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Evaluation of Gene Expression of some Toll-like Receptors Genes among Iraqi Meningitis

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Abstract: Meningitis is an important infectious disease with a high morbidity and mortality rate. The present study characterized the Toll-Like (receptors) (TLRs) role in the host immune response and as markers for bacterial and viral meningitis. The aim of this study evaluate the gene expression of Toll-like receptors TLR2, TLR3, TLR 4, TLR 7 and TLR9 genes the delta delta ct method among patients with meningitis infectious pathogens (*Streptococcus pneumoniae*, *Staphylococcus spp*, Herpes simplex virus1 and Epstein-Barr virus) and compared the results with the results of healthy control. The results of gene expression of TLR2 revealed that this receptor expression was increased among blood cells for infected patients with *Staphylococcus spp* (4.8 fold) in comparison with the control. The results of gene expression of TLR3 demonstrated that this receptor was increased among meningitis patients with Herpes simplex virus1 (5.1 fold). The receptor TLR4 was increased among patients with *S. pneumoniae* (6.2 fold). Also it was found that the gene expression of TLR7 and TLR9 was increased among patients through Epstein-Barr virus (6.2 fold), and Herpes simplex virus1 (4 fold), respectively. The findings of the study revealed that the mRNA expression levels of TLRs 2 and 4 had increased in infected patients with bacteria, while in the patients infected with viruses, there was increasing in the levels of TLRs 3, 7 and 9. Also thelevels of TLR2 were exhibited low increasing among patients with viruses. In conclusion, TLRs perform an important function in immune system response. to viral and bacterial meningitis and by way of genetic markers for identifying high-risk patients with meningitis and perhaps leading to novel treatment methods.

Keywords: Gene expression, Meningitis, TLRs.

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Introduction

Meningitis is dangerous illness that impacts the CNS. It refers to Meningitis is an inflammation of the meninges, the membrane that covers the spinal-cord and brain. Bacteria, viral, or fungal diseases all can cause meningitis. This neurological condition can be caused by a wide range of viruses, such enteroviruses, herpesviruses, and flu viruses. Nevertheless, enteroviruses have been identified as the root cause of the majority of viral meningitis infections globally (1). Bacterial meningitis, a potentially fatal infection, causes 1.2 million new cases every year and kills 135,000 persons. Viral meningitis, on the other hand, typically has a fair prognosis and can be treated in one or two weeks with no treatment. It is rarely feasible to distinguish between viruses and bacterial meningitis, which adds to the excessive use of antibiotics in order to promote resistance (2). Pathogen recognition receptors (PRRs) are found on a variety of cells that include microglia and astrocytes inside the Central Nervous System, and realize (PAMPs). Following genetic transcription of pro-

inflammatory cytokines, nuclear factor kappa B (NFB) is activated.. Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain containing proteins are the two types of PRRs (NODs). TLR4 senses LPS, TLR2 senses bacterial lipoproteins, and TLR9 senses unmethylated Cytosine-phosphate-Guanine (CpG) patterns (4) which are TLRs and serve critical role in pathogen-associated molecular patterns identification (PAMPs). A substantial inflammatory element is induced by a range of acute neurological and neurosurgical disorders, and it is notably difficult to identify imposed nosocomial against a backdrop of an active innate immune system response. The nonexistence of appropriate diagnostic examinations at the moment of clinical equipoise is a crucial unmet need with severe clinical and healthcare cost consequences (5).

Materials and methods

Collection of samples

During the period from January 2020 to June 2021 one hundred CSF sample has been collected regardless sex and age. The samples were taken from different hospitals which are Pediatric teaching hospital, Baghdad teaching hospital at Medical City of Baghdad, Child's Central Teaching Hospital and Al-Kadhimiya Teaching Hospital. The samples are divided to two groups. One hundred samples were taken from patients who suffer from meningitis symptoms and the twenty five samples were taken from patients who carry leukemia unsuspected to be infected with microbial meningitis. The samples were stored in a special media that maintain genomic DNA. After DNA extraction from CSF specimens, Primers (Forward and Reverse) and probes used in this study for detecting *Streptococcus pneumoniae*, *Staphylococcus* spp, Herpes simplex virus1 and Epstein-Barr virus by Multiplex Real-time PCR. Also, blood samples were used for RNA extraction in order to evaluate the gene expression of Toll-like receptors (TLRs) (2, 3, TLR4, 7, and 9) was evaluated in blood cells (by delta delta ct) method.

