aerogenes* and found that in the presence of glucose the organism produced acetoin and 2,3-butanediol. When testing both compounds in the presence of KOH, neither produced the pinkish-red color described by Voges and Proskauer (16). However, addition of peptone to the reaction yielded the described coloration in the presence of acetoin (4). Harden suggested that acetoin was oxidized to diacetyl which in turn reacted with compounds in the peptone mixture forming a colored product. Later it was found that the presence of a guanidine group (on a compound in the peptone) is essential for the color reaction (5).

In 1915 Clark and Lubs published a new method for the differentiation of bacteria of the colon-aerogenes family (3). Until then bacteria of this group were differentiated by the gas ratio (CO₂/H₂) produced during fermentation. When grown in buffered peptone-glucose broth, bacteria could be differentiated into "low-ratio organisms" and "high-ratio organisms," depending if CO₂ and H₂ were produced in nearly equal amounts or if the amount of CO₂ was much higher. A third group "∞ ratio group" refers to organisms not producing H₂. As Clark and Lubs pointed out, the methods used for determination of the gas ratios could not be adapted to routine testing (3), so they tested other methods to differentiate between the different groups. Three years earlier Michaelis and Marcora (10) found that *Escherichia coli* produced hydrogen ions when grown in lactose broth. Using this information, Clark and Lubs tested low-ratio organisms and high-ratio organisms for their ability to produce hydrogen ions when growing on dextrose. They found that in the glucose-peptone medium the low-ratio organisms produced 100 times more hydrogen ions than the high-ratio cultures (3).

Clark and Lubs optimized the culture medium (0.5% peptone, 0.5% dextrose, 0.5% K₂HPO₄) to allow discrimination of low-ratio organisms and high-ratio organisms. In this medium, the quantity of hydrogen ions generated by low-ratio organisms created an acidity level that inhibited their growth, whereas high-ratio organisms produced significantly fewer hydrogen ions. After growth in this media, the final hydrogen ion concentration allowed distinction between high-ratio organisms and low-ratio organisms. As an assay for hydrogen ion concentration, pH indicators were used. The two pH indicators tested were paranitrophenol and methyl red, the

latter is used today in the methyl red (MR) test.

In 1916 Levine observed that bacteria which tested negative in the methyl red test were positive in the Voges-Proskauer reaction. This observation established the general observed correlation between the production of hydrogen ions and acetoin: organisms that ferment glucose producing high levels of hydrogen ions do not produce acetoin as a fermentation intermediate (7). In 1916, the Voges-Proskauer (VP) test was not yet optimized. After the addition of KOH to the test media, a positive reaction was distinguished by a faint eosin pink color and required approximately 24 hours to develop. The method was improved by the addition of ferric chloride prior to adding NaOH (instead of KOH). The improved assay was more sensitive and resulted in a stable copper tone instead of the original eosin pink (17). O'Meara further improved the VP test by adding creatine and NaOH to the grown cultures. Within a couple of minutes after addition and agitation, a red color appeared, indicating the presence of acetoin and a positive Voges-Proskauer test (11). The time reduction and intensification of the reaction lead to wide acceptance of O'Meara's modification as the standard for the VP test of the time. However, O'Meara's modification did not give consistent results. In 1936 Barritt published his modification of the Voges-Proskauer test in which he showed that addition of a-naphthol intensified the positive reaction and made it more sensitive (2). Today, Barritt's modification of the Voges-Proskauer test is the standard procedure used to detect the presence of acetoin as a metabolic intermediate in the fermentation of glucose via the butanediol pathway.

During the development of the methyl red and Voges-Proskauer tests to differentiate *Escherichia* from the *Enterobacter-Klebsiella* group of Enterobacteriaceae, it seemed that there was an inverse relationship between the outcomes of the two tests: *Escherichia* was MR+ (positive) and VP- (negative), whereas members of the *Enterobacter-Klebsiella* group showed the opposite results of being MR-and VP+. The MR-VP test was used in clinical labs to differentiate among these two groupings of the Enterobacteriaceae. With Barritt's modification and increased sensitivity of the assay, it was found that some members of the Enterobacteriaceae could not be distinguished with the paired MR-VP tests. These organisms tested positive for both the methyl red as well as the Voges-Proskauer test. In 1939 Vaughn and coworkers analyzed the effect of incubation temperature and time on the MR-VP results of this so called "intermediate" group" (15). This group includes members of the genera *Enterobacter*, *Klebsiella*, *Hafnia*, *Proteus*, and *Serratia*.

