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Pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines for differentiating *Haemophilus influenzae* type b from other meningitis bacterial agents

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Favorable treatment outcome is depending on accurate detection of meningitis etiological agent. Lately, increasing significance is attributed to the role of cytokines in bacterial meningitis differentiation. Herein, we aimed to evaluate the diagnostic significance of tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) in *Haemophilus influenza* type b (Hib) meningitis. Ninety-six patients clinically suspected of bacterial meningitis (60 with Hib and 36 with non-Hib meningitis) were enrolled in this study. CSF and serum level of TNF-α and IL-10 were quantified by ELISA. Diagnostic performances of single and combined cytokines were evaluated using area under ROC. Significant increase in IL-10 and TNF-α levels was noted in both CSF (*P*=0.004 and 0.009; respectively) and serum (*P*=0.007 and 0.010; respectively) in Hib meningitis patients compared to non-Hib meningitis patients. IL-10, and TNF-α had a discriminative capability for Hib meningitis in CSF (AUC=0.75 and 0.77; respectively) as well in serum (AUC=0.72 and 0.78; respectively). Combined use by multiplying these cytokines (TNF-α × IL-10) exhibited a valuable power for Hib meningitis detection in CSF (AUC=0.88, sensitivity=84% and specificity= 71%). Interestingly, this index (TNF-α × IL-10) showed also comparable discriminative power in serum (AUC=0.85, sensitivity=83.3% and specificity= 78.6%). In conclusion, TNF-α and IL-10 cytokines are potential biomarkers for differentiating Hib meningitis from meningitis causing by other bacterial agents and the convenient combined of them can improve diagnostic efficiency of Hib meningitis particularly in serum, less invasive sample, to help or even replace lumbar puncture.

Keywords: *Haemophilus influenza* type b; meningitis; tumor necrosis factor alpha; interleukin-10; cytokines; index.

INTRODUCTION

Despite the effectiveness of antimicrobial therapy, bacterial meningitis remains a serious global health problem that could be fatal in both adults and children (Wang et al., 2016). New and simple methods to determine meningitis causative agents will be of great importance to obtain a more reliable bacterial meningitis picture worldwide (Peltola et al., 2010).

Haemophilus influenza type b (Hib) is one of the most frequent agents causing bacterial meningitis. Rapid identification is significant for the early administration of Hib vaccine and chemoprophylaxis (Kimberlin et al., 2015).

However, diagnosis of Hib meningitis is difficult; lumbar punctures and microbiological analysis of cerebrospinal fluid (CSF) including cytochemical analysis, microscopy and cell count must be performed. But, these techniques require an experienced microbiologist, and detected bacteria in large amounts only (Peltola et al., 2010). Furthermore, Hib pathogen has fastidious growth requirements and CSF culture has diminished sensitivity in specimens from persons who have received antibiotics (Başpınar et al., 2017; Coleman et al., 2003). In addition, latex agglutination for Hib antigen detection is costly with varies specificity and reported false both positive and negative results (Seehusen et al., 2003). Thus, the identification of biomarkers for Hib meningitis is highly required.

Previously, it has been indicated that pro- and anti-inflammatory cytokines can be valuable in differentiating bacterial from viral meningitis as sensitive markers of bacterial disease (Hsieh et al., 2009; Tang et al., 2001). Also, it is becoming increasingly evident that Hib meningitis is attributed to elevated different mediators levels in CSF, like IL-6, soluble IL-2 receptor, IL-8 and TNF- α (Khair et al., 1994; Nishimura et al., 2000). Nevertheless, the assessment of these cytokines during Hib meningitis and their diagnostic value are still not known (Hsieh et al., 2009; Tang et al., 2001).

In this study we aimed to assess the clinical value of a combined measurement of pro-and anti-inflammatory cytokines (TNF-α and IL-10) in CSF and serum to facilitate Hib meningitis differentiation from meningitis causing by other bacterial agents.

