Archives of Razi Institute, Vol. 66, No. 1, June (2011) 43-49

Full Article

Comparison of bacterial biomass and PRP production between different isolates of *Haemophilus influenza* type b (Hib) under different culture conditions

Esmaily[*1], F., Aminian[2], M., Tavangar[1], A.R., Hadi[1], A.

1. Department of Human Bacterial vaccine, Razi vaccine and serum research institute, Karaj, Iran

2. Department of Biochemistry, Tehran University of Medical Science, Tehran, Iran

ABSTRACT

Heamophilus influenzae type-b (Hib) is a gram - negative pleomorphic bacterium that causes meningitis infections in children with the age of less than 5 years particularly in two years old infants. In the present study various isolates of *Heamophilus influenzae* from infants suspected to meningitis were collected, identified, characterized and were used throughout our experiments. Different culture media namely Brain Heart Infusion Broth (BHIB), Tryptic Soy Broth (TSB) and GC medium Base (GCB) which this medium was modified and prepared in our own laboratory, were compared to determine the highest bacterial yield. All media were added supplements 10mg/ml hemin & 0.01/ml IsovitaleX containing V factor. The bacterial yield for all Hib strains present in our laboratory were measured with an initial inoculums of 10^4 cfu per ml. The result showed very closed amount of biomass for all isolates however, GCB had slightly higher yield and ultimately we chose this medium for cultivation and extraction of capsular polysaccharide (CPS-b). In our laboratory we have adapted the PRP production according to our technical and instrumental availabilities which exists in our laboratories replacing ultra centrifugation to phenol chloroform to remove contaminants like endotoxin and proteins to the minimum level and also decreased the number of some chemical treatments while some steps were added in purification process. Our study showed although there were not significant differences between the PRP extract of the three isolates with average amount of 108 mg/lit, however, isolate ATCC10210 (ATF2) showed the highest amount with 192mg/lit and the least PRP was produced by isolate H.inf.1, with 16 mg/lit. It seems that the data can be categorized to a normal distribution with the mean of 106.4 and standard deviation of 6.25. This result was confirmed by one sample kolmogorov-Smirnov test, hence the PRP ≥192 mg/lit is statistically significant at a significant level of α =0.10 (P=0.085). The amount of contaminants e.g protein along with nucleic acid present in PRP was estimated at optical density 280 and 260nm, were under 10% and 5% respectively.

Keywords: *Haemophoilus influenzae* type b (Hb)**,** isolation, identification, polyribosylribitol phosphate (PRP), bacterial biomass

INTRODUCTION

Systemic infections by *Heamophilus influenzae* are almost always due to type b strains, for which the capsule is the principal virulence factor (Norden 1982). Before the introduction of effective vaccines Hib was the leading cause of bacterial meningitis and other invasive bacterial diseases eg. otitis media, epiglotitis, cellulites among children less than 5 years

[*]Author for correspondence.Email: fesmaily@yahoo.com

old(Bergeron 1987, Wenger 1998). Despite the availability of broad-spectrum antibiotics, the mortality rate had remained at 5 to 10% while 30 to 50% of the survivors developed chronic neurological complications such as blindness, deafness, convulsions, mental retardation and hydrocephalus (Nyhan & Richardson 1963, Sell *et al* 1972).

Antibodies to type b capsular polysaccharide which is composed of a linear polymer of ribosylribitol phosphate (PRP) confer protection against all Hib infections. It has been well demonstrated that vaccines which have been prepared from purified type b polysaccharide, although highly immunogenic in adults, are not as immunogenic in children less than two years of age. However, by conjugating the capsular polysaccharide to an immunogenic protein it possible to obtain a vaccine preparation capable of inducing high levels of antibodies against the type b polysaccharide and protecting vaccinated children (Peltola *et al* 1990).