Purpose

The methyl red and Voges-Proskauer tests are part of a battery of biochemical tests known as IMViC used in the clinical lab. The acronym IMViC stands for indole, methyl red, Voges-Proskauer and citrate (13). The "i" in the acronym is added for pronunciation purposes.

Originally the paired MR-VP tests were used to distinguish between members of the family Enterobacteriaceae, but now they are used to characterize other groups of bacteria including Actinobacteria (6,14).

Theory

Both the methyl red and Voges-Proskauer tests are commonly used in conjunction with the indole and citrate tests, to form a group of tests known as IMViC which aid in the differentiation of Enterobacteria.

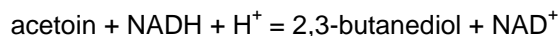
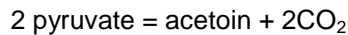
Methyl red

Escherichia coli and other members of the low-ratio organisms described by Clark and Lubs ferment sugars by the mixed acid pathway resulting in a low ratio of CO₂ to H₂ gas produced by fermentation. The mixed acid pathway gives 4 mol of acidic products (mainly lactic and acetic acid), 1 mol of neutral fermentation product (ethanol), 1 mol of CO₂, and 1 mol of H₂ per mol of glucose fermented (9). The large quantity of acids produced causes a significant decrease in the pH of the culture medium. In contrast, *Enterobacter aerogenes* and other members of the high-ratio organisms (those that produce a high ratio of CO₂ to H₂ from the fermentation of glucose) ferment sugars via the butanediol fermentation pathway, producing only 1 mol of acid per mol of glucose. This pathway results in a lower degree of acidification of the culture medium. The pH indicator methyl red (p-dimethylaminoaeobenzene-O-carboxylic acid) has been found to be suitable to measure the concentration of hydrogen ions between pH 4.4 (red) and 6.0 (yellow) (12).

When the culture medium turns red after addition of methyl red, because of a pH at or below 4.4 from the fermentation of glucose, the culture has a positive result for the MR test (Fig. 1A). A negative MR test is indicated by a yellow color in the culture medium (Fig. 1B), which occurs when less acid is produced (pH is higher) from the fermentation of glucose.

Voges-Proskauer

Bacteria fermenting sugars via the butanediol pathway produce acetoin (i.e., acetyl methyl carbinol or 3-hydroxybutanone) as an intermediate which can be further reduced to 2,3-butanediol.



In the presence of KOH the intermediate acetoin is oxidized to diacetyl, a reaction which is catalyzed by α -naphthol (2). Diacetyl reacts with the guanidine group associated with molecules contributed by peptone in the medium, to form a pinkish-red-colored product (Fig. 2A). The α -naphthol in the Barritt's modification of the VP test serves as a color intensifier.

RECIPES (1, 8)

The media and reagents listed below can be made or purchased commercially from biological suppliers.

Buffered peptone-glucose broth

(commercially available as MR-VP broth)

Buffered peptone 7.0 g (can be replaced by 3.5 g of peptic digest of animal tissue and 3.5 g of pancreatic digest of casein)

Dipotassium phosphate 5.0 g

Dextrose 5.0 g

Add 1 liter of deionized water for a final pH of 6.9 (\pm 0.2) at 25°C.

Gently heat to completely dissolve the ingredients and distribute 5 ml aliquots into culture tubes. Sterilize via autoclaving at 121°C and 15 psi for 15 minutes. After the medium cools down it can be stored in a refrigerator until use.

This medium is a modification of the original Clark and Lubs medium using the decreased concentration of glucose (0.5%) as well as peptone. It was shown that this amount of glucose allowed for an identification of MR-positive organisms (3). The reduced amount of peptone results in a less-colored medium, making the interpretation of the color reaction easier.

Methyl red solution

Completely dissolve 0.1 g of methyl red in 300 ml of ethanol (95%). Add 200 ml of deionized water to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol. Store the prepared methyl red solution at 4°C.

Voges-Proskauer reagents

Barritt's reagent A: 5% (wt/vol) α -naphthol in absolute ethanol

Barritt's reagent B: 40% (wt/vol) KOH in deionized water (this might be replaced by a 40% (wt/vol) NaOH solution)

Reagents must be prepared fresh. Reagents are also referred to as VP-1 and VP-2 or VP-A and VP-B.

