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Different Preservation Methods for Long Term Maintenance of *Haemophilus influenzae*

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Abstract

Haemophilus species are Gram-negative coccobacilli that require factor X and factor V for growth. Beyond this, it is a finicky bacterium to culture, and any modification of culture procedures greatly reduces isolation rates. Poor quality of laboratories in developing countries results in its poor isolation rates. This study was done with the objective of finding out the optimal cultural environment and media so that it could be maintained for a longer period in economical settings like ours which was done using *H. influenzae* ATCC 49,766. In this study, several culture media were tested as a means to preserve *H. influenzae* ATCC like TSB + glycerol + sheep blood, BHI broth, BHI broth + glycerol, BHI broth+ glycerol + sheep blood, Chocolate agar slant and satellitism plate. Three sets of respective media were inoculated with 18 - 24 hours growth of *H. influenzae.* They were incubated at 37˚C 48 hours in a candle extinction jar. The media were checked for growth by subculturing them on chocolate agar plates and identified by biochemical reactions. Each set was maintained at 2˚C - 8˚C, −20˚C and at room temperature and checked for the viability 24 hourly by subculturing them on chocolate agar. Results showed best growth of *H. influenza* on chocolate agar slants for 15 - 20 days, followed by BHI + glycerol + sheep blood broth and satellitism plate for 4 - 6 days followed by BHI broth for 2 - 4 days. There was no growth in TSB + glycerol + sheep blood broth and BHI + glycerol broth media. Present study showed similar results as done by NS Srikanth *et al.* 2003 with growth on chocolate agar & satellitism plate for 3 - 5 days but no growth in TSB + Glycerol + Sheep blood broth media. Chocolate agar slant is by far the most long term preserving media for *H. influenzae.* However, growth on BHI broth with various modifications is also showed a good preservation for 3 - 5 days, so with further experiments we can hope to maintain the organism in these media also.

Keywords

H. influenzae, Preservation Methods, Culture Media

1. Introduction

The genus *Haemophilus* includes a number of species that cause a wide variety of infections but share a common morphology and a requirement for blood-derived factors during growth that has given the genus its name. *Haemophilus influenzae*, the major pathogen, can be separated into encapsulated or typable strains, of which there are seven types ("a" through "f" including "e") based on the antigenic structure of the capsular polysaccharide, and unencapsulated or nontypable strains. Type b *H influenzae* is by far the most virulent organism in this group, commonly causing bloodstream invasion and meningitis in children younger than 2 years. Nontypable strains are frequent causes of respiratory tract disease in infants, children, and adults [1].

Haemophilus species are Gram-negative coccobacilli that share common ultrastructural features with other Gram-negative bacilli. Their cell walls contain lipo-oligosaccharide, which resembles the lipo-polysaccharide of Gram-negative bacilli but has shorter side chains. *Haemophilus* species have generally been thought not to make toxins or other extracellular products that account for their ability to produce infection. These organisms require hemin (factor X) and/or nicotinamide adenine dinucleotide (NAD+) (factor V) for growth. Whereas NAD+ is released into the medium by red blood cells and is available to the bacteria in blood agar, hemin is bound to red blood cells and is not released into the medium unless the cells are broken up, as in chocolate agar. *Haemophilus influenzae* requires both factors X and V; accordingly, it grows on chocolate agar but not on blood agar. It may appear on a blood agar plate as tiny satellite colonies around the colonies of other bacteria that have lysed red blood cells [2]. All *Haemophilus* species grow more readily in an atmosphere enriched with CO_2. As shown in **Figure 1**, colonies of *H. influenzae* appear as convex, smooth, pale, grey or transparent colonies. Beyond this, *H. influenzae* is a finicky bacterium to culture, and any modification of the standard culture procedures can greatly reduce isolation rates. Lack of proper facilities in laboratories of developing countries has resulted in poor isolation rates of *H. influenzae*.

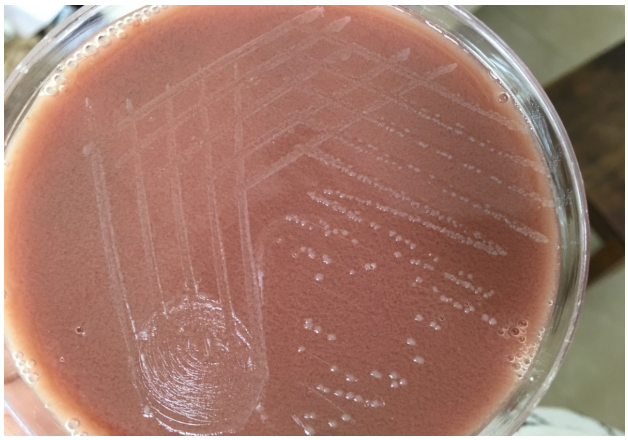

Figure 1. Large, round, smooth, convex, colorless-to-grey colonies of *H. influenzae* on chocolate agar plate after 18 - 24 hours incubation in a candle extinction jar.

