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RESEARCH ARTICLE

NEW ENRICHED CULTURE MEDIA FOR CULTURING HAEMOPHILUS INFLEUNZAE

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Background and aim: Fastidious bacteria require special nutrients and growth factors to grow on enriched culture media. Many enriched culture media were developed for culturing and isolating fastidious bacteria in pure cultures. A number of defined media have been described, but their use often leads to frustration. The present study investigates the ability of spirulina powder to support the fastidious bacteria growth in pure culture compared to ordinary enriched media, e.g Blood agar and Chocolate agar. **Methods**: Spirulina powder was used as a nutrient source with some additives to prepare different types of Spirulina media. Reference strain of Haemophilus infleunzae (H.imfleunzae) ATCC (49347) and three clinical isolates were examined for their growth on the developed candidate medium. Three formulations of spirulina powder were used: Medium 1, Medium 2, and Medium 3. **Results**: spirulina media type 2 and 3 supported the growth of H infleunzae and there was no significant difference in the morphology and cultural characteristics on chocolate agar media. Colonies size of H infleunza were slightly smaller size and white in color on spirulina media. **Conclusions**: Spirulina media type 2 and 3 are a possible candidate that can be used as enriched culture media for culturing and isolating fastidious bacteria such as H.infleunzae.

Key words: Bacteria, Haemophilus Infleunzae, Nicotinamide adenine nucleotide, Pantothenate, Spirulina Powder.

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INTRODUCTION:

H. influenza is a Gram-negative bacterium responsible for severe pneumonia, meningitis, sinusitis, otitis media and other invasive diseases, almost exclusively in children aged less than five years old. *H. influenzae* isolates are usually classified according to their polysaccharide capsule into six capsular types a to f. *H. influenzae* type b populations are often heterogeneous [1]. *H. influenza* is an obligate parasite of human mucous membranes; it is not found in another animal species. It colonizes the throat and nasopharynx, and to a lesser extent the conjunctivae and genital tract [2]. Identification of organisms of the *Haemophilus* group depends on demonstrating the need for certain growth factors called X and V. Factor. X acts physiologically as hemin; factor V can be replaced by nicotinamide adenine nucleotide (NAD) or other coenzymes. *H influenzae* is differentiated from related Gram-negative bacilli by its requirements for X and V factors and by its lack of hemolysis on blood agar [3]. Human blood is not an acceptable substitute. For *H. influenzae,* because it is a fastidious organism requiring media containing (X factor) and (V factor). Growth occurs on CAP because of haemin released during the heating process in the preparation of chocolate agar. Hamelin is available from non-haemolyzed as well as haemolyzed cells.[4]

H. influenzae has a number of specific nutritional requirements for growth which permit unusual control of the transformability of this organism. Growth is reduced by omission of any of several essential components. The requirements for growth include arginine or citrulline; ornithine cannot be substituted for them. NAD and pantothenate are also specific requirements, as is heme or hemin. It has been reported that hemin is necessary only for aerobic growth, Glutamic acid, cystine and tyrosine can be replaced, although they seem to be more effective than other amino acids, inosine or other purine nucleosides and lactate or pyruvate must be present during growth of *H. influenzae*.[5] A number of defined media have been described, but their use often leads to frustration.[6].

Ordinary culture media, for example: Blood agar and chocolate agar contains inhibitors for certain bacteria, such as members of the *Neisseria* and *Haemophilus genera*, and the blood must be heated to inactivate these inhibitors [7]. There was also the problem of collecting sufficient blood from the smaller animals. Although cow blood is easily available, there is variability of the hemolytic reactions by some of the organisms. The most commonly used blood for the isolation of Microorganisms from human tissue and fluids. However, expired blood from blood banks is still used despite the risk of exposure to HIV and other blood –borne infection, human blood may contain antibodies and anti–microbial agents which may also inhibit growth or cause false hemolysis [8]. When making blood or chocolate agar plates, the formation of bubbles during mixing and pouring can be removed by applying the flame directly to the surface of the agar plate, but this increases the possibility of contamination. It is believed that bacteria floating in the air enter the agar medium.[9]

In this research, Spirulina powder was used as a nutrient source with some additives like Pericarp powder (high non-heme iron supplement). Were used to prepare different types of Spirulina media, and *H. infleunzae* species were examined for their growth on the developed candidate medium.

MATERIAL AND METHODS:

Organisms used:

Reference *H. infleunzae* ATCC (49247) strain and three clinical isolates (full identified) collected from hospitals in Khartoum were used in this study.

Formulation and inoculation on solid media

Spirulina powder was placed in warm water, mixed, and boiled at 100°C. Filtered and 12gms agar were added to 250 ml distilled water. NaCl, peptone water, and pericarp powder were used as additives to formulate different culture media. In all experiments pH of the media was adjusted to 6.5 – 7.0. The dissolved media were sterilized in a water bath at 100°C for 30 minutes, then were poured into sterile Petri dishes separately **(Table 1)**.