TLR gene expression was measured using the RT PCR method

This experiment had been planned via the usage of Fifty-four clinical samples as peripheral blood from infected patients with diagnosed meningitis who identified with special predominant microorganisms (*Streptococcus pneumoniae*, *Staphylococcus spp*, Herpes simplex virus1and Epstein-Barr virus). RT-PCR was the six genes' gene expression measures in the clinical samples according to delta delta ct method.

RNA isolation via TRIzol and cDNA synthesis

Blood cells extracted was used to isolate Total RNA byTRIzol® reagent according to (6). RNA (1 µg) was reverse transcribed to gain cDNA via a Revert Aid™ cDNA Synthesis kit Given the process conditions as in Table (1).

Table (1): Program PCR converted RNA to cDNA.

Steps	Temperature (^{o}C)	Time (min)	No. of cycles
Step (1)	37	15	1
Step (2)	60	10	1
Step (3)	95	3	1

Primer preparation

Certain primers were used (Table 2) in accordance with references mentioned in the table for detection of the *TLR*, gene expression.

Table (2): Primers that use for gene expression of TLR and GAPDG genes.

Gene Name	Direction	Oligonucleotide primer Seq (5'-3')	Prodsize (bp)	The referenc
GAPDH	F	GAAGGTCGGAGTCAACGGATT	228	(7)
	R	CGCTCCTGGAAGATGGTGAT		
TLR2	F	GGCTTCTCTGTCTTGTGACC	294	(8)
	R	GGGCTTGAACCAGGAAGACG		
TLR3	F	AGCCACCTGAAGTTGACTCAGG	270	
	R	CAGTCAAATTCGTGCAGAAGGC		
TLR4	F	TTGTATTCAAGGTCTGGCTGG	438	
	R	GCAACCTTTGAAACTCAAGCC		
TLR7	F	GATAACAATGTCACAGCCGTCC	321	
	R	GTTCCTGGAGTTTGTTGATGTTC		
TLR9	F	TACCAACATCCTGATGCTAGACTC	233	
	R	TAGGACAACAGCAGATACTCCAGG		

Quantitative Real-time PCR Assay (RT-PCR) protocol

There are seven PCR tubes for each sample, one tube for each gene, *TLR2, 3, 4, 7, TLR9,* and *GAPDH*. Fluorescent power of SYBR Green was based on the detection of quantity. The component is composed of the reaction mix with their quantity as mentioned in Table (3)

Table (3): Volumes and concentrations of RT-PCR reaction mix

Component	Volume(µl)
Master Mix SYBR green (promega)	12.5
F primer	1
R primer	1
cDNA	4
Nucleasefree Water	6.5
Total volume	lµ 25

Table (4) is shown that PCR tubes were quickly spinned in order to collect the liquid in 1 minute at 200g and remove the bubbles and the thermocycling protocol was used to set up the program for Real-Time PCR.

Table (4): RTPCR cycling program.

Cycle Step	Temperature	Time	Cycles number
First Denaturation	95 °C	60sc	1
Denatration	95 °C	15sc	40
Anealing	60 °C	30sc	
Extenson	72 °C	40sc	1

Calculations of gene expression

Therefore as direct comparison of the aim and reference Ct values (housekeeping) genes, the data findings of qRT-PCR were calculated. The following equations ΔΔCt illustrate how the genes were examined utilizing the relative measurement of gene

expression levels (fold change) using the Ct method defined by (9)

Primarily, the ΔCt amid the reference gene and aim gene was premeditated for each taster (the unknown sample were collected and the reference samples).

ΔCt = Ct target – Ct reference gene

Secondary, the variance among the unknown's ct value and the calibrator's Δct value were determined, giving the ΔΔCt value:

ΔΔCt = (Ct target – Ct reference) sample – (Ct target – Ct reference) Control

Thirdly, as measured by the sample's normalized target quantity was equalized to 2-ΔΔCt. The samples' expression levels were contrasted using this value: Fold change = 2-ΔΔCT

Thus, The CT (compare threshold cycles) value technique (2- ΔΔCt) was used to compare the relative changes in mRNA expression levels among the illness besides the seemingly healthy.