MATERIALS AND METHODS

Haemophilus influenzae type-b strains coded as H.inf.1, H.inf.2, H.inf.3, ATF1 & ATF2 which have been isolated from infants under two years of age (from various children Hospital), were subsequently identified and characterized by bacteriological, biochemical and serological methods and were used in this study. ***H. influenzae*** serotype b was determined by direct slide agglutination with polyvalent and monovalent specific type sera (Sigma). The strains for further investigation were kept either as lyophilized powder or in vials of cryobank containing glass beads at -70 or in glycerol consisting of glycerol and bacterial culture with the ratio of 3 to 1 at -20 ^{o}C, and used for cultivation. The cryobank was used as a source of bacteria for our investigation.

Cultivation of bacteria. one bead of each Hib isolates from vials of cryobank (Mast Diagnostics) was put into each of five flasks containing 1lit of GCB which is modified and prepared in our own laboratory, consists of 7.5g pancreatic digest of casein, 7.5g peptic digest of animal tissue, 1g corn starch, 4g dipotasium phosphate, 1g mono potassium phosphate, 5g sodium chloride and 5g glucose (all from Merck) per lit, supplemented with hemin (Becton, Dickinson) 10mg/ml and IsovitaleX (Becton, Dickinson) containing 0.01/ml NAD, and incubated (Binder incubator, Germany) at 37 ^{o}C with 5-10% CO_2 for 18 hr with vigorous shaking (GFL, Germany). The pH of the culture was maintained at 7.5 during cultivation. The bacterial suspension were taken out at the late logarithmic – growth phase and checked for purity by Gram staining, also optical density was measured by spectrophotometer (LKB) at 560nm and the number of bacteria in each flask was calculated using serial dilution method (number of colonies on plate multiply by reciprocal dilution of simple equal to number of bacterial per ml). The same process of cultivation was carried out in BHIB (Merck) and TSB (Difco) supplemented with hemin 10mg/ml and IsovitaleX containing 0.01/ml NAD. All bacterial suspension were centrifuged at 6000 × g for 20 min at 4 ^{o}C and the wet weight of all strains were measured (0.01-Precisa) on GCB.

PRP was preparatd by modified method of Rachel et al 1980 the pellet (20g wet weight of cells/liter) were mixed with %1cetavlon (hexadecyltrimethyl ammonium bromide) (Merck), 0.5 M NaCl and stirred vigorously at 37 ^{o}C for 2 hours The suspension was centrifuged at 16000 × g for 2 hours at 4 ^{o}C and the bacterial sediment discarded, the supernatant was brought to 25% ethanol, stored overnight at 3-8 ^{o}C and centrifuged at 16000 × g for1 hour at 4 ^{o}C. This time the pellet was discarded, and the supernatant brought to 70% ethanol and stored overnight at 3-8 ^{o}C. The precipitate was removed by centrifugation at 2500 × g, at 4 ^{o}C and treated first with DNAase and RNAase followed by phospholipase, pronase and cold phenol chloroform pH 7.0 to remove residual protein, nucleic acid and lipopolysaccharide (LPS). The suspension then dialyzed with 12-14 KDa molecular weight cutoff dialysis tubing extensively against double-distilled

water at 3-8 ^{o}C and the product was finally analyzed and assayed by Bial method using ribose as standard.

Analytical methods of CPS-b (PRP) concentration. Protein was estimated with the Folin phenol reagent and bovine serum albumin as standard (Lowry *et al* 1951). Protein was also estimated along with nucleic acid at optical density 280 and 260 nm LKB Biochrom, Novaspec respectively and were dialyzed (Takagi *et al* 2006). Pentose then assayed by the orcinol reaction with D-ribose as standard using modified Bial method (Ashwell 1957).

Concentration of CPS-b, polyribosylribitol phosphate. was estimated using a conversion factor in which 1 mg of ribose corresponded to 2.55 ml of polyribosylribitl phosphate. The value for the conversion factor was based on the polyribosylribitol phosphate structural formula reported by (Crisel *et al* 1975).