MATERIALS AND METHODS

Patients

This study was carried out on a total of 96 patients clinically suspected of bacterial meningitis who were admitted in Abbassia Fever Hospital, Cairo, Egypt. All patients had laboratory and clinical criteria, symptoms and physical signs of meningitis, and their CSF were examined. Patients were grouped into two cohorts according to the identified infectious microorganisms, group I: 60 patients with Hib meningitis and group II: 36 non-Hib meningitis patients had other infecting microorganism (i.e *Streptococcus pneumoniae* (21 cases) and *Neisseria meningitidis* (15 cases)). Bacterial meningitis was diagnosed by CSF cultures and/or polymerase chain reaction. Data were collected from hospital records, including demographics, clinical characteristics, laboratory results, comorbidities, and treatment administered. Patients who received treatment (included antibiotics and steroids) and human immunodeficiency virus infected individuals were excluded from the study. Written informed approval was obtained from all included patients and this work protocol was approved under the Helsinki Ethics declaration.

Sample collection and cytokines assay

All participated patients were subjected to CSF collection by lumbar puncture; one part was used for routine microbiological and cytochemical examination and the other part was centrifuged and frozen for cytokines measurement. Simultaneously, serum venous peripheral blood was obtained from all included patients, permitted to clot then centrifuged at 4,000 rpm for fifteen minutes and stored at -20°C until analysis. Afterwards, serum and CSF samples were investigated simultaneously for TNF-α and IL-10. Cytokines were quantified according to the manufacturers' instructions of commercially available ELISA kits (Quantikine Kit, R&D Systems, Inc., Minneapolis, USA).

Statistical analysis

All data analyses were carried out using the GraphPad Prism package; v.5.0 and SPSS; v.15.0 software. Significant differences were performed by Student's *t* test and variables were expressed as mean ± standard deviation. The cytokines ability to diagnose and differentiate Hib meningitis was evaluated by receiver operating characteristic (ROC) curve. The value that maximized the area under the curve (AUC) was selected as the best cutoff. Multivariate discriminant analysis (MDA) was used to calculate the predicted probabilities of various combinations of cytokines. The result revealed that the original regression coefficients were not estimable, so that a simplified score was calculated after dropping these coefficients by multiplying the single markers.(Attallah et al., 2015) Correlations were evaluated by Spearman's rank correlation co-efficient. The specificities and sensitivities were derived from a 2 × 2 contingency table. $P<0.05$ is considered significant.

RESULTS

Patients' characteristics

Medical chemistry and cytological parameters in CSF of the studied populations are reported in

Table 1.

Table 1. Patients' characteristics

Variables	Patients infected with Hib	Patients non-infected with Hib	*P* value
No. (%)	60 (62.5%)	36 (37.5%)	—
Gender (male/female)	27/9	30/8	0.449
Age (years)	20.6±2.8	23.6±2.4	0.414
Cerebrospinal fluid analysis			
Protein (mg/dL)	201.8 ± 44.3	186.8 ± 31.4	0.700
Glucose (mg/dL)	38.5 ± 8.4	40.8 ± 9.8	0.762
WBCs (cells/µL)	3135.8 ± 363.7	823.6 ± 112.5	0.251
Lymphocytes (%)	46.5 ± 9.2	55.9 ± 11.2	0.427
Neutrophils (%)	57.1 ± 12.9	63.6 ± 13.6	0.557
NLR	11.6 ± 4.3	11.2 ± 4.1	0.954

Abbreviation: WBCs: white blood cells count; NLR: neutrophils / lymphocytes ratio; Hib*: Haemophilus influenzae* type b. Student *t*-test was used to compare between studied groups. Data were expressed as mean ± SD and *P*<0.05 is considered significant.

