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IMMUNOCHEMICAL STUDIES ON PATIENTS WITH MENINGITIS

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ABSTRACT

Meningitis is an infection of the membranes (meninges) and cerebrospinal fluid (CSF) surrounding the brain and spinal cord. There are many types of meningitis: bacterial meningitis, viral meningitis, fungal (cryptoccal) meningitis and tuberculous meningitis.

Haemophilus influenzae type b (Hib) is a common respiratory pathogen and an important cause of morbidity in humans. Early diagnosis and treatment of *H. influenzae* infection can greatly improve the likelihood of recovery. Detection of *H. influenzae* circulating antigen may be responsible approach for the diagnosis of meningitis. *Hib* antigen was detected in 102 CSF and serum samples of meningitis patients using ELISA and western blotting techniques. Western blot analysis showing a single immunoreactive band only in the sera and CSF of meningitis patients at 95-kDa. The Quantitation detection of *Hib* antigen was performed by assaying serial fivefold dilution of the antigen and calculating the result from a standard curve performed with each experiment. A significant correlation was shown between the concentration of *Hib* antigen in serum and CSF and no significant difference ($p > 0.05$) was shown between detection rate of Hib antigen in serum (58%) and CSF (61%) samples.

We concluded that, ELISA facilitates investigations concerning pathogenic effects in human *Hib* disease so it can be used successfully in the detection of circulating *H. influenzae* antigen in CSF and Serum of meningitis patients. It is possible to use serum samples in the detection of the *H. influenzae* antigen other than CSF samples.

INTRODUCTION

Bacterial meningitis is still an important cause of morbidity and mortality in children worldwide **[Corvec *et al.*, (2005)].** The early signs of meningitis are entirely nonspecific in the new born and unless the disease is positively excluded when they

appear or before any antibiotic therapy is started mortality and morbidity rates will continue to be high so the first essential for improved results is early diagnosis **[Smith *et al.*, (1973)]; Hambleton & Davies (1975)]**. Intensive research has been carried out to find new and rapid diagnostic methods for differential diagnosis of bacterial meningitis **[Taskın *et al.*, (2004)]**. rapid antigen detection is a very useful tool for the rapid etiological diagnosis and guideline for the choice of antimicrobials in systemic infections due to Hib. It is necessary to diagnose bacterial etiology as a routine procedure using not only Gram stain and culture but also rapid antigen detection technique in patients with suspected Hib systemic infection **[Ohkusu & Nakamura (1999)]**. The enzyme-linked immunosorbent assay (ELISA) is highly sensitive and specific in detecting *H. influenzae* antigen; the necessity to perform the blocking assay on all sera limits its usefulness for the examination of these specimens. However, it should prove valuable for the detection of the antigen in cerebrospinal fluid **[Wetherall *et al.*, (1980)]**.

The aim of the present study was, identify and purify *H. influenzae* circulating antigen in serum and CSF samples of meningitis patients using biochemical and immunochemical techniques.

SUBJECTS AND METHODS

Samples:

CSF and Serum samples of 102 Egyptian individuals (70 males and 32 females, aged 3 months–80 yr, mean age 22.43 ± 18.59), kindly provided by staff of Abbassia Fever Hospital, Cairo, Egypt, were included in the present study before antibiotic therapy and after approval from hospital ethics committee. The signs and symptoms in all groups included fever, and CNS dysfunction, such as meningeal signs (bulging of the anterior fontanelle, nuchal rigidity, pathologic reflexes and abnormal behavior like irritability and lethargy. We also collected serum samples from 25 healthy volunteers as negative controls. All samples were stored at – 20 °C until analyses.

SDS-PAGE and Western Blotting:

Serum and CSF samples of selected patients were separated by SDS-PAGE **[Laemmli (1970)]**. Resolved samples were then electrotransfered onto the nitrocellulose filter (0.45 µm pore size, Sigma) in protein transfer unit (BioRad Laboratories, Inc.1000 Alfred Nobel Dr.Hercules, CA 94547) according to **[Towbin *et al.*, (1979)]**. The nitrocellulose filter was blocked using a blocking buffer composed of 3% (w/v) dry milk dissolved in 0.05 M tris-buffered saline (TBS), containing 0.15 M NaCl, pH 7.4, rinsed in TBS, and incubated with a monospecific rabbit anti- *H. influenzae* IgG antibody (ABC Diagnostics, New Damietta, Egypt) diluted in blocking buffer with constant shaking overnight. The blots were washed three times (15 min/wash) in TBS followed by 2 hr incubation with anti-rabbit IgG alkaline phosphatase conjugate (Binding site) diluted in TBS. The blots were then washed three times with TBS. The reaction was visualized by incubating the NC filter and soaked in premixed BCIP/NBT alkaline phosphatase substrate (ABC Diagnostics, New Damietta City, Egypt). And stopped by distilled water after color development within 10 min.

