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ORIGINAL ARTICLE

Production of haemophilus growth factor disks in Iran

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ABSTRACT

Background: Haemophilus influenza may cause severe infections in children and adults. The isolation and identification of *Haemophilus* spp. are not appropriately achieved in Iran, while numerous infections are ascribed to the different members of this genus. Lack of growth factors disks is our main shortage in this regard.
Materials and methods: We obtained haemin (X-factor) from human blood, and nicotinamidadenine di nucleotide (V factor) from yeast cells. The products were absorbed to the filter paper disks and a comparative analytical study was performed using prepared disks in comparison to commercial disks from Oxoid Company.
Results: Results revealed that the prepared disks were as useful, sensitive and potent as the Oxoid disks in isolation and identification of *H. influenzae and H. parainfluenzae*.
Conclusion: We have prepared growth factor disks according to the original formulations. They could be commercially produced and uses in microbiological laboratories.

Keywords: *Haemophilus, Growth factor,Factor V, Factor X*.
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INTRODUCTION

The Genus *Haemophilus* includes a large number of non-pathogenic and pathogenic bacteria, which may cause severe infections in children and adults with a high mortality rate if remained untreated (1).

H. influenzae was first described by Pfiffer in 1892, and was thought to be the causative agent of influenza pandemic occurred during 1889-1892 (2-4). He finally grew the bacillus on a blood supplemented medium. It is a fastidious organism which requires good quality nutrient medium, especially two "growth factors" that are present in blood and determine the hemophilic nature of Pfeiffer's bacillus (2-7). Identification and characterization of growth factors were achieved during the first few years after introducing the microorganism. The phenomenon of "Satellatism" was first described by Grossberger in 1897, and then Ritter showed that the growth promoting substances from staphylococcus can be used for the growth of H. influenzae (8).

Pittman classified H. influenzae in capsulated and non-capsulated strains. Six different serotypes (a-f) were demonstrated among the capsulated strains, while type b was considered as the most important pathogen. Study of biochemical features of H. influenzae was postponed because of

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difficulties in growth media till 1974, when Kilian demonstrated different biotypes on the basis of indole, ornithine decarboxylase and urease tests, meanwhile, it was found that H. parainfluenzae could be identified by ONPG test (9-13).

Haemophilus Genus is now defined as: small to medium-sized spherical, oval, or rod shaped cells; generally less than 1µm in width and variable in length, sometimes forming threads of filaments and showing marked pleomorphism, gram negative, non-motile and facultative anaerobe. Almost all species require preformed growth factors in the blood, particularly X factor (protoporphyrin IX or protoheme) and/or V factor (nicotinamide adenine dinucleotide (NAD) or NAD phosphate (NADP) (14,15). Necessary growth factors for cultivation of *Haemophilus* Genus include nicotinamide adenine di nucleotide (NAD) and haemin that are designated as V and X factors, respectively (16). Some species require one of these factors while others require both; a fact that help to identify different species.

These growth factors are produced by different factories and are provided in the forms of disks containing V-, X-, or XV-factor(s) commercially. Unfortunately, the disks are not available easily in Iran and are quite expensive.

We have intended to prepare these growth factors disks in Labbafinejad hospital, affiliated to Shaheed Beheshti University of Medical Sciences, according to the original formulation and recommendation described by Marshall (15) and subsequently designed a study to compare their potency and efficacy with the available commercial disks.

PATIENTS and METHODS

To prepare X factor the following protocol was observed: forty milliliters of human blood was centrifuged and 100ml of acetone containing 1.2ml of concentrated hydrochloric acid was added while shaking the solution. Then it was filtered and 120ml of distilled water was added to the filtrate to precipitate the haemin. The mixture was filtered and washed with water and dissolved in 25ml of 0.1M $NaHPO_4$ and sterilized at 115^oC for 10 minutes.

V factor was prepared as follow: fifty grams of yeast was suspended in 100ml of 0.2M KH_2PO_4 and heated to 80^oC for 20 minutes. The supernatant was clarified and sterilized by filtration and stored in refrigerator.

Finally, the following protocol was observed to prepare X-, V- and X/V factor disks: filter paper (Whatman No. 3) was cut into disks of 10mm in diameter. The disks were soaked with X-, V-, as a mixture of X- and V- factors solutions separately. The disks were drained and dried at 37^oC, then stored in a refrigerator.

Single, non-motile, oxidase-negative, gram-negative coccobacilli isolated from sputum (on chocolate or blood agar) were suspended in 3ml of peptone water and inoculated into a duplicate of plain agar plate. The prepared X-, V-, and XV-disks were placed on the plate with a distance of 2cm apart from each other while commercial disks of Oxoid Company were placed on the next plate (control). The plates were incubated at 37^oC for 24 hours in a candle jar. Finally, the growth pattern around each disk was recoded and compared with its duplicate (control).

RESULTS

Totally, 75 gram-negative, oxidase-negative coccobacilli were obtained from sputum samples after homogenization and culture on chocolate agar plates, and were tested for their growth factor requirements using our disks in parallel with Oxoid disks as a control. The results are shown in table 1.

Results revealed that the prepared disks were as useful, sensitive and potent as the Oxoid disks in isolation and identification of H. influenzae and H. parainfluenzae.

Table 1. *Comparative study of growth factors requirement of H influenzae and H parainfluenzae using prepared (test) and Oxoid disks as control*

		H. influenzae	*H. parainfluenzae*
Factor V	Oxoid	0	47
	Test	0	47
Factor X	Oxoid	0	0
	Test	0	0
Factor X/V	Oxoid	28	47
	Test	28	47

DISCUSSION

Determining the requirements of growth factors is the initial step in identification of species within the *Haemophilus* genus. Some of these species require haemin (X) and nicotinamide adenine dinucleotide (NAD) (V) in their culture medium.

X factor could be supplied by heat-stable iron containing pigments that provide protoporphyrins which is essential for catalase, peroxidase, and cytochrome oxidase of electron transport chain. The heat-labile V factor is a coenzyme that may be supplied by nicotinamide adenine dinucleotide phosphate or nicotinamide nucleoside (2,3,6,9).

H. influenzae, H. suis, H. hoemolyticus, H. gallinarum and H. aegypticus are within the X/V-depended group. The next *Haemophilus* species require only NAD as growth factor, thus, they can grow around both X/V- and V-containing disks. H. parainfluenzae, H. parasuis, H. parahaemolyticus, H. paragallinarum, H. paraphrophilus and H. parophrohaemolyticus are classified in this group. Finally, the third group is composed of species require haemin. H. Ducreyi, H. haemoglobinophilus, H. influenzaemurium, H. aphrophilus and H. ovis fall in this group. They grow around X- and X/V-containing disks. Therefore, the identification of various haemophili isolated from patients requires the initial determination of the growth factor requirements.

Growth factors are usually purchased from different factories and are not routinely available in Iran; meanwhile, they are quite expensive when ordered privately.

We have prepared these growth factors according to the original formulation of Marshal (15) and compared them with the growth factor disks supplied by Oxoid Company. Fortunately, we found our products as sensitive and potent as the control disks. Thus, they could be commercially prepared with lower expenses.

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