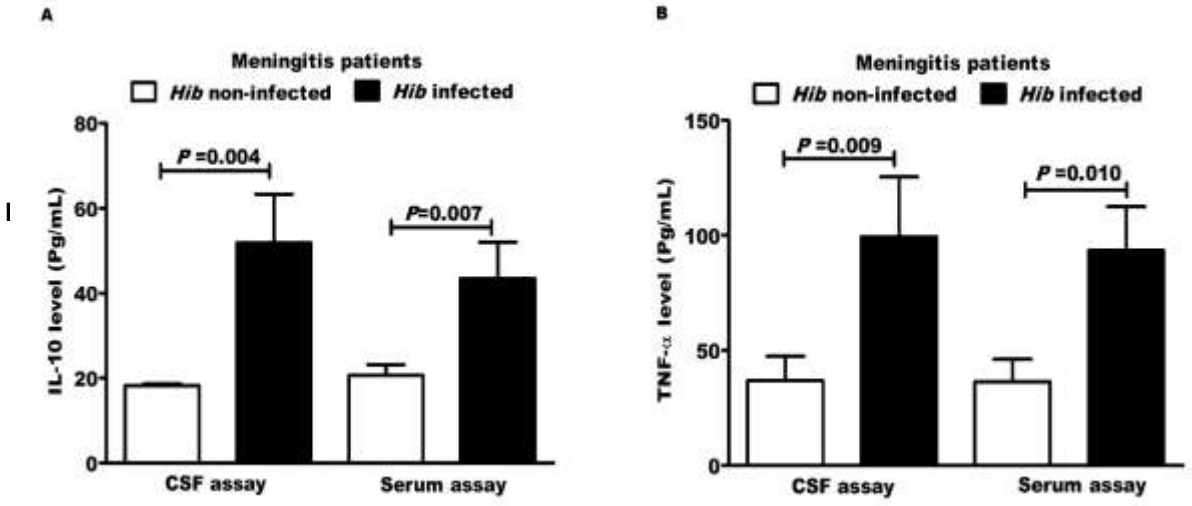

Figure 1; Detection of (A) Interleukin-10 (IL-10) and (B) Tumor necrosis factor alpha (TNF-α) in serum and CSF of meningitis patients with and without Hib infection. Student *t*-test was used to compare between studied groups. Data were expressed as mean±SD and *P*<0.05 is considered significant. Hib=*Haemophilus influenzae* type b.

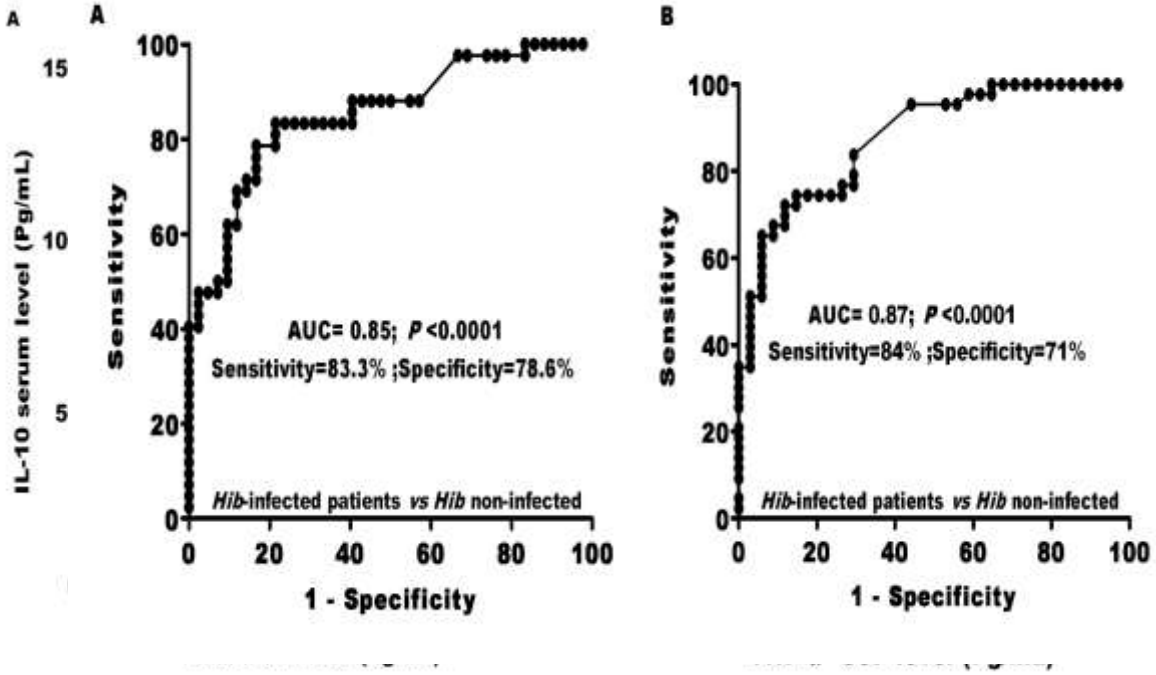

Figure 2; Correlation between serum and CSF levels of (A) Interleukin-10 (IL-10) and (B) Tumor necrosis factor alpha (TNF-α) in meningitis patients. Pearson's correlation was used and *P*<0.05 is considered significant.