Quantitative detection of *H. influenzae* type b antigen using ELISA:

After optimization of reaction condition, polystyrene microtiter plate were coated with 50 µl/well of each serum and CSF sample diluted 1: 400 and 1:3 respectively in coating buffer (pH 9.6) and incubated overnight at 4 °C. After washing, 50 µl/well of 1: 150 diluted monospecific anti-*H. influenza* antibody (ABC Diagnostics) in PBS-Tween 20 (PBS-T20) were added and incubated at 37 °C for 2 h. After washing, 50 µl/well of anti-rabbit IgG alkaline phosphatase conjugate (Binding site) diluted 1:300 in 0.2% (w/v) BSA in PBS-T20, was added, and incubated for 1 h at 37 °C. The amount of coupled conjugate was determined by incubation with p-nitrophenyl phosphate substrate (Sigma). The reaction stopped by NaOH and the absorbance read at 490 nm using ∑960 microplate autoreader (Metreiteck, Germany). Quantitation of *H. influenzae* antigen in unknown samples was performed by assaying serial fivefold dilution of the antigen and calculating the result from a standard curve performed with each experiment.

Statistical analysis:

All parameters were analyzed using the statistical analysis program (SPSS). The unpaired student t-tests were used to compare the difference between means of variables. P values are two-tailed and the significance of difference $p > 0.05$ is considered not significant.

RESULTS

SDS-PAGE of serum and CSF samples from meningitis patients and healthy individuals sera:

The resolved bands were identified in CSF and serum after staining the gel by Coomassie Brilliant Blue R-250 dye. Several resolved protein bands were located within the high and low molecular weight regions as shown in Fig. (1&2).

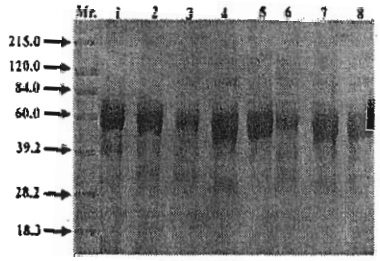

Fig. (1): Coomassie blue stained SDS-PAGE of CSF from meningitis patients under reducing conditions. Samples were loaded per well and electrophoresed under 200 volts for 45 minutes. **Lane 1-4:** CSF samples from meningitis patients infected with bacteria other than *H. influenzae*. **Lane 5-8:** CSF sample from meningitis patients infected with *H. influenzae*. **Molecular weight marker (Mr)** includes: Myosin (215.0 kDa), phosphorylase B, (120.0 kDa), Bovine serum albumin (84.0 kDa), Ovalbumin (60.0 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28.2 kDa), and lysozyme (18.3 kDa).

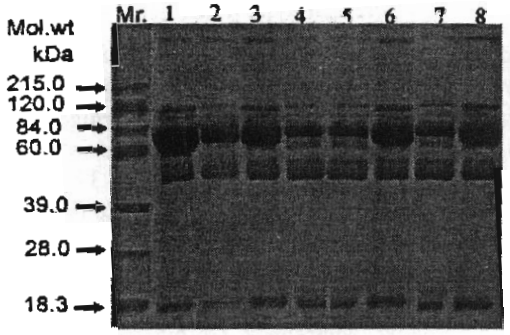

Fig. (2): Coomassie blue stained SDS-PAGE of sera from meningitis patients and non infected individuals under reducing conditions. Samples were loaded per well and electrophoresed under 200 volts for 45 minutes. **Lane 1-4:** Serum samples from healthy individuals. **Lane 5-8:** Serum samples from meningitis patients. **Molecular weight marker (Mr)** includes: Myosin (215.0 kDa), phosphorylase B, (120.0 kDa), Bovine serum albumin (84.0 kDa), Ovalbumin (60.0 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28.2 kDa), and lysozyme (18.3 kDa).

Identification of *H. influenzae* type b antigen using western blotting:

A specific *anti- H. influenzae* antibody was used as an immunological probe for identification of target *H. influenzae* antigen using western blot. The specific anti-*H. influenzae* antibody reacted against *H. influenzae* in CSF and serum samples at molecular weight of 95 kDa Fig. (3&4).