2. Material and Method

As maintenance of *Haemophilus influenzae*on chocolate agar plates every 2 - 3 days is a tedious job [3], it is important that a culture media and its maintenance method be found so that the organism can be maintained for longer periods. Keeping this in mind, this study tried to maintain the organism on various culture media as mentioned below. The study was started after obtaining approval from the Institute Ethical committee.

Following media were used for the study [4] [5] as shown in **Figure 2**.

1. TrypticSoya Broth + glycerol + sheep blood
2. Brain Heart Infusion broth
3. Brain Heart Infusion broth + glycerol
4. Brain Heart Infusion broth + glycerol + sheep blood
5. Chocolate agar slant
6. Satellitism plate

These media were prepared as follows [4] [5]:

1) **TSB + Glycerol + Sheep blood:** 30 grams media is suspended in 1000 ml purified/distilled water. It is then sterilized by autoclaving at 15 lbs pressure (121˚C) for 15 minutes. After autoclaving the media, 10% w/w glycerol is added along with heated sheep blood and then about 2 - 3 ml media is dispensed into autoclaved glass containers.
2) **BHI broth:** 52 grams media is suspended in 1000 ml purified/ distilled water. It is then sterilized by autoclaving at 15 lbs pressure (121˚C) for 15 minutes. The autoclaved media are allowed to cool and then about 2 - 3 ml media are dispensed into autoclaved glass containers.

Figure 2. Different media like Brain Heart Infusion broth, Brain Heart Infusion broth + glycerol, Brain Heart Infusion broth + glycerol + sheep blood, Tryptic Soya Broth + glycerol + sheep blood prepared and stored in glass screw-capped bottles.

3) **BHI broth with glycerol:** 52 grams media is suspended in 1000 ml purified/ distilled water. It is then sterilized by autoclaving at 15 lbs pressure (121˚C) for 15 minutes. After autoclaving the media, 10% w/w glycerol is added along with heated sheep blood and then about 2 - 3 ml media is dispensed into autoclaved glass containers.
4) **BHI broth with glycerol with added sheep blood:** 52 grams media is suspended in 1000 ml purified/ distilled water. It is then sterilized by autoclaving at 15 lbs pressure (121˚C) for 15 minutes. After autoclaving the media, 10% w/w glycerol is added along with heated sheep blood and then about 2 - 3 ml media is dispensed into autoclaved glass containers.
5) **Chocolate agar slant:** Chocolate agar is prepared by adding 28 grams of nutrient agar media in 1000 ml distilled water. It is then autoclaved at 15 lbs pressure at 121˚C for 15 mins. After autoclaving, heated sheep blood is added to the media at 45˚C - 50˚C. The media is then poured in glass tubes and kept at an angle such that a slant is formed.
6) **Satellitism plate:** A loopful of *Haemophilus* colonies is suspended in 2ml of sterile physiologic saline. Using a sterile swab, the organism is then inoculated on blood agar plate. A pure culture of *Staphylococcus aureus* ATCC No 25923 is streaked across each of the inoculated plate.

The above mentioned media were prepared and stored at 2˚C - 8˚C until use. The containers used for storage of media were glass bottles with 10ml capacity. The bottles were washed with extran, glass cleaning solution, and kept overnight. After that the bottles were washed for about 5 - 6 times with running tap water and again 5 - 6 times with distilled water and then dried. Before filling the media in the bottles, they were autoclaved at 15 lbs pressure and 121˚C for 15 minutes. Three sets of all the media mentioned above were taken. Each set of media was inoculated with 18 - 24 hrs growth of *H. influenzae* on chocolate agar plates. The media were incubated at 37˚C, for 48 hours in a candle extinction jar. After the incubation, the media were checked for growth by subculturing them onto chocolate agar plates and then identified by biochemical reactions like oxidase and catalase test positive and fermentation of glucose [2]. Each set of media were then maintained at room temperature, at 2˚C - 8˚C and at −20˚C and checked for viability of organism by sub culturing them on chocolate agar plates every 24 hourly.