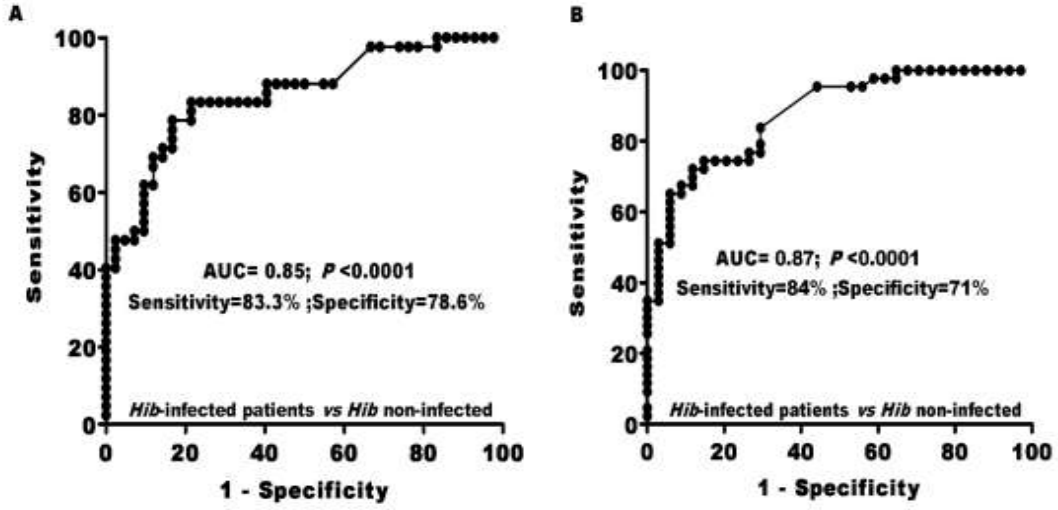

Figure 3; Receiver-operating characteristic curve analysis of interleukin-10 (IL-10) and tumor necrosis factor alpha (TNF-α) combined use (TNF-α × IL-10) in (A) serum and (B) CSF for Hib=*Haemophilus influenzae* type b meningitis differentiation

Table 2; Parameters independently associated with presence of *Haemophilus influenzae* type b

Variables	AUC	Beta	Standard error	OR (95% CI)	*P* value
Protein (mg/dL)	0.51	-0.131	0.004	0.996 (0.987-1.006)	0.461
Glucose (mg/dL)	0.56	0.033	0.021	1.031 (0.950-1.120)	0.463
WBCs(cells/µL)	0.61	-0.047	0.002	1.000 (0.999-1.001)	0.867
Neutrophils (%)	0.63	0.015	0.017	0.655 (0.410-1.047)	0.077
Lymphocytes (%)	0.59	-0.036	0.063	0.927 (0.841-1.022)	0.127
NLR	0.54	0.117	0.083	0.673 (0.310-1.460)	0.361
CSF IL-10 (pg/mL)	0.75	0.039	0.016	1.945 (0.898-3.795)	0.015
serum IL-10 (pg/mL)	0.72	0.077	0.020	1.460 (0.932-2.189)	0.030
CSF TNF-α (pg/mL)	0.77	0.008	0.012	1.554 (0.029-2.995)	0.029
serum TNF-α (pg/mL)	0.78	0.004	0.009	1.246 (0.908-2.286)	0.034

Abbreviation: WBCs: white Blood Cells count; NLR: neutrophils / lymphocytes ratio; IL-10: interleukin-10; TNF-α: tumor necrosis factor alpha; AUC: area under curve.

Table 3; Capability of combined TNF-α × IL-10 use in CSF and serum for discriminative Hib infected meningitis patients from those non-infected in comparison to each parameter alone

Variables	Cutoff (pg/mL)	AUC	Sen (%)	Spe (%)	PPV (%)	NPV (%)	Eff (%)
CSF IL-10	30	0.75	61.9	66.7	68.4	60	64.1
CSF TNF-α	35	0.77	66.7	72.5	71.8	67.4	69.5
CSF TNF-α × IL-10	740	0.88	84.0	71.0	86.5	73.0	80.0
Serum IL-10	27.5	0.72	62.9	73.8	70.3	66	67.9
Serum TNF-α	35	0.78	67.4	72.9	70.7	68.9	69.8
Serum TNF-α × IL-10	720	0.85	83.3	78.6	82.5	74.4	81.4

Abbreviation: IL-10: interleukin-10; TNF-α: tumor necrosis factor alpha; AUC: area under curve; Sen: sensitivity; Spe: specificity; NPV: negative predictive value; PPV: positive predictive value; Eff: efficiency.