RESULTS

The results of Gram stain for purity and colony morphology showed coccobacilli with some pleomorphic bacilli on GC agar containing both X and V factor. The need for X (hemin) and V (NAD) factors showed to be positive for all isolates. The result of capsule staining test, quelling (capsular swelling) examined as described by Alexander 1958 and the observation under electro- microscopic are all shown in figure 1(a, b, c, d, e). The polyvalent and mono specific type b antibodies which were used for recognition of the genus and type b showed positive agglutination results for all isolates, the list and source of five strains of *H.influenzae* including two ATCC and their code number used in this study are shown in Table 1**.** The bacterial mass was measured by dilution method (Milse & Mizra 1938) on GCB, BHIB, TSB, wet weight (only for GCB), optical density, colony morphology and also the retabulation of all Tables are shown in Tables 2, 3, 4 and 5. As it is shown in table 5 although there was not much difference between the biomass of the isolates however, strain H.inf ATF 2 showed the highest yield with the amount of 3.3 × 10^{11} cfu/ml and OD of 0.665 nm. The PRP amount of the strains were determined by multiplying the ribose concentration by 2.55 (Crisel 1975), the strain ATF2 showed the highest amount with 192 mg/lit of culture and the least amount was belong to strain H.inf1 with 16mg/lit.The protein and nucleic acid measurement of the strains showed to be under 10% and 5% respectively.

Table1.source and code number of *Heamophlus influenzae* type-b isolates

Source of bacteria	Isolates and code number
Mofid children Hospital, Tehran	H.Inf. 1
Emam Khomeini Hospital, children section, Tehran	H.Inf. 2
Mofid children Hospital, Tehran	H.Inf. 3
ATCC 35540	H.Inf ATF1
ATCC 10210	H.Inf ATF2

Table2. The cfu/ml, optical density (OD), wet weight and morphology of all isolates on GCB.

GCB medium	Cfu/ml after 18h.	Optical density (OD)	wet weight (g/lit)	Gram stain and colony orphology
H.inf 1	8× 10^{9}	0.480	23	Gram negative, cocobacilli
H.inf 2	1.24×10^{10}	0.525	25	Gram negative, cocobacilli
H.inf 3	9× 10^{9}	0.521	21	Gram negative, cocobacilli
H.inf ATF1	5× 10^{10}	0.600	20	Gram negative, cocobacilli
H.inf ATF2	3.3× 10^{11}	0.665	24	Gram negative, cocobacilli

DISCUSSION

Over the process of choosing the best culture media for highest bacterial yield of *H. influenzae* there are many factors to be considered. In 1945 Johnston described a medium that could successfully produce colonies of *N. gonorrhoeae in* 24 rather than 48 hours. The accelerated growth rates were primarily due to the decreased agar content (solidity) of the medium (Johnston 1945). GC medium base was introduced in 1947 with reduced agar containing glutamine and

cocarboxylase to improve recovery (Lankford & Snell 1943).

Table 3. The Cfu/ml, optical density (OD) and morphology of all isolates on BHIB.

BHIB medium	Cfu/ml after 18h.	Optical density (OD)	Gram stain and colony morphology
H.inf 1	1.6×10^9	0.480	Gram negative, cocobacilli
H.inf 2	1.5×10^{11}	0.639	Gram negative, cocobacilli
H.inf 3	5×10^8	0.390	Gram negative, cocobacilli
H.inf ATF1	1.32×10^{11}	0.606	Gram negative, cocobacilli
H.inf ATF2	6×10^9	0.583	Gram negative, cocobacilli

To achieve the best growth rate of *H.influenzae* type b(Hib) we modified and produced GC broth with optimum amount of 10g/lit of hemin(X factor) and IsovitaleX supplement which consists of V factor 10mg/lit together with vitamins, amino acids, coenzymes, dextrose and other factors to improve the detection and growth rate of H. inf.

Table 4. The cfu/ml,optical density (OD) and morphology of all isolates on TSB

TSB medium	Cfu/ml after 18h.	Optical density (OD)	Gram stain and colony morphology
H.inf 1	2.2×10^9	0.495	Gram negative, cocobacilli
H.inf 2	8.5×10^9	0.554	Gram negative, cocobacilli
H.inf 3	7.6×10^8	0.486	Gram negative, cocobacilli
H.inf ATF1	3.5×10^{10}	0.592	Gram negative, cocobacilli
H.inf ATF2	$4.9\ 10^9$	0.500	Gram negative, cocobacilli

The other process like addition of glucose to neutralize acidity (Dingel & Fothergill 1939) of the culture, time of cultivation presence of 5 – 10% Co_2 shaking, sparged aeration and pH control can increase the growth rate of Hib (Takagi *et al* 2003).