3. Results

The media were checked for viability of *H. influenzae* every 24 hours by subculturing on chocolate agar plates in a candle extinction jar at 37˚C for 18 - 24 hours. *H. influenzae* was best preserved in the media that were kept at room temperature compared to lower temperatures that is at 2˚C - 8˚C and at −20˚C. Average viability of *H. influenzae* in different media kept at room temperature is shown in **Figure 3**.

The growth on chocolate agar plate was identified by Gram staining and biochemical reactions like oxidase and catalase test positive and fermentation of

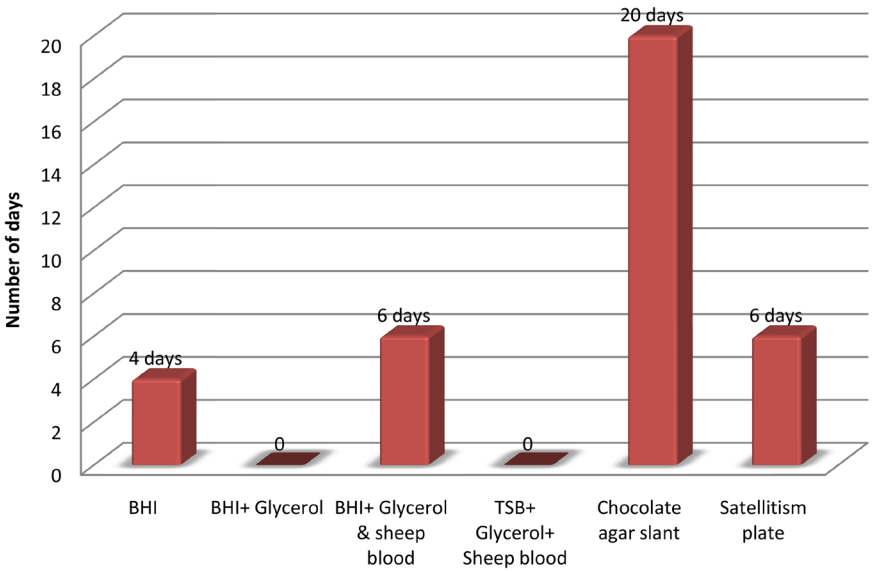

Figure 3. Figure showing the average number of days of viability of *H. influenzae* in different culture media like BHI broth (4 days), BHI with glycerol broth (no growth), BHI with glycerol and sheep blood (6 days), TSB with glycerol and sheep blood (no growth), chocolate agar slant (20 days)and satellitism plate (6 days).

glucose. *H. influenzae* remains viable at room temperature for 15 - 20 days in chocolate agar slants, 4 - 6 days in satellitism plate and in BHI + Glycerol & sheep blood. No growth was observed in glycerol containing media like BHI+ Glycerol and TSB + Glycerol + Sheep blood.

4. Discussion

Preservation of medically important isolates or ATCC strains is an important task for Microbiological laboratories for the purpose of doing further studies or analysis, to perform biochemical reactions or for internal quality control purposes. *H. influenzae* is also one of such important organism, the ATCC of which is used for doing internal quality control of chocolate agar plates and as a quality control strain for certain antimicrobial susceptibility tests [6]. So its preservation is an important task. The present study has shown that chocolate agar slant is by far the best preservation method with the organism being viable for 15 - 20 days. This study showed similar results as done by NS Srikanth *et al.* 2003 with growth on chocolate agar & satellitism plate for 3 - 5 days but no growth in tryptic soya broth with glycerol and sheep blood [4]. As lyophilization is not always available in limited resource laboratories, preservation in chocolate agar slants can be helpful in such cases. The media mentioned above were filled earlier in polypropylene autoclavable containers, but the organism was unable to grow in any of the media. Then the media were made in glass bottles after proper washing which then showed the growth of *H. influenzae.* So another observation from this study was that, glass containers used for preservation are superior to polypropylene containers, may be due to inhibitory factors present in plastic, which could affect the growth of organism.

5. Limitations

Contamination of media while storing them at room temperature is a major limitation for maintaining the organism for longer periods. This study was done using *H. influenzae* ATCC, but it would have been better if the study could be performed on different isolates from clinical samples. Also due to limitation of resources, lyophilization, cryoprotective agents like milk, sucrose or lactose and other culture media such as BHI with milk, glucose and yeast extract (MGY) and BHI with blood, glucose and yeast extract (BGY) which can preserve the organism for weeks to months could not be tried [5].

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