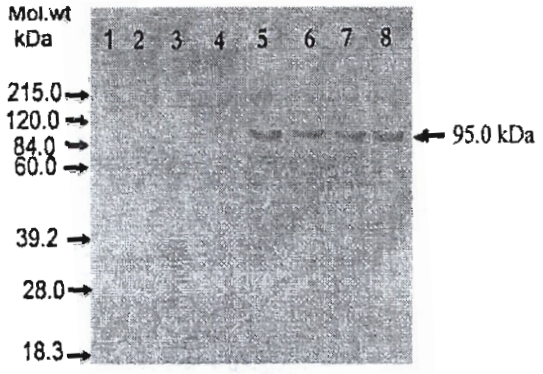

Fig. (3): Western blot based on mono-specific anti- *H. influenzae* antibody in CSF samples from meningitis patients. **Lane 1-4:** CSF samples from meningitis patients infected with Bactria other than *H. influenzae* (Hib). **Lane 5-8:** CSF sample from meningitis patients infected with *H. influenzae* (Hib). **Molecular weight marker (Mr)** includes: Myosin (215.0 kDa), phosphorylase B, (120.0 kDa), Bovine serum albumin (84.0 kDa), Ovalbumin (60.0 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28.2 kDa), and lysozyme (18.3 kDa).

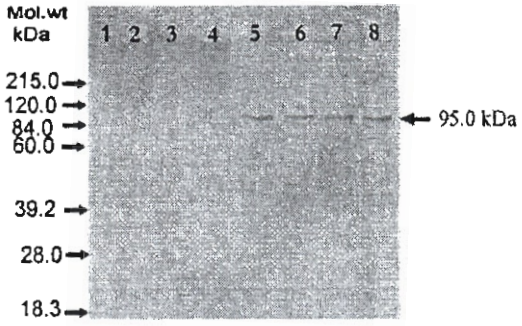

Fig. (4): Western blot based on specific anti- *H. influenzae* antibody in serum samples from meningitis patients. **Lane 1-4:** Serum samples from healthy individuals, **Lane 5-8:** serum samples from meningitis patients. **Molecular weight marker (Mr)** includes: Myosin (215.0 kDa), phosphorylase B, (120.0 kDa), Bovine serum albumin (84.0 kDa), Ovalbumin (60.0 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28.2 kDa), and lysozyme (18.3 kDa).

Detection of *Hib* antigen in serum and CSF using ELISA:

The cutoff level of ELISA was calculated as the mean ELISA optical densities of 25 serum samples from non-infected individuals ± 3 standard deviation (i.e. 0.147 ± [3 × 0.02]) = 0.20), and serum samples from 10 infected patients showed optical densities above the cut off level, The *Hib* antigen was detected in 59 sera of 102 (58%) meningitis patients, Fig (5); While, the *Hib* antigen was detected in 62 CSF of 102 (61%) of total meningitis patients, Fig. (6).

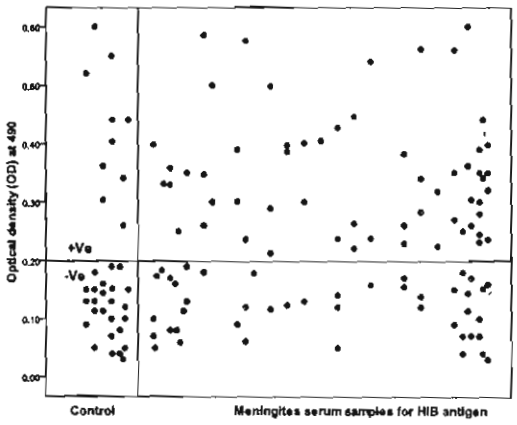

Fig. (5): Detection of *Hib* antigen in serum samples of meningitis patients.

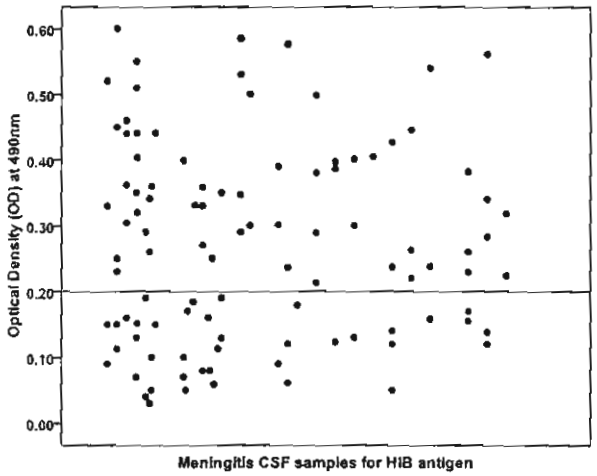

Fig. (6): detection of *Hib* antigen in CSF samples of meningitis patients.

