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Full Article

A study on *Haemophilus influenzae* type b growth rate and capsule production in different media

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ABSTRACT

In the present study, seven isolates of Haemophilus influenzae type b were cultured in four different media to compare growth rate and capsule production. Four liquid media namely brain heart infusion broth (BHI), trypticase soy broth (TSB), Mueller Hinton Broth (MHB) and Gonococci broth (GC) with added supplements (1% hemoglobin, 1% Isovitalex) were used. The growth was measured by colony counting (CFU/ml) using serial dilution. Four of the isolates showed the highest growth rate with the average of 10^{13} CFU/ml on BHI broth while TSB had the second highest growth of more than 10^{10} CFU/ml in an 18-hour culture at 37 ºC culture. In the next step, the amount of capsular polysaccharide (CPS-b) antigen which is made of Polyribosyl ribitol phosphate (PRP) was assessed all isolates by two methods: modified Indirect Sandwich Enzyme-linked immunosorbent assay (ELISA) and Bial method to select the isolate producing the highest amount of PRP. The antisera used in sandwich ELISA were prepared by immunization of Rabbit and Rat. The maximal amount of PRP was produced by isolate Hib (5s) with the amount of 321 mg/lit.

Keywords: *Haemophilus influenzae* type b, bacterial yield, polyribophosphate(PRP), supernatant, Bial method, Indirect sandwich ELISA

INTRODUCTION

Haemophilus influenzae serotype b (Hib) is well established as one of the leading causes of bacterial meningitis in children between 3 months to 5 years old (Adams *et al* 1993, Bergeron et al 1987, Wenger 1998). The capsular polysaccharide antigen (CPS-b) of Hib was characterized as a polymer of D-ribose phosphate and named polyribophosphate(PRP) (Zamenhof 1953, Rosenberg 1962). Subsequent studies identified ribitol as an additional constituent (Kroll 1991, Argaman 1974) and proposed a structure based upon equimolar ribose, ribitol, and phosphate (Crisel 1975). CPS-b appears to be the principle virulence determinant in systemic infections by Hib (Alexander 1965, Robbins 1973). Antibody to PRP although protective in adults cannot give immunity to children under 2 years of age (Murphy and Apicella, 1987, Murphy 2003). However the purified PRP

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conjugated to a protein is effective vaccine for children less than five years old (Anderson 1972, Wenger 1990). In these days of automated microbiology, *Haemophilus influenzae* is still difficult bacterium to grow and automated systems have not yet been successful in providing adequate identification results (Bergeron 1987). Much still remains to be discovered about the influence of different media on the bacterial yield of Hib. Four liquid media have been used throughout our study to compare the growth rate of 7 different isolates. The purpose of this study was to evaluate the influence of mentioned media on growth rate and polysaccharide capsule production.

MATERIALS AND METHODS

Bacterial isolates. Throughout the period from January 2009 to June2010, two strains of Hib were isolated from Bahrami children Hospital in Tehran. Three of 7 isolates had been isolated in the past by previous workers from various hospitals in Tehran (2002, unpublished). The strains were isolated from CSF specimens of children suspected to meningitis; also two ATCC (Table 1) strains were used as standard in all experiments.

Identification procedures. Isolates were identified as Hib by Gram and capsule staining, typical colony morphology on chocolate agar, failure to grow on blood agar, requirement for hemin and NAD(X, V and XV factor disks; HUGO™) and fermentation of sugars. Pittman serotype (a to f) was determined by direct slide agglutination and Quellung reaction using polyvalent and monovalent specific typing sera (Difco).

Test media and supplements. There are lots of media that have culture conditions for *Haemophilus*. In this study, the growth rate was compared between four different basic media: brain heart infusion broth (BHIB) (Difco), trypticase soy broth (TSB) (Difco), Mueller-Hinton broth (MHB) (Difco), and Gonococci broth(GCB), which this particular medium was modified in our own laboratory GCB consisted of : Pancreatic digest of caseion, peptic digest of animal tissue, Corn starch, Di potassium phosphate, Mono potassium, Sodium chloride and Agar. The added supplements were 1% hemoglobin (BBL) and 1% Isovitalex (BBL) consisting NAD, vitamins and minerals.