Table 1: Formulation of media 1, 2 and 3 where Spirulina Powder is the main constituent:

Ingredients	Medium 1	Medium 2*	Medium 3**
Spirulina powder	5 gm	10 gm	15 gm
NaCl	05 gm	05 gm	05 gm
Agar	12 gm	12 gm	12 gm
Pericarp powder	-	02 gm	02 gm

*The mixture was dissolved in250 ml distilled water
** The mixture was dissolved in 250 ml distilled water and 10ml of peptone water

H. infleunzae reference strain ATCC (49247), and three clinical isolates were inoculated on to the spirulina media 1, 2 and 3 and incubated under an anaerobic atmosphere with CO_2 (10%CO_2) at 37 °C. Significant bacterial growth was checked every 12 hours for three days. Colonies size, shape, and color were reported and compared with those produced on Chocolate agar.

RESULTS:

Growth on solid media:

Spirulina media 2 and 3 supported the growth of *H. infleunzae* in the formulated culture media. There was no significant variation in the colony morphology. Colony size was slightly smaller than that produced on ordinary enriched media (CA) (**Table 2**). (**Figure 1 and 2)**

Figure 1. Hemophilus infleunzae on chockalott agar medium

Figure 2. Heamophilus infleunzae on Spirullina Medium

Table 2: Growth of *H. infleunzae* on different spirulina media (1, 2, and 3), C.A .media.

Type of culture media	Beginning of growth	Complete growing	Colony Size	Colony Shape & Color	Condition of the growth
Medium1	No Growth	→	→	→	37 °C anaerobic with a CO_2 atmosphere
Medium 2	At 24 hours		Smaller size colonies than that produce on Chocolate agar(4-4.5mm)	Moist, convex, white color	37 °C anaerobic with a CO_2 atmosphere
Medium 3	At 24 hours	At 48 hours	Smaller size colonies than that produce on Chocolate agar(4-4.5mm)	Moist, convex, white color	37 °C anaerobic with a CO_2 atmosphere

Chocka lott agar media	At 24 hours	At 48 hours	Mediu m size colonie s	Moist, convex, transpar ent	37 °C anaerobi c with a CO_2 atmosph ere

DISCUSSION:

This study was constructed to investigate and assess the capability of spirulina powder to provide nutrients and growth factors required to support fastidious bacteria's growth. Three formulations of spirulina media were examined for their ability to support *H.infleunzae* growth as an example of a fastidious organism. The growth of *H.* infleunzae on spirulina media indicates that this candidate medium provides the essential elements and growth factors to support *H. infleunzae* recovery. Commonly used enriched culture media like chocolate agar media contain heated blood as enriched nutrients to support the growth of fastidious bacteria. However, these media are prone to contamination during the pouring process of blood. Dried spirulina is a good nutritional source with high protein content and significant lipid content. Spirulina is high in unsaturated and polyunsaturated fatty acids in particular (25% - 60% of the total fatty acids), such as oleic acid, linolenic acid, gamma-linolenic acid and docosahexaenoic (DHA).It also contains amino acid with a high nutrient digestibility. In addition, spirulina contains substances such as pigments (for example carotenoids such as β-carotene and zeaxanthin), phycobiliproteins (for example phycocyanin, which is unique in the cyanobacteria), vitamins, polysaccharides, macro and micro mineral elements and antioxidants [10,11].

Spirulina media prepared in this study contained a small amount of spirulina powder, NaCl, agar for solidification, and other additives (peptone water and pericarp powder). This study showed that: Spirulina media supported *H. infleunza* growth on formulated culture media 2 and 3, with no significant variation in the colony morphology, but the colony size was slightly smaller than that produced on ordinary enriched media (CA). However, white colonies produced in spirulina media. This result indicated that spirulina powder with same additives could be used as an enriched medium to support *Heamophilus infleunzae* culturing. However, growth inhibited in culture media 1, because it lacking Factor X (hemin) and contain only little amount of non-heme iron source Takagi *et al* reported that *H. influenzae* culture medium improved by studying the effect of hemin (X factor) and Isovitale X supplement containing V factor, specific requirements, as is or hemin. It has been reported that hemin is necessary only for aerobic growth. [12,13]. Roger et al reported that the components had no appreciable effect on the growth properties of *H. influenzae*. Neither the L-histidine nor nitrilotriethanol is needed by the organism, but are used to keep hemin in suspension. There are two types of iron: heme iron and non-heme iron. Heme iron is typically found in meat products, while non heme iron is found in nuts, seeds, fruits and veggies and dark green plants. For most part. Heme and non-heam iron are the same, however. Heam is typically absorbed well than non- heme–iron and absorption rate is indicated5 to 6 times higher than non- heam iron. The absorption of non-heme iron could be inhibited by dietary fiber during their absorption. However, Iron ions are surrounded by prophyrin rings, and there for, it is less likely to damage [5,14,15]. Pericarp powder used as additives in Spirulina medium type 2 and 3 support *H. influenza* growth through high non-heme iron supplement.

CONCLUSION:

In conclusion, the present study showed that spirulina medium with addition of peptone water, NaCl and Pericarp powder can support *Haemophilus influenza* growth compared to ordinary CA media. Based on these findings, The proposed spirulina medium 2 and 3 formulation will be a possible medium candidate to support the growth of fastidious bacteria.

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