Results and discussion

The results of Real-Time PCR for identification of some meningitis pathogens (*Streptococcus pneumoniae*, *Staphylococcus* spp, Herpes simplex virus1 and Epstein-Barr virus) demonstrate that the prevalence of these pathogens among 100 patients was 44 cases (44%), and the distribution ofthese agents was (*Streptococcus pneumoniae* (22), *Staphylococcus* spp (10), Herpes simplex virus1 (15) and Epstein-Barr virus (7)), where *Streptococcus pneumoniae* was the predominant infection agent among patients. In a previous study included a total 1303 CSF samples were taken from 863 patients, of which 130 CSF specimens were positive for bacterial growth. As a result, 101 bacterial strains that were isolated from the CSF of 96 individuals were examined. *S. pneumoniae* was the most common isolated species (36 strains, 35.6%), and five patients experienced a relapse of BM (10). TLR (Tolllike receptor) gene expression (TLR2, TLR3, TLR4, TLR7, and TLR9) was evaluated (via delta delta ct) method among patients with meningitis infectious pathogens (*Streptococcus pneumoniae*, *Staphylococcus* spp, Herpes simplex virus1 and Epstein - Barr virus) and compared the results by the results of healthy control. TLR mRNA expression levels were determined in the blood cells of infected patients. The results of the gene expression as fold change of TLRs were summarized in the figures 1 to 5.

The results of gene expression of TLR2 revealed that this receptor was increased among blood cells for *Staphylococcus* spp (4.8 fold) infected patients followed by *S. pneumoniae* (2.7 fold) in comparison with the healthy control (1 fold) and viruses (Figure 1).

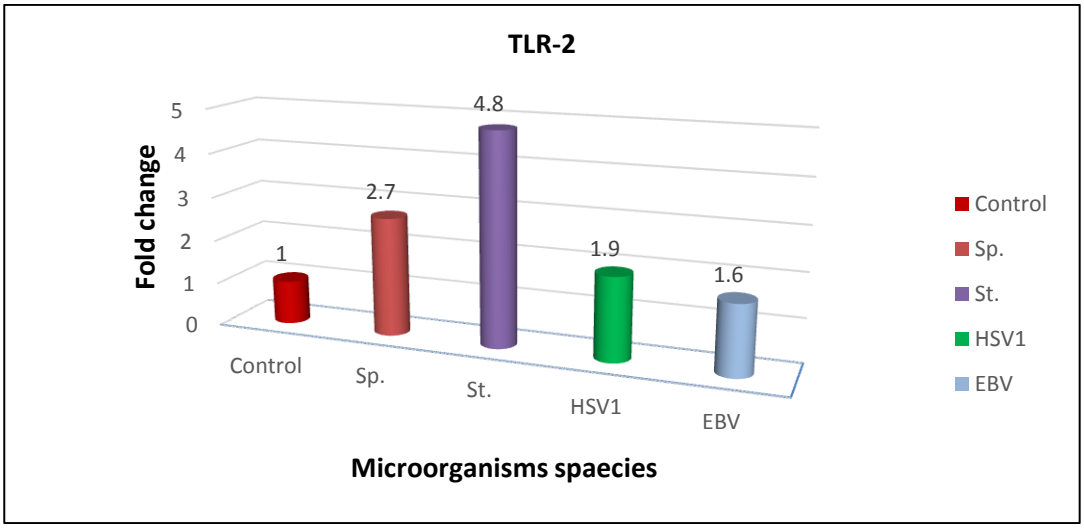

Figure (1): The (mRNA) expression level of TLR2 in the blood cells obtained from infected patients with meningitis compared to the control group (Sp: Streptococcus pneumoniae; St: Staphylococcus spp; HSV1: Herpes simplex virus1; EBV: Epstein-Barr virus.

The results of gene expression of TLR3 demonstrated that this receptor was increased among blood cells of Herpes simplex virus1 (5.1 fold) of infected patients followed by Epstein - Barr virus (3.1 fold) in comparison with the healthy control (1 fold) and bacteria which exhibited very low expression levels (Figure 2).