In our study although there were not much differences between the liquid media which were used, however, our modified GC broth showed the highest yield with the amount of 3.3×10^{11} cfu/ml for Hib strain ATF2.

Table 5. Retabiulation of tables 2, 3, 4.

strains	Liquid medium	CFU/ml	Optical density (OD)
H.inf ATF2	GC	3.3×10^{11}	0.665
H.inf ATF1	TSB	3.5×10^{10}	0.592
H.inf 2	BHI	1.5×10^{11}	0.639

Table 6. The amount of PRP/mg for all strains.

strains	Liquid medium	PRP mg/lit
H.inf 1	GC	16
H.inf 2	GC	116
H.inf 3	GC	108
H.inf ATF1	GC	100
H.inf ATF2	GC	192

For extraction of PRP, although investigators have isolated PRP from culture for several decades but underlying physiology of synthesis has not been completely described (Anderson *et al* 1976).

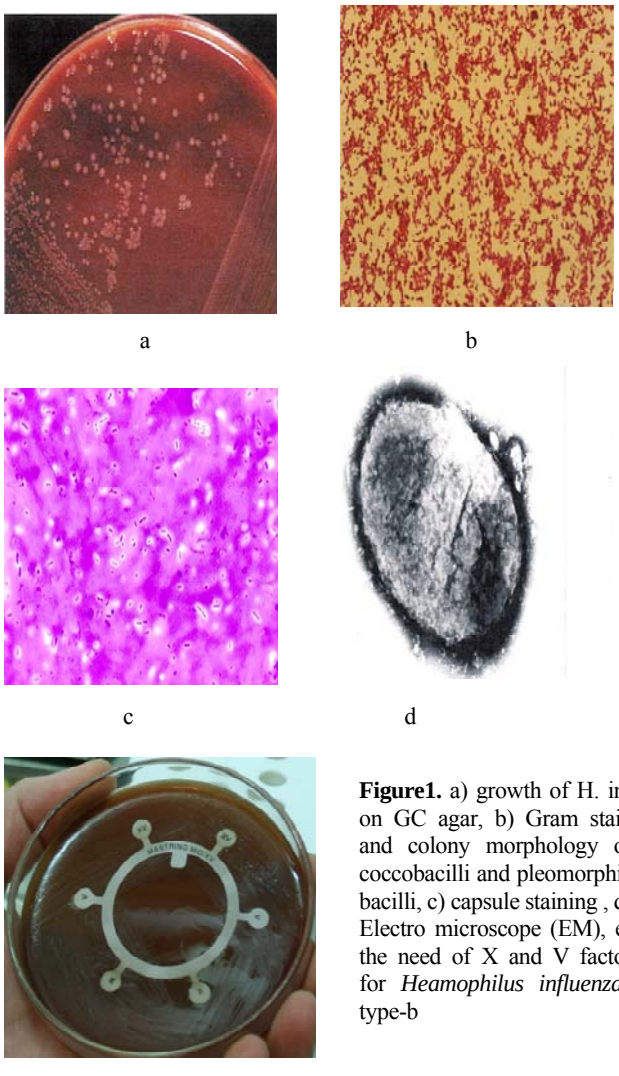

Figure1. a) growth of H. inf on GC agar, b) Gram stain and colony morphology of coccobacilli and pleomorphic bacilli, c) capsule staining , d) Electro microscope (EM), e) the need of X and V factor for *Heamophilus influenzae* type-b

It has been shown that the capsulation of the organism began to deteriorate after certain bacterial density on the surface of solid medium, however, this is much less on broth culture (Alexander 1958). Since the production cost of conjugate vaccine is high we have modified a method which is easy and more economic than conventional procedures. We have replaced ultrafiltration by phenol chloroform and decreasing some of chemical steps while using pronase can decrease the protein contaminant to minimum level. These factors can give us some advantages for large scale preparation of PRP.