Growth rate (bacterial yield) and conditions. The growth rate of the 7 isolates of Hib in four different basic media enriched with 1% Isovitalex and 1% hemoglobin was compared. Each medium was inoculated with initial inoculum of 10^4 CFU per milliliter of each isolate in a volume of 10 ml of each culture, and incubated for about 24 h at 37 °C with 5-10% CO_2 and vigorous shaking. Optical density of each culture was measured at 640 nm, before and after the cultivation. The method used for determination of the bacterial yield, was serial dilution bacterial colony counting used for measurement of CFU/ml. To calculate the CFU/ml this formula was used: Number of colonies on the plates × reciprocal of dilution.

Measurement of PRP amount. The PRP released by isolates of Hib was prepared from the supernatant of each media. Flasks containing 10mL of culture were withdrawn from incubator and centrifuged at 3220 × *g*, 4 °C for 60min[i] and the supernatant were obtained. Two different methods were used, a serological and a biochemical assay to measure the amount of capsular polysaccharide or PRP in the supernatant of each broth culture medium.

Antisera. For Indirect sandwich ELISA used in this study, two different sources of anti PRP antibody were prepared; rat and rabbit anti PRP antibodies. These antisera were prepared by 5 times injections of Hib vaccine(Vaxem Hib,Chiron)into 14 days old infant rats Dutch breed, and 3 to 4 months old rabbits of Vista breed. The interval of injections was 7 days. The rabbits and rats were injected subcutaneously (sc) and intraperitonealy(ip) respectively. The animals were bled 7 days after the last injection and sera were collected .

1) Indirect sandwich ELISA. Since sticking process of polysaccharide antigens to polystyrene plates is not as easy as protein antigens, we used indirect sandwich

ELISA. In this method, the first antibody (rabbit anti PRP) is bound to the solid phase and the antigen (PRP) is captured (Crowther 2001). In the next step, antigen would be detected using the second antibody (rat anti PRP). Finally the second antibody is bound by goat anti rat conjugated with peroxidease (goat anti rat ~ PO, MAST™).

Assay steps. Step1. Plates were coated with100 µl of 1: 80 dilution of rabbit anti PRP antiserum in carbonate buffer, based on results of checker board. Step2. After washing three times in PBS-Tween80, wells were blocked with 250 µl of 5% skimmed milk dissolved in PBS as blocking buffer and incubated at room temperature (RT) for 2-3 h . Step3. All plates were washed and 100 µl of each diluted samples(1:200 and 1:1000 in blocking buffer)were added to each well and incubated for about 24h at RT. Positive control, negative control and standard sera were added to each plate. Step4. After washing 3 times, 100 µl of rat anti PRP was added (1:40 was selected according to checker board and incubated for 3h at RT. For checker board the antigen concentration was 50ng/ml and the antisera dilution were started from 1: 20. Step5. After washing, detection of rat antibody was developed by addition of 100 µl of 1:2000 goat anti rat ~ PO to all wells. Step6. Plates were incubated for 2h at RT, and then washed 5 to 6 times with PBS-Tween 80. Step7. 100 µl of TMB-ethanol as substrate was added to each well and incubated for 30 minutes at RT. The reaction was stopped with 100 µl of 2M sulfuric acid and read at 450 nm with ELISA reader. (Figure 1)

2) Bial method. (i) Phenol chloroform extraction; to decrease contaminants like proteins to minimum level, 0.5 ml of the supernatants obtained from each culture were submitted to the same volume of phenol chloroform. The resulted precipitates was discarded after centrifugation at 10,000 x g for 15 min at 4 °C and the liquid phase was used as samples for measurement of PRP.

(ii) Procedure for Bial test; 0.4 ml of each sample was diluted with equal volume of PBS. Then, 200 µl of above sample was taken and mixed with the same volume of distilled water plus 1 ml of Bial's reagent (orcinol – HCl). In the last step, the mixtures were heated for 45 min in a boiling-water bath, and optical density was read at 670 nm. The PRP concentration was determined using ribose (Sigma) as standard. The concentration of PRP, was estimated using a conversion factor in which 1 mg of ribose corresponded to 2.55 mg of polyribosylribitol phosphate (Pavia 2005).

Data Analysis. Mann-Whitney Test was employed to compare the average of CFU for the isolates in MHB with other media using the statistical package SPSS 13.0 and p-value less than 0.05 considered as a significant level.

