

## Addition of isovitalex in chocolate agar for the isolation of *Haemophilus influenzae*

S.K. Saha<sup>\*,\*\*</sup>, A.H. Baqui<sup>\*\*,+</sup>, G.L. Darmstadt<sup>\*\*</sup>, M. Islam<sup>\*</sup>, S.E. Arifeen<sup>++</sup>, M. Santosham<sup>\*\*</sup>, T. Nagatake<sup>†</sup> & R.E. Black<sup>\*\*</sup>

*\*Department of Microbiology, Dhaka Shishu (Children) Hospital, Dhaka, Bangladesh Institute of Child Health, Dhaka, Bangladesh; \*\*Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA; ++Child Health Programme, ICDDR,B, Dhaka, Bangladesh & †Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan*

Received September 11, 2007

**Background & objectives:** The reason for lack of data on burden of *Haemophilus influenzae* type b (Hib) in developing countries was mainly failure of detection of this fastidious organism in laboratories. Use of isovitalex (IVX) was suggested as an essential supplement for growing this organism. This study was carried out to investigate the impact of IVX supplementation to chocolate agar for detection of Hib.

**Methods:** Chocolate agar with and without supplementation of IVX was prepared. Clinical samples as well as reference strains of Hib were simultaneously cultured on both the media.

**Results:** *H. influenzae* isolates (N=194) were simultaneously grown on chocolate agar (CA) with and without isovitalex (IVX). Average colony size of *H. influenzae* on CA with IVX (CA-IVX) was larger only by 0.10 cm (range 0.05 to 0.16 cm) compared to CA alone. Addition of IVX to CA increased the cost of media by 2.1-fold.

**Interpretation & conclusion:** Isovitalex is not essential for the isolation and growth of *H. influenzae* almost halving the cost.

**Key words** Growth - *Haemophilus influenzae* - isovitalex

Benefits of *Haemophilus influenzae* type b (Hib) vaccine are primarily limited to children of the developed part of the world mainly for lack of convincing local disease burden data from the developing countries. A principal reason for the underestimation of Hib is the failure of most laboratories in developing countries, especially in Asia, to detect this fastidious micro organism<sup>1-3</sup>.

Hib has appeared as a predominant cause of meningitis and pneumonia whenever explored applying standard microbiological procedures and rigorous quality controls<sup>4-6</sup>. However, use of special base media and/or supplement(s) is being recommended for investigating Hib burden, instead of emphasizing on good laboratory practices mentioned in the text books<sup>7,8</sup>. In 1994, Gellert *et al*<sup>1</sup> reported isovitalex (IVX) as an

essential nutrient for the growth of *H. influenzae* and that lack of this supplement in the culture medium led to failure of the Latvian National Bacteriology Laboratory to isolate the organism. They also reported that plain chocolate agar (CA) could not support growth of *H. influenzae* on subculture, unless supplemented with IVX. This suggestion has been well taken by the public health practitioners and policy makers, and the addition of IVX as an essential supplement for the isolation of *H. influenzae* has been included as a standard procedure in the laboratory manuals<sup>9</sup>. It is now widely believed that isolation of *H. influenzae* is difficult and requires substantial resources<sup>2</sup>, which has served to discourage laboratories of many developing countries from exploring the burden of Hib diseases.

We therefore used plain CA and IVX-supplemented (1%) CA (CA-IVX) simultaneously to isolate *H. influenzae* and other fastidious organisms, *Neisseria meningitidis* and *Streptococcus pneumoniae*, and compared for their colony size. Comparative cost of CA with and without IVX were also calculated and compared.

#### Material & Methods

The study was conducted at the Department of Microbiology of Dhaka Shishu Hospital, Dhaka, Bangladesh; during the period of 1995-1998. CA was prepared by heating blood agar base (Oxoid Ltd. Hampshire, England) with 5 per cent sheep blood at 75-80°C for 15 min in a water bath. IVX (Becton Dickinson, Sparks, USA) was added to CA at 45°C, and equal amounts (10 ml) of CA or CA-IVX were dispensed into respective separate chambers of the same petriplate. Each batch of CA media with and without supplement was tested for adequate growth of reference strain *H. influenzae* ATCC 49247. Cerebrospinal fluid (CF) specimens were collected from the children with suspected meningitis and sent to the department of microbiology as part of their clinical care. The specimens were simultaneously cultured on both media on the same plate and incubated at 37°C in a CO<sub>2</sub> jar for 18-20 h, following the standard procedure<sup>8</sup>. In addition to culturing the clinical specimens, Hib isolates collected from meningitis and pneumonia case of Japan (n=5) and the USA (n=10) were also cultured on both the media. Temperature and time of incubation were identical for both type of plates, and laboratory personnel remained blinded to the media type while culturing and observing bacterial growth. Impact of IVX on

colony size of *H. influenzae* was tested by culturing them on CA, with and without IVX. After 18-20 h of incubation in CO<sub>2</sub> jar, isolated colonies of the same isolate, at the distant part of streaking, on each of the media were digitalized and the average of two-way diameters of a colony was taken with the scale generated by Photoshop Adobe Program Version 6.0 (Adobe systems Incorporated, Sanjose, CA, USA). Diameters of 5 separate colonies of the same isolate on each media were measured in the same way and average value calculated.