Other materials

- sterile culture tubes containing 5 ml of MR-VP broth
- sterile culture tubes
- test and control bacterial strains (commonly used controls are *Escherichia coli* and *Enterobacter aerogenes*) from trypticase soy agar or broth
- inoculation loop (disposable or have additional equipment available for sterilization of the inoculation loop)
- transfer pipettes

PROTOCOL (1, 8)

A. Inoculation of medium

Prepare MR-VP broth as described above or as directed by the manufacturer. Prior to use, allow the medium to come to room temperature.

Inoculate one tube of MR-VP broth from a fresh (18 to 24 hours) pure culture (e.g., grown on trypticase soy agar) of the test culture. Transfer a light inoculum from an isolated colony and resuspend it in the 5 ml MR-VP broth tube. Note that the use of a heavy inoculum may result in aberrant results.

For comparison it is suggested that *Escherichia coli* (MR+, VP-) and *Enterobacter aerogenes* (MR-, VP+) be used as control cultures.

B. Incubation

Incubate test and control cultures at 35°C (+/- 2°C) for 48 hours.

C. Methyl red test and interpretation of results

1. Transfer 2.5 ml of culture into a new sterile culture tube.
2. Add 5 drops of the methyl red reagent.
3. Compare the test organism to the control cultures to immediately interpret the result, MR positive (e.g., *E. coli*) (Fig. 1A) or MR negative (e.g., *E. aerogenes*) (Fig. 1B).



A

B

FIG. 1. Methyl red reaction. *E. coli* (A) and *E. aerogenes* (B) were grown for 48 hours at 35°C in MR-VP medium. After incubation, 5 drops of a 0.05% methyl red solution (per 2.5 ml) were added to each culture. The MR-positive *E. coli* (A) shows a red coloration as a result of high acid production and a decrease in the pH of the culture medium to 4.4. The MR-negative *E. aerogenes* culture (B) has a yellow color indicating a less acidic medium.

False-negative results or an inconclusive orange color may occur due to insufficient length of incubation. In this case, it is recommended that the test be repeated with a culture that was incubated an additional 24 to 48 hours.

An increase in glucose concentration in the medium might lead to false-positive results in the MR test. Clark and Lubs showed that increased glucose concentration also led to high hydrogen ion concentration in high-ratio cultures which, under standard test conditions, are MR negative (3).

D. Voges-Proskauer test and interpretation of results

1. Use the remaining 2.5 ml of culture grown in MR-VP broth.
2. Add 0.6 ml (or 12 drops) of Barritt's reagent A.
3. Add 0.2 ml (or 4 drops) of Barritt's reagent B.
4. Carefully shake the tube for 30 seconds to 1 minute to expose the medium to atmospheric oxygen (necessary for oxidation of acetoin to obtain a color reaction).
5. Allow the tube to stand for at least 30 minutes.
6. Within 1 hour, compare the test result to control cultures to determine if the culture is VP positive like *E. aerogenes* (Fig. 2A) or VP negative like *E. coli* (Fig. 2B). Delayed reading of the result may lead to an erroneous reading; over time a-naphthol and KOH may react to give a copper-like color.



A

B

FIG. 2. Voges-Proskauer reaction. *E. aerogenes* (A) and *E. coli* (B) were grown in MR-VP-broth for 48 hours at 37°C and Barritt's reagents A and B were added. VP-positive *E. aerogenes* (A) shows red coloration on top of the culture, whereas VP-negative *E. coli* (B) has a yellowish color.

Incubation times shorter or longer than 48 hours may result in false-negative results since either the amount of acetoin accumulated is too low to be detected or acetoin will be further reduced to 2,3-butanediol.

It should be noted that some bacteria (e.g., *Hafnia alveii* and *Enterobacter*) are VP variable when grown at 37°C but VP positive when grown at lower temperatures (25°C to 30°C).

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

- An alternative to completing the MR-VP test from one culture tube: inoculate two tubes of MR-VP broth with the same organism. Use one tube for the MR test and one tube for the VP test.
- Use small screw cap tubes for the MR-VP assay to decrease leakage during shaking.

Material safety data sheets links:

[MR-VP broth \(BD\)](#)

[Voges-Proskauer reagent A \(BD\)](#)

[Voges-Proskauer reagent B \(BD\)](#)

[a-naphthol \(MPbio\)](#)

[Methyl red \(JTBaker\)](#)

[Ethanol \(Fisher Scientific\)](#)

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