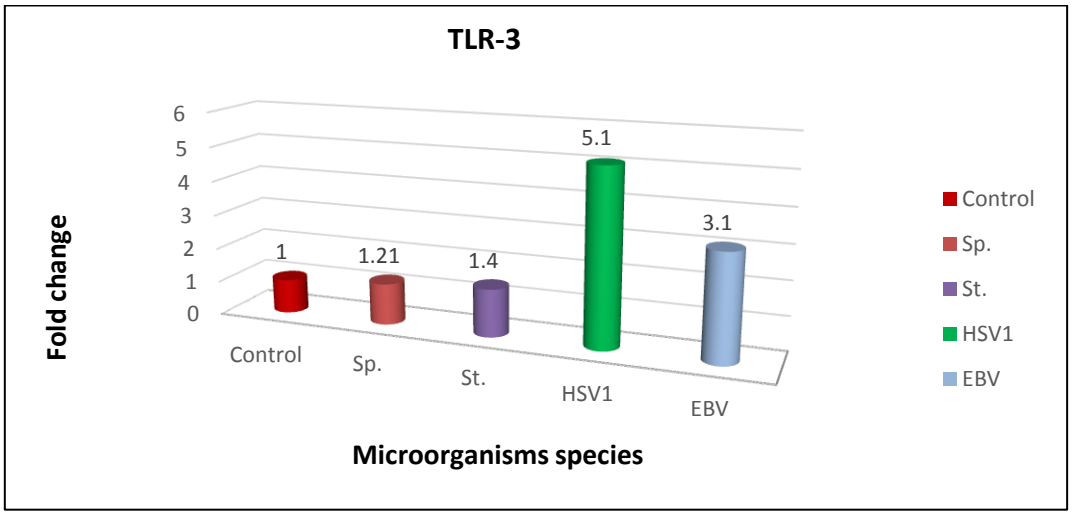

Figure (2): The mRNA expression levels of TLR3 in the blood cells obtained from infected patients with meningitis compared to the control group (Sp: *Streptococcus pneumoniae*; St: *Staphylococcus* spp; HSV1: Herpes simplex virus1; EBV: Epstein-Barr virus).

The results of gene expression of TLR4 revealed that this receptor was increased among blood cells of S. pneumoniae (6.2 fold) infected patients followed by *Staphylococcus spp*. (3 fold) in comparison with the healthy control (1fold) and viruses which exhibited expression levels similar to control (Figure 3).

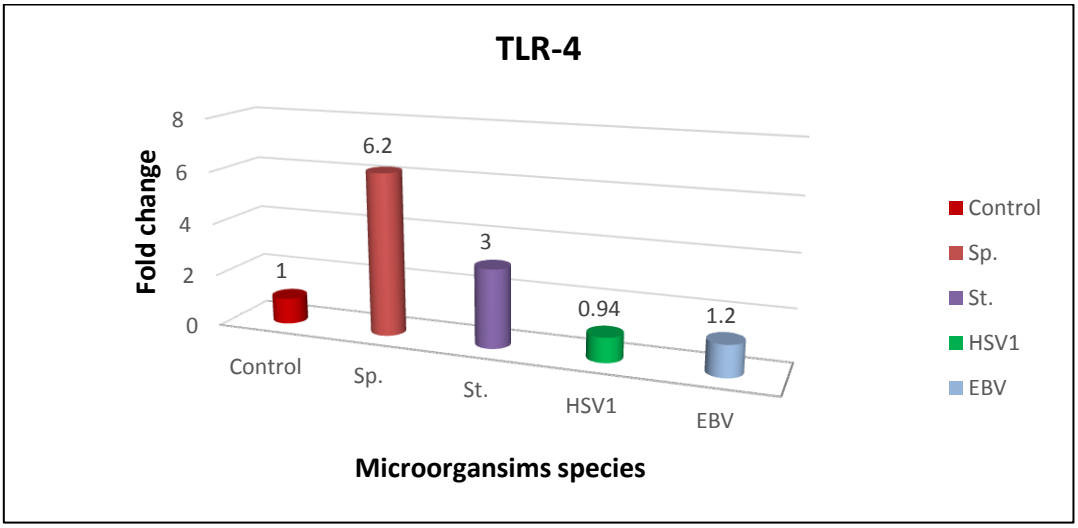

Figure (3): The (mRNA) expression level of TLR4 in the blood cells obtained from infected patients with meningitis compared to the control group (Sp: *Streptococcus pneumoniae*; St: *Staphylococcus* spp; HSV1: Herpes simplex virus1; EBV: Epstein-Barr virus).

The results of gene expression of TLR7 revealed that this receptor was increased among blood of Epstein-Barr virus (6.2 fold) infected patients followed by Herpes simplex virus1 (3.7 fold) in comparison with the healthy control (1 fold) and bacteria which exhibited low expression levels (Figure 4).