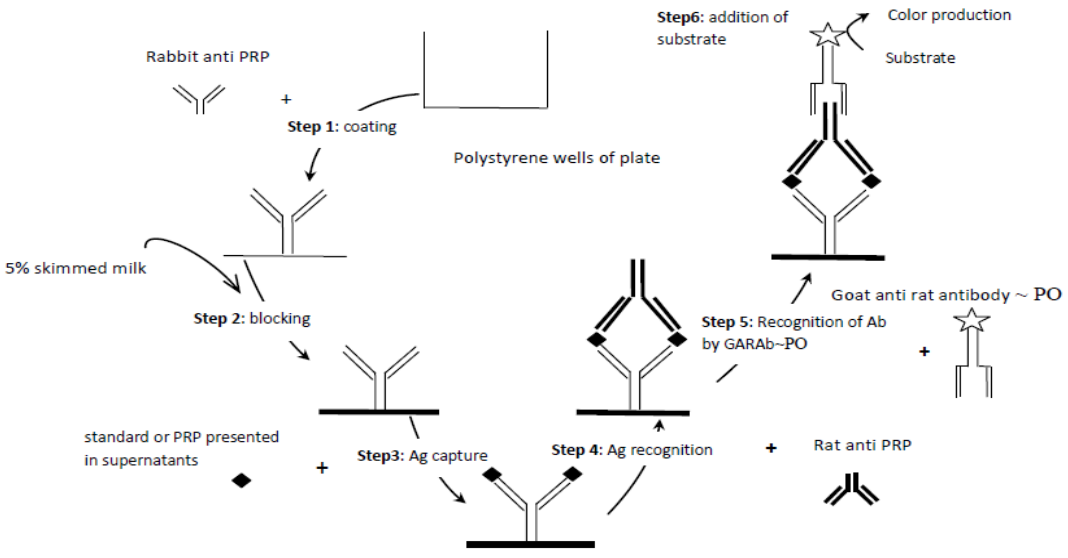

Figure1. Schematic representation of ELISA indirect sandwich assay.

RESULTS

Identification of the isolates. Two strains isolated from Bahrami children hospital in Tehran were taken to Razi Vaccine and serum research institute, Karaj Hib Lab, and were identified as *Haemophilus influenza* type b (Hib), and the rest of strains used in our study had been identified previously (Esmaily *et al* 2011). Two ATCC strains used as positive control. The list of strains and their code number are shown in Table 1.

Table 1. source and code number of *H. influenzae* type b isolates.

Source of bacteria	Strains and code number
Mofid children Hospital	Hib(1)
Mofid children Hospital	Hib(2)
Mofid children Hospital	Hib(3)
Bahrami children Hospital	Hib(4b)
(ATCC 35540)	Hib(5s)
(ATCC 10210)	Hib(6s)
Bahrami children Hospital	Hib(7b)

Growth studies. As it is shown in Table 2, five out of 7 isolates showed the highest bacterial yield in BHIB with the rate of > 10^{12} CFU/ml after incubation for 24 h. The rest of isolates had the maximal growth rate in TSB. However our study showed the strain Hib(2) with the highest CFU/ml among all the isolates. The average of CFU for the isolates in MHB were significantly lower than those used in other media ($p<0.005$), varied from 10^3 to 10^6 CFU/ml. All media were enriched with 1% hemoglobin and 1% IsovitaleX as supplements.

PRP measurement. Two methods were used to determine the extent of PRP that each isolate had released. The Indirect sandwich ELISA produced irreproducible results and high background levels due to various reasons. In checker board the optimum dilution of rabbit anti PRP and rat anti PRP antibody was determined by testing dilutions ranging from 1:20 to 1:2560 for first antibody and 1:20 to 1:40960 for second antibody. The 1:80 dilution of rabbit anti PRP and 1:40 of rat anti PRP antibody were selected on the basis of OD and dilution (Table 3). Several procedures used were found to be unreliable and quickly forsaken. Using rabbit anti PRP for coating polystyrene plate and PRP as antigen in supernatants followed by second antibody (rat antiPRP) and ultimately conjugated antidody showed negligible OD and highly irreproducible results. The outcome of checker board may have been as a result of non specific binding of antibodies and antigen. Various reasons could have interfered to the results of ELISA assay e.g; the small size of the antigen (PRP), impurity of antibodies and samples and non specific binding of antibodies. But the second method which was Bial reaction worked well.