Laboratory features of patients with Hib meningitis were compared with those with other bacterial meningitis by univariate analysis (student's *t*-test). As a result, no laboratory and clinical alterations were found between the 2 groups. CSF characteristics such as leukocytosis, neutrophilic pleocytosis, decreased glucose and increased proteins concentrations were similar in Hib meningitis and non-Hib meningitis patients (*P*>0.05).

Value of inflammatory cytokines in identifying Hib meningitis

As shown in Figure 1(A), a significant increase was noted in both CSF and serum concentrations of IL-10 in Hib meningitis groups (51.9±11.4 pg/mL and 43.4±8.9 pg/mL; respectively) as compared to non-Hib meningitis patients (20.7±2.5 pg/mL and 18.2±0.4 pg/mL; respectively). Also, for TNF-α there was also a significant increase in Hib meningitis patients in both CSF (99.4±26.1 pg/mL *vs* 36.9±10.6 pg/mL, *P*=0.009) and serum (93.4±19.1 pg/mL *vs* 36.5±9.7 pg/mL, *P*= 0.010), Figure 1(B). In addition to, there was weak positive correlation between CSF and serum of both IL-10 (*r*= 0.380, *P* <0.001) and TNF-α (*r*= 0.401, *P* <0.0001), Figure 2.

Factors related to the presence of Hib infection

Multivariate analysis of factors related to presence of Hib infection was presented in Table 2. As a result, the higher levels of CSF or serum IL-10 (*P*=0.015 and 0.030; respectively) and TNF-α (*P*= 0.029 and 0.034; respectively) were remained significantly related to the presence of Hib infection in multivariate analysis. Moreover, these cytokines yielded the highest AUCs for identifying Hib meningitis.

Diagnostic performance and model development

Interestingly, CSF IL-10, and TNF-α had a good discriminative capability between Hib meningitis patients and non-Hib meningitis patients (AUC= 0.75 and 0.77; respectively). Serum cytokines had also this discriminative capability (AUC= 0.72 for IL-10 and 0.78 for TNF-

α). Thus, serum and CSF levels of IL-10 and TNF-α could be useful to identify Hib meningitis, as presented in Table 3.

Then in an attempt to improve cytokines diagnostic accuracy, MDA analysis revealed that their combined use (TNF-α × IL-10) in CSF and serum improves their diagnostic performance for Hib meningitis detection. This index had AUC of 0.88 in CSF and 0.85 in serum, Figure 3. Combined use of these cytokines exhibited improved sensitivities (83.3% and 84%) and specificities (78.7 and 71%) for Hib differentiating whether in serum or CSF respectively which were higher than each parameter alone, Table 3.

DISCUSSION

The identification of the bacterial meningitis pathogen is considered the most critical stage in the disease management. However, the used techniques for bacterial meningitis diagnosis have serious limitation (Wang et al., 2014). Also, Hib meningitis can't be distinguished from other bacterial meningitis by clinical examination alone (World Health Organization, 2013).

Number of new, rapid, relatively inexpensive diagnostic markers in CSF or circulating system are becoming available to aid in meningitis diagnosis as well as being of some value in separating this entity from viral/aseptic meningitis (Abro et al., 2009). Among these markers, cytokines have been proposed to be useful in differential diagnosis of bacterial meningitis from aseptic meningitis (Ye et al., 2016). Herein, it was the first time to assess the ability of both pro- and anti-inflammatory cytokines (TNF-α and IL-10) for differentiating Hib meningitis from other bacterial agents. Levels of TNF-α and IL-10 in serum and CSF were found to be elevated in Hib meningitis patients than those with other meningitis causative agents.

Bacterial meningitis is linked with inflammatory cascade activation and production of pro- and anti-inflammatory cytokines (Kim, 2003; Srinivasan et al., 2016). For instances, TNF-α is a vital pro-inflammatory cytokine that secreted in response to microbial or endotoxin stimulation and involved in neuro-infection pathogenesis (Sulik et al., 2014). This response is mediated by anti-inflammatory cytokines including IL-10 (Srinivasan et al., 2016). It has been reported that IL-10 has important role in the anti-inflammatory activities which can directly inhibit T-cell proliferation, activation and chemotaxis and also production of cytokine (Ye et al., 2016).