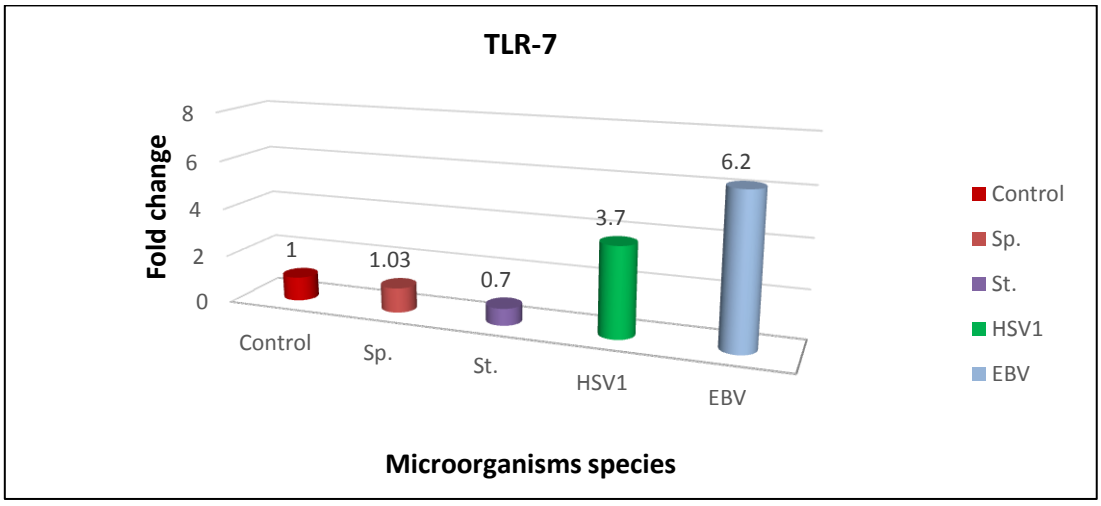

Figure (4): The mRNA expression levels of TLR7 in the blood cells obtained from infected patients with meningitis compared to the control group (Sp: *Streptococcus pneumoniae*; St: *Staphylococcus* spp; HSV1: Herpes simplex virus1; EBV: Epstein-Barr virus).

The results of gene expression of TLR9 indicted that this receptor was increased among patients with Herpes simplex virus1 (4 fold) followed by Epstein-Barr virus (3.6 fold) in comparison with the healthy control (1 fold) and bacteria which exhibited low expression levels (Figure 5).

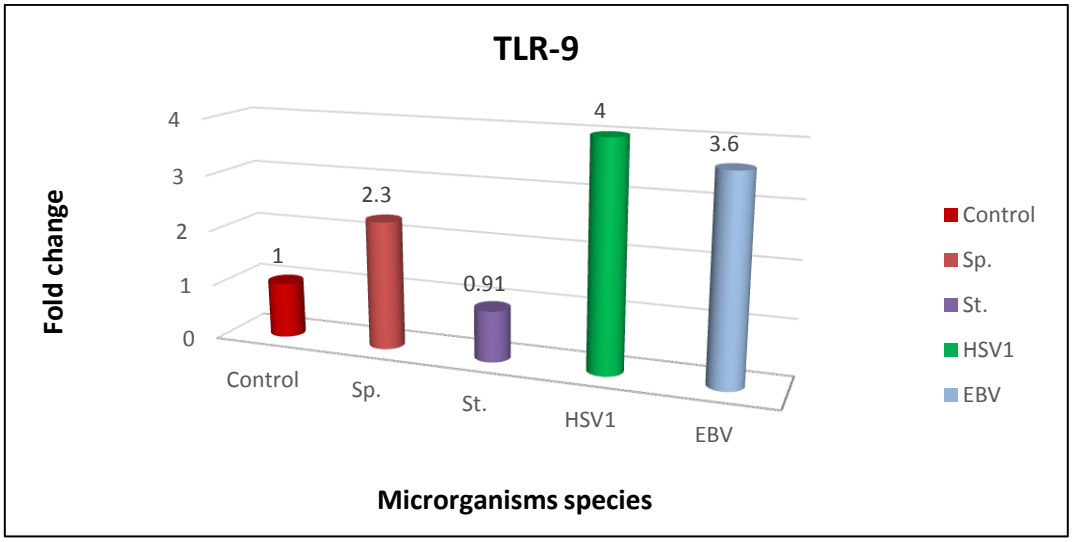

Figure (5): The (mRNA) expression level of TLR9 in the blood cells obtained from infected patients with meningitis compared to the control group (MH: *Streptococcus pneumoniae*; St: *Staphylococcus* spp; HSV1: Herpes simplex virus1; EBV: Epstein-Barr virus).