The *H. influenzae* antigen was detected in sera of 58% of total meningitis patients; while, the *H. influenzae* antigen was detected in CSF of 61% of total meningitis patients as depicted in Fig. (7&8).

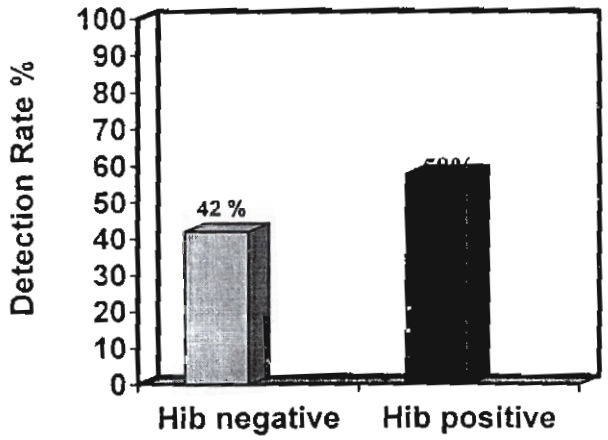

Fig. (7): Detection rate of *Hib* antigen in sera of 102 meningitis patients using ELISA.

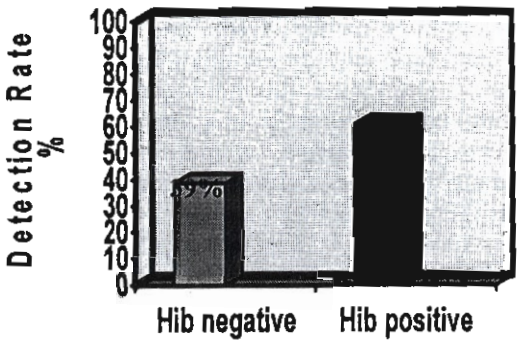

Fig. (8): Detection rate of *Hib* antigen in CSF of 102 meningitis patients using ELISA.

Correlation between *Haemophilus influenzae* antigen levels in serum and CSF of patients with meningitis:

A very significant correlation (r = 0.342; p= 0.001) was shown in the concentrations of *Haemophilus influenzae* antigen (ng/ml) between serum and CSF of total meningitis patients Fig. (9).

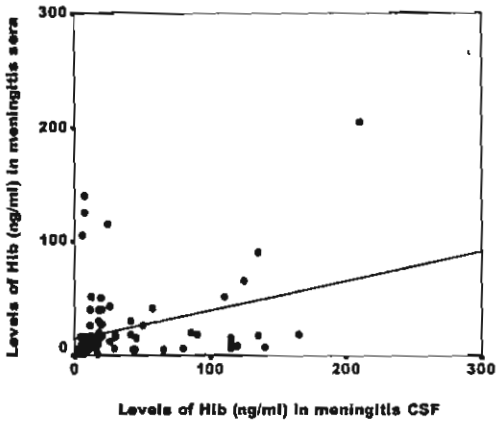

Fig. (9): Correlation between *Haemophilus influenzae* type b antigen levels in serum and CSF, (r=0.342; p=0.001).