The comparison between the PRP which we prepared from the isolates showed that strain ATCC10210 had the highest amount of PRP 192 mg/lit and the least amount was belong to H.inf.1 with 16mg/lit. By using the GC liquid medium and the above process for extraction of PRP, this procedure can be easily adopted and optimized for scale up production of PRP in future.

References

Alexander, H.E. (1958). *Haemophilus influenzae*, p. 470-485. In R. J. Dubos (ed.), Bacterial and mycotic infections of man. J. B. Lippincott Co., Philadelphia.

Anderson, P., J. Pitt, and D. H. Smitth. (1976). The synthesis and release of polyribophosphate by Haemophilus influenzae type b in vitro. *Infection Immunology*13:581-589.

Ashwell, G. (1957). Colorimetric analysis of sugars. *Methods Enzymol* 3:73-105.

Bergeron, M.G, Simard P., and Provencher P. (1987). Influence of growth medium and supplement on growth of *Haemophilus influenzae* and on antibacterial activity of several antibiotics, *Journal of clinical microbiology* 25: 650-655.

Crisel, RM, Baker RS, and Dorman DE (1975) Capsular polymer of *Haemophilus influenzae*, type I – structural characterization of the capsular polymer of strain Eagan. *Journal of Biological Chemistry* 250: 4926-4930.

Dingle, J.H., and L.D. Fothergill. (1939). Purification of the type-specific polysaccharide from Hemophilus influenzae, type b. *Journal of Immunology* 37:53-68.

Johnston,. J. (1945). Veneral Disease Infromation. 26:239. Cited by Rafael Llanes, Calixto Domínguez, Alberto Prat, Claudio Rodríguez, Eduardo A. Valdés, Oderay Gutiérrez and Daymi.

Guzmán, V.J.T, Antonano, F.J.L., Salgado, N.R.(2005). In: *Archives of Medical Research* 36, 4, 344-349.

Lankford, C.E. and Snell< E.E. (1943). Glutamine as a growth factor for certain strains of *Neisseria gonorrhoeae*. *Journal of Bacteriology* 45:410.

Lowry, O. H., Rosebrough, N.J. Farr, A.L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent, *Journal of Biological Chemistry* 193:265-275.

Milse, A.A. & Misra, S.S. (1938). Estimation of the bacterial power of blood. J.38:732-749.

Norden, C.W.(1982). In Bacterial infectious of humans.ed by A.s. Evans and H.A. Feldman. Plenum, New York, 259-273.

Nyhan, W.L., and F. Richardson. (1963). Complications of meningitis. *Annul. Review Medicine* 285:666-667.

Peltola, H., Kaythy H, Virtanen M, Makela.(1990). PH. Prevention of Haemophilus influenzae type b bacteremic infections with the capsular polysaccharide vaccine. New *England Journal of Medicine*310:1561-6.

Porter, A., Jane P., and David H, Smith. (1956). Synthesis and release of polyribophosphate by *Haemophilus influenzae* type b in vitro. *Infection and immunity* 581-589.

Rachel, S.,Osmar B.,Ann S., John, B.R.(1980). Preparation, characterization, and immunogenicity of Haemophilus Influenzae Type b polysaccharide protein conjugates. *Journal of Experimental medicine* 152: 361-375.

Sell, S. H. W., Merrill, R. E. Doyne, E. O. and Zimsky, E.P. (1972). Long-term sequellae of *Hamophilus influenzae* meningitis. *Pediatrics* 49:206-211.

Takagi, M, Cabrea-Crespo J, Zangirolami TC, Raw I, Tanizaki MM (2003). Characterization of polysaccharide production of *Haemophilus influenzae* type b and its relationship to bacterial cell growth. *Journal of Applied biochemistry and biotechnology* 110:602-608.

Takagi, M, Cabrea-Crespo J, Zangirolami TC, Raw I, Tanizaki MM (2006). Improved cultivation conditions for polysaccharide production by H. influenzae type b. *Journal of Chemistry Technology Biotech*nology 81:182-188.

Wenger, J.D. (1998). Epidemiology of Haemophilus influenzae type b *disease* and impact of Haemophilus influenzae type b conjugate vaccines in United States and Canada. *Journal of pediatric infectious disease* 17, S132 602-608.