The findings of this study confirmed that the (mRNA) expression level from TLRs 2 and 4 were increased in infected patients with bacteria, while in the patients infected with viruses, there was increasing in the levels of TLRs 3, 7 and 9. Also the levels of TLR2 were exhibited low increasing among patients with viruses (1.6-1.9 fold).

Different bacterial ligands are bound by TLR4 and TLR2. TLR4 is able to detect lipopolysaccharide (LPS). Even though recognition lipopeptides can happen autonomously of TLR1 and 6, TLR2 binds tri- and di-acetylated lipopeptides, respectively, in combination with either TLR1 or TLR6. TLR2 and TLR4 stimulation causes the release of TNF and IL-8 in addition to activation of NF-B, which binds to the B cis-acting component (11).

TLR2 and TLR4 play a crucial role in controlling the inflammatory response of the host during pneumococcal meningitis, might mediate a variety of compensatory mechanisms shield the host not just from mortality, also from long-term neural squeal. Pattern recognition receptors (PRRs), which are present in innate immune cells including microglia in addition to astrocytes, can detect pneumococci in the central nervous system (CNS). The important PRRs include the lipotechoic acid-activated TLR2 and the pneumolysin-activated TLR4 (12).

The meningitis caused by *Streptococcus pneumoniae* increased the expression of TLR2, TLR4, and TLR9 mRNA. TLR2, TLR4, and TLR7 mRNA expression were increased in *Escherichia coli* meningitis, whereas TLR4 mRNA increased in Herpes simplex encephalitis. Treatment with S. pneumoniae R6 boosted the expression of TLR2 and TLR3 mRNA in organotypic hippocampus cells. The particular response to the causal infection and non-specific activation of the innate immune system are likely combined to produce the TLR mRNA regulation that has been seen. (13).

The endosome TLRs identify the herpes simplex virus (HSV) (TLR3, 7, and TLR9). Which are TLRs and serve a significant function in the detection of pathogen-associated pattern recognition receptors (PAMPs). A substantial

inflammatory element of CNS injury is induced by a variety of factors. RLR (retinoic acid inducible gene, RIG-like receptors) are cytoplasmic receptors that identify viral RNA and participate with TLR in the production of IFN- after activation an adapter protein, toll/interlukin-1 receptors domains having an adapter protein (TRIF), after attaching to its cognate (14).

The previous findings indicted that protection against genital HSV-2 challenge was brought on by mucosal, but not systemic, administration of TLR-3 ligands, which did not result in any local inflammation. Unexpectedly, TLR4 m RNA expression was found to be higher in murine vaginal mucosa than TLR3 or TLR9. Likewise, it was discovered that murine RAW264.7 cells express higher TLR4 mRNA than TLR3 or TLR9 mRNA. These findings suggest that TLR3 ligand generates a more robust antiviral response than TLR4 and TLR9 ligands, suggesting that it may be a less dangerous method of preventing viral infections (15). After HSV-1 infections, human neurons that express TLR-3 can mount a variety of innate immune reactions. TLR-3, one of the 10 TLRs found in humans, has been found to react to dsRNA (16).

Dendritic cells are also induced by TLR9 to produce type I IFN responses following EBV infection. It also controls the release of IL-17 following intraperitoneal injection of EBV DNA. EBV dUTPase, possibly released in (exosomes), plus/or a virion component both trigger TLR2 signaling (17). Several local studies revealed the importance of molecular detection of bacterial meningitis and the role of Toll-like receptors as immunological markers (18, 19).

Conclusion

This study revealed the associated between meningitis pathogens and TLRs and their activities against microbial causative agents of CSF infections may help us to prevent, control and treat meningitis at a higher quality level.