DISCUSSION

Haemophilus influenzae type b causes a variety of serious infections in infants and young children, including bacteremia, meningitis, cellulitis, pericarditis, septic arthritis, pneumonia, empyema, and epiglottitis. Early treatment is critical, and antibiotics are often prescribed empirically because the physician cannot wait a day or longer for definitive culture results. In addition, cultures may not be helpful because many pediatric patients are already taking oral antibiotics when invasive haemophilus disease is first suspected. Moreover, in some infections, such as pneumonia in infants, culture material is difficult to obtain. Therefore, there has been considerable interest in developing rapid diagnostic tests that can detect the presence of haemophilus infection even when bacteria are no longer viable or when appropriate specimens for culture are unavailable. ELISA has a valuable potential for the rapid diagnosis of meningitis and is of particular help in patients who have been pre-treated with antibiotics **[Salih *et al.*, (1995)]**. By using western blot analysis we found that, the specific *anti-H. Influenzae* antibody reacted against *H. influenzae* in CSF and serum samples at an apparent molecular weight of 95 kDa. Several authors uses Western blot technique in identification of *H. influenzae* antigen in both CSF and serum samples. Others **[Johnson *et al.*, (1993)]** observed that the specific antibody for the *H. influenzae* antigen of an apparent molecular mass of 49 kDa by western blot analysis in CSF samples. On the other hand, **[Langford *et al.*, (1992)]** Identified bands in in-vivo-grown organism at 51 and 92 kDa by using SDS-PAGE in serum samples. The enzyme-linked immunosorbent assay (ELISA) is highly sensitive and specific in detecting *H. influenzae* antigen; the necessity to perform the blocking assay on all sera limits its usefulness for the examination of these specimens. However, it should prove valuable for the detection of the antigen in cerebrospinal fluid **[Wetherall *et al.*, (1980)]**. We analyzed the clinical significance of the presence of *H. influenzae* antigen in sera and CSF of meningitis patients with different manifestation by ELISA. We noted that, percentage of positivity was high in both CSF (61%) and serum (59%) samples and no significant difference ($p > 0.05$) was shown between detection rate of *H. influenzae* antigen in serum and CSF ($P > 0.05$). The positive results of *H. influenzae* antigen in another study was (44.7 %), The percentage of positivity was higher in CSF samples (57.0 %) than in serum (33.8 %) or blood (33.3 %) samples by using PCR technique **[Kalmusova *et al.*, (2004)]**.

A significant correlation ($r = 0.195$; $p= 0.010$) was shown in the concentrations of *Haemophilus influenzae* antigen between serum and CSF of total meningitis patients. Our findings suggested that The 95 KDa antigen may have a diagnostic potential for diagnosis of *H. influenzae* infection. Also, ELISA can be used successfully in the detection of CSF and serum circulating *H. influenzae* antigen in meningitis patients. However, the use of serum samples are superlative CSF samples due to the spinal tab process is painful of meningitis patients.

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دراسات كيميائيه مناعيه على مرضي مصابين بالالتهاب السحائي

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يعرف الالتهاب السحائي بأنه التهاب الاغشيه المحيطه بالمخ وسائل النخاع الشوكي المحيط بالحبل الشوكي وهناك أنواع عديده من الالتهاب السحائي فمنه الالتهاب السحائي البكتيري والفيروسي والفطري. أما بالنسبه للالتهاب السحائي البكتيري فمن أهم البكتريا المسببه له بكتريا الهيموفيليس انفلوانزا من النوع بي التى تعتبر من أهم البكتريا الممرضه التى تهاجم الجهاز التنفسي وخاصة للإنسان وعليه فان التشخيص والعلاج المبكر لهذه الاصابه يمكن أن تساعد علي التغلب علي المرض بشكل كبير. إن الكشف عن انتيجين الهيموفيليس انفلوانزا بي مسئول بشكل كبير عن تشخيص الالتهاب السحائي.لقد تم الكشف عن انتيجين الهيموفليس انفلوانزا بي في ١٠٢ عينه سيرم وسائل نخاع شوكي لمرضى مصابين بالالتهاب السحائي باستخدام تقنية الادمصاص المناعى الانزيمى (ELISA) والشفط المناعى الغربي (Western blot). إن الشفط المناعى الغربي (Western blot) قد اظهروجود باند منفرده وحيده فقط عند الوزن الجزيئي ٩٥ كيلو دالتون في عينات السيرم وسائل النخاع الشوكى للمرضى المصابين بالاتهاب السحائي. لقد تم عمل كشف كمي عن انتيجين الهيموفليس انفلوانزا بي عن طريق عمل تخفيفات متسلسله من الانتيجين المفصول وحساب التركيز عن طريق عمل المنحنى القياسي لكل تجربه. لقد وجد أيضا أن هناك علاقه ذات دلاله احصائيه بين تركيز انتيجين الهيموفيليس انفلوانزا بي في كلا من السيرم والسائل الشوكي حيث انه لا يوجد فرق بين معدل تواجد الانتيجين في السيرم (٥٨%) وبين تواجده في السائل الشوكي (٦١%).

ونستخلص مما سبق :أن تقنية الادمصاص المناعى الانزيمى (ELISA) تسهل الكشف عن انتيجين الهيموفليس انفلوانزا بي في كلا من عينات السيرم وسائل النخاع الشوكى لمرضى الالتهاب السحائي وأيضا توصلنا انه يمكن استخدام عينات السيرم للكشف عن انتيجين الهيموفليس انفلوانزا بي بدلا من عينات السائل الشوكى التى يصعب الحصول عليها بسبب الخطوره والالم اللذان يتعرض لهما المريض .