#### Results & Discussion

During the period of 1995 to 1998, 540 CSF specimens were simultaneously cultured on both the media and 194 *H. influenzae* isolates were obtained. Of these, 189 were type b, 2 were type a and 3 were nontypable. All strains were isolated on both CA and CA-IVX after the same period of incubation, irrespective of their serotype and biotype. The ATCC strain 49247 always showed confluent growth on both the media. Although Oxoid blood agar base was mainly used in this study, we transiently switched to blood agar base of Remel (Lenexa, USA), and Mast Diagnostics (Merseyside, UK), and found that the CA made from any of these basal media, either with or without IVX supplementation, supported confluent growth of *H. influenzae*. All isolates of *H. influenzae* collected from Japan and the USA also grew well on CA and CA-IVX.

Average colony size of *H. influenzae* on CA-IVX was larger by 0.10 cm (range 0.05 to 0.16 cm) compared to CA. Calculation of cost showed that addition of isovitalex to CA increased its price by 2.1-fold.

One of the major reasons for the low usage of Hib vaccines particularly in developing countries, is the perception that Hib is not an important cause of disease<sup>2,6</sup>. This perception is a result of low isolation rates of Hib in developing countries, in part due to poor laboratory facilities and limited resources. It is, therefore, important to use methods that are inexpensive as well as sensitive for the detection of Hib.

We have successfully grown all 194 *H. influenzae* isolates on plain CA, irrespective of their sero- or biotype, without the addition of IVX. This indicates that CA is sufficient to support the growth of this organism.

Failure of other laboratories in Bangladesh<sup>3</sup>, India<sup>10</sup>, and Guatemala (Dr Edwin Esturias, JHU, personal communication) to isolate this fastidious organism was possibly due to faulty preservation of specimens before culturing, use of poor quality base media or source of blood, faulty preparation of media or a combination of these factors. Our success in isolating all *H. influenzae* isolates further emphasizes that the most important factors in preparing media for isolation of this organism are to (i) use a good quality and appropriate basal medium; (ii) addition of blood from an animal source (not human blood); (iii) use of correct temperature and time to chocolate the media; (iv) check the quality of each batch for its optimum ability to grow a set of test strains; and (v) use the prepared plates in a timely fashion. Minimal difference in colony diameter (0.10 cm) and growth of all isolates of *H. influenzae* indicates that IVX is not essential for primary isolation of this organism.

In conclusion, we recommend that isovitalex is not essential for the growth of *H. influenzae*, and the resource-poor institutions, with good laboratory practice, can continue their effort to isolate Hib without wasting their resources for IVX.

### Acknowledgment

The study was partially funded by Ministry of Health and Labor of Japan. Authors acknowledge Ms. Tania Nasreen for technical support and Prof. Gary Doern, University of Iowa, USA, for providing the strains.

### References

1. Gellert GA, Wenger JD, Brilla A. *Haemophilus influenzae* type b disease in Latvia. *Lancet* 1994; 344 : 959.
2. Levine OS, Wenger JD. Defining the burden of Hib disease in India. *Indian Pediatr* 2002; 39 : 5-11.
3. Setarunnahar, Chowdhury AA, Farida H. Aetiological agents of meningitis in Bangladeshi children. *Indian J Med Microbiol* 1988; 6 : 81-5.
4. Baqui AH, Arifeen SE, Saha SK, Person L, Zaman K, Gessner BD, *et al.* Effectiveness of *Haemophilus influenzae* type B conjugate vaccine on prevention of pneumonia and meningitis in Bangladeshi children: a case-control study. *Pediatr Infect Dis J* 2007; 26 : 565-71.
5. Saha SK, Abdullah AH, Gary DL, Amin MR, Hanif M, Arifeen SE, *et al.* Invasive *Haemophilus influenzae* type b diseases in Bangladesh, with increased resistance to antibiotics. *J Pediatr* 2005; 146 : 227-33.
6. Watt JP, Levine OS, Santosham M. Global eradication of Hib disease: What are the next steps? Proceedings of the Meeting. Scottsdale, Arizona. September 22-25, 2002. *J Pediatr* 2003; 143 : S163-87.
7. Campos JM. *Haemophilus*. In: Murry PR, Baron EJ, Pfaller MA, Tenover FC, Tenover RH, editors. *Manual of clinical microbiology*. 8th ed. Washington, D.C: American Society for Microbiology; 1995. p. 556-65.
8. Cheesbrough M. Microbiological tests. In: *Medical laboratory manual for tropical countries*. London: Tropical Health Technology and Butterworth-Heinemann; 2000; 2 : p.201-3.
9. Levine OS, Schuchat A, Schwartz B, Wenger JD, Elliott J. Generic protocol for population based surveillance to assess the local burden of meningitis due to *Haemophilus influenzae* type b among children less than 5 years old. Geneva: World Health Organization (WHO/VRD/GEN/95.05); 1996.
10. Panjarathinam, R, Shah RK. Pyogenic meningitis in Ahmedabad. *Indian J Pediatr* 1993; 60 : 669-73.

*Reprint requests:* Dr Samir K. Saha, Professor & Head, Department of Microbiology, Dhaka Shishu (Children) Hospital Bangladesh Institute of Child Health, Dhaka 1207, Bangladesh  
e-mail: sksaha@bangla.net