In the central nervous system (CNS), evolution of the inflammatory process depends on cytokines set generated during infection first steps. It has been suggested that a porin produced by Hib could trigger the early release of cytokines (IL-1, TNF-α), within 6 hours, that amplifying the inflammatory response. This expression is increased after 24 hours and correlated with the injury of blood–brain barrier (BBB) which proved by existence of leukocytes and serum proteins in CSF (Galdiero et al., 2001). Also, in an early study it was found that, during the course of experimental bacterial meningitis, Hib and *Streptococcus pneumoniae* induce different intracerebral mRNA cytokine patterns. The brain of Hib-inoculated rats showed higher level of TNF-α when compared with *Streptococcus pneumoniae* -inoculated rats, while IL-10 exhibited similar patterns of induction (Diab et al., 1997). This can imply that different immunomodulating approaches should be considered, depending on etiology.

The production of cytokines is dependent on CNS bacterial load that could explain their higher levels in CSF than in circulating system (Arditi et al., 1990; Carrol et al., 2007). In this study, we find a weak correlation between CSF and serum levels of both IL-10 and TNF-α (r= 0.380 and 0.401; respectively), indicating that CSF can't be the source of serum circulating cytokines. Once bacteria enter the CNS through the bloodstream, they multiply and induce the release of some mediators include cytokines and chemokines that contributed to pleocytosis and increased BBB permeability. Although, cytokines cannot easily cross BBB, promoting the local inflammatory process and neuronal tissue damage (Nau and Brück, 2002). Also, cytokines such as TNF-α and IL-10 that have transporters from CNS to circulation are quickly saturable (Banks et al., 2009).

Using ROC analysis, IL-10 and TNF-α yielded AUCs of 0.75 and 0.77 respectively for identifying Hib meningitis in CSF and 0.72 and 0.78 respectively in serum. Furthermore, it was found that the combined use of these cytokines in CSF can improve their performance for Hib detection. Indeed, CSF TNF-α × IL-10 combination yielded AUC (0.88), sensitivity (84%) and specificity (71%) better than that for each parameter alone. Also, their combination in serum revealed valuable power (AUC=0.85, 83.3% sensitivity, 78.7% specificity) which can facilitate Hib detection without performing lumbar punctures.

Our obtained results are superior to the conventional test; CSF Gram stain, which

sensitivity range 25-65% for Hib meningitis diagnosis (Brouwer et al., 2010). Also, our index is in comparable with other Hib diagnostic techniques like latex agglutination test, blood cultures and PCR. Published studies have discordant results about the latex agglutination test sensitivity that ranged from 78 to 100% for CSF detection of Hib (Gray and Fedorko, 1992). If CSF cultures are unavailable or negative, blood cultures may be useful to detect causative organism. But, it also has varies positivity (50-90%) for *H. influenzae* meningitis patients (Brouwer et al., 2010; Pedersen et al., 2010). In patients with bacterial meningitis, nucleic acid amplification tests have demonstrated efficacy in detecting bacterial DNA in CSF. some studies have been noted that PCR assay has sensitivity of 92% for *H. influenzae* detection, (Corless et al., 2001) however others have found that its sensitivity may be considerably lower 88% and may reach to 72% (Parent du Chatelet et al., 2005; Tzanakaki et al., 2005).

CONCLUSION

In conclusion, cytokine response can be varying depending on the type of the bacterial aetiological agent. The combined TNF-α × IL-10 use could improve the powerful of these parameters for more accurate and reliable Hib detection whether in CSF or in serum without undergoing invasive lumbar puncture procedure. This work represents an advance in biomedical science because it sheds light on the combined use of pro and anti-inflammatory cytokines, TNF-α and IL-10, to differentiate Hib meningitis from other meningitis bacterial agents.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Omran MM, Abdel Aziz MH, and Abou-Dobara MI were chief investigators who conceptualised and designed the study. Abdelrazek MA, Aggag MM and Khedr FM were investigators who collected data from the literature, collected samples and carried on with different experiments and techniques. Omran MM, Abdelrazek MA and Khedr FM aquired data and performed all data and statistical analysis. Omran MM, Abdelrazek MA and Khedr FM interpreted data and wrote final manuscript. All authors read, reviewed and approved the final manuscript.

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