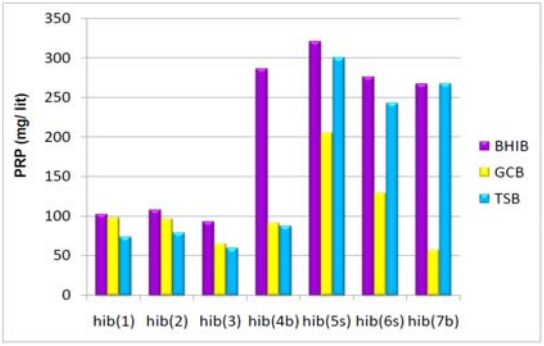

Figure 2. PRP amount released from each isolate cultured in four media

Bial reaction: The amounts of PRP obtained from each isolate by this method are shown in Table 3. All isolates produced the highest amount of PRP in BHIB compared to TSB and GCB. The average amount of PRP released by 3 of the isolates was < 200 mg/lit, 3 of other isolates showed the amount of <300 mg/lit, however isolate Hib(5s) had the highest amount of PRP with 321 mg/lit.

DISCUSSION

To achieve high amount of CPS-b released into the media, that would be beneficial to choose the proper culture medium and also the proper PRP producing isolate for vaccine production against Hib, our laboratory studied some culture media and conditions to choose one of the studied media. Our results showed that isolate Hib(2) which was one of the local isolates

Table2. The growth rate of isolates on the basis of CFU/ml and optical density

Strains	CFU/ml in BHI	OD of BHIB: 0.141 OD in BHIB	CFU/ml in GC	OD of GCB: 0.151 OD in GCB	CFU/ml in TSB	OD of TSB: 0.081 OD in TSB	CFU/ml in MHB	OD of MHB: 0.134 OD in MHB
Hib(1)	4.3×10^{12}	0.625	4×10^{12}	0.301	1.9×10^{12}	0.312	1.7×10^{4}	0.01
Hib(2)	6.6×10^{16}	0.462	8.2×10^{13}	0.397	1.2×10^{14}	0.862	8.8×10^{7}	0.07
Hib(3)	9.9×10^{13}	0.666	5.0×10^{11}	0.353	1.51×10^{10}	0.352	7.5×10^{6}	0.03
Hib(4b)	4.9×10^{13}	0.542	2.0×10^{11}	0.444	2.25×10^{11}	0.419	9×10^{4}	0.02
Hib(5s)	5.0×10^{10}	0.321	1.32×10^{11}	0.330	3.55×10^{11}	0.723	$10^{7}\times5.6$	0.08
Hib(6s)	1.53×10^{12}	0.839	1.24×10^{10}	0.420	8.5×10^{9}	0.322	4.3×10^{6}	0.04
Hib(7b)	1.6×10^{9}	0.469	8.0×10^{9}	0.412	1.13×10^{10}	0.643	1.1×10^{5}	0.02

from CSF specimens from a child suspected to meningitis,had the high rate of growth with 6.6×10^{14}CFU/ml in BHIB. However the amount of PRP produced by this isolate was <200 mg/lit. The results obtained from indirect sandwich ELISA for measurement of PRP was not very conclusive. There are many reasons which can explain the outcome of vague results. The most important one is the presence of various other antigens e. g; proteins, lipopolysaccharides, phosphates, etc. as well as capsular polysaccharide antigen released into culture supernatant. Other factors like molecular size of antigen, impurity of prepared antibodies, or low levels of anti-capsular antibodies in the antisera, due to the weak or lack of antibody response to vaccination regimen used in animals. In 1979, Doris L. Drow and et al. described an indirect sandwich ELISA method which used polystyrene balls instead of microtiter plates. Use of these balls as the solid phase may be a key factor in this assay's specificity and sensitivity. Their diameters are extremely uniform. They have found the ease of manipulation of the balls together with their minimal space requirements for storage to be a distinct advantage over the use of microtiter plates. The measurement of PRP using bial method gave us a conclusive result (Figure2). The strain Hib(5s) showed the high amount of PRP with > 300 mg/lit.

These results showed that the rate of growth is not always in correlation with the amount of PRP, as it was shown by two isolates of Hib(2) and Hib(5s). The increase of PRP amount was seen in BHIB for most of the isolates which also showed the effect of culture media on the total synthesized of some antigens such as PRP.

Table 3. amount of PRP released by each isolate in three media.

strains	PRP(mgr/l)		
	BHIB	GCB	TSB
Hib1	102.7	98	73.8
Hib2	108	96.8	79
Hib3	93.4	65.6	59.7
Hib(4b)	286.7	91.4	87.3
Hib(5s)	321	205.3	300
Hib(6s)	276.4	129	242.6
Hib(7b)	267.2	57.3	111.2

Our result showed that BHI broth with some modification can be used to produce high bacterial yield .It was also shown that Bial method can be used for detection and measurement of PRP production.

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