References

1. Kohil, A.; Jemmieh, S.; Smatti, M. K. and Yassine, H. M. (2021). Viral meningitis: an overview. Archives of Virology, 166(2): 335–345.
2. Li, D. and Wu, M. (2021). Pattern recognition receptors in health and diseases. Signal Transduction and Targeted Therapy, 6(1): 291.
3. Xia, P.; Wu, Y.; Lian, S.; Yan, L.; Meng, X.; Duan, Q. *et al.* (2021). Research progress on Toll-like receptor signal transduction and its roles in antimicrobial immune responses. Applied Microbiology and Biotechnology, 105(13): 5341–5355.
4. Carbonnelle, E. (2009). Apport des examens biologiques dans le diagnostic positif, la détermination de l'étiologie et le suivi d'une méningite suspectée bactérienne [Laboratory diagnosis of bacterial meningitis: usefulness of various tests for the determination of the etiological agent]. Médecine et Maladies Infectieuses. 39(7-8): 581-605
5. Menke, A.; Rex-Haffner, M.; Klengel, T.; Binder, E. B. and Mehta, D. (2012). Peripheral blood gene expression: it all boils down to the RNA collection tubes. BMC Research Notes, 5: 1-8.
6. Dai, C.; Sun, L.; Yu, L.; Zhu, G.; Wu, S. and Bao, W. (2016). Effects of porcine MyD88 knockdown on the expression of TLR4 pathway-related genes and proinflammatory cytokines. Bioscience Reports, 36(6): 409-413.
7. Liu, S.; Gallo, D. J.; Green, A. M.; Williams, D. L.; Gong, X.; Shapiro, R. A., *et al*. (2002). Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide. Infection and Immunity, 70(7): 3433–3442.
8. Schmittgen, T. D. and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C (T) method. Nature Protocols, 3(6): 1101–1108.
9. Mares, D.; Simoes, J. A.; Novak, R. M. and Spear, G. T. (2008). TLR2-mediated cell stimulation in bacterial vaginosis.

Journal of Reproductive Immunology, 77(1): 91–99.

10. Mirecka, A. (2018). Etiological agents of bacterial meningitis in adults and antibiotic susceptibility of Streptococcus pneumoniae isolated between 2009-2016 from patients of Regional Specialist Hospital of Dr Wł. Biegański in Lódź. Przeglad Epidemiologiczny, 72(3): 313–324.
11. Too, L. K.; Yau, B.; Baxter, A. G.; McGregor, I. S. and Hunt, N. H. (2019). Double deficiency of toll-like receptors 2 and 4 alters long-term neurological sequelae in mice cured of pneumococcal meningitis. Scientific Reports, 9(1): 16189.
12. Böttcher, T.; von Mering, M.; Ebert, S.; Meyding-Lamadé, U.; Kuhnt, U.; Gerber, J. *et al*. (2003). Differential regulation of Toll-like receptor mRNAs in experimental murine central nervous system infections. Neuroscience Letters, 344(1): 17–20.
13. Govindan, B. (2014). Toll like receptors: Sensors for herpes simplex virus infection. Indian Journal of Sexually Transmitted Diseases and AIDS, 35(2): 170–171.
14. Ashkar, A. A.; Yao, X. D.; Gill, N.; Sajic, D.; Patrick, A. J. and Rosenthal, K. L. (2004). Toll-like receptor (TLR)-3, but not TLR4, agonist protects against genital herpes infection in the absence of inflammation seen with CpG DNA. The Journal of Infectious Diseases, 190(10): 1841–1849.
15. Préhaud, C.; Mégret, F.; Lafage, M. and Lafon, M. (2005). Virus infection switches TLR-3-positive human neurons to become strong producers of beta interferon. Journal of Virology, 79(20): 12893–12904.
16. Gaglia, M. M. (2021). Anti-viral and pro-inflammatory functions of Toll-like receptors during gamma-herpesvirus infections. Virology Journal, 18(1): 218-229.
17. Gaglia, M. M. and Munger, K. (2018). More than just oncogenes: mechanisms of tumorigenesis by human viruses. Current Opinion in virology, 32: 48–59.
18. Al-Banae, H.Z.; Khalid, K.A.; AL-Awadi, S.J. (2012). Detection of autolysin, pneumolysin and pneumococcal surface adhesion genes among *Streptococcus pneumoniae* causing bacterial meningitis in children. Iraqi Journal of Biotechnology, 11(2): 519-528.
19. Khalaf, M.A Al-Saadi, B.Q.H., Mohammed, H.Q. (2022). Evaluation of TLR-3, TLR4, IL-7, and IL37 Immunological Markers in β-Thalassemia Major Iraqi Patients. Iraqi Journal of Biotechnology, 21(1): 115-123.