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RESEARCH ARTICLE

Comparison between Gram′s stain, culture, Real – time PCR for diagnosis of Neisseria meningitidis in CSF Cases

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Abstract

Objective : to compare characteristics of the gram stain , culture, Real-time PCR to detection pathogenic bacteria in CSF samples of patients were suspected of acute bacterial meningitis.
Methods: 100 CSF sample were suspected of acute bacterial meningitidis Gram stain , bacterial culture, Real-time PCR based on SYBR Green I fluorescent dye for detection of Nisseria meningitidis in all samples.
Results: Gram stain and bacteria culture for all samples were positive in 5 case (5%) including two bacterial genus Listeria monocytogenes in four case (80%) and Staphylococcus aureus in one case (20%) while in RT-PCR based on SYBR Green for detection of SodC gene which is specific gene to diagnosis of N. meningitidis were positive in 42 case (42%) .
Conclusion: RT-PCR method is rapid , sensitive and specific diagnostic test for acute meningitis . RT- PCR test was positive in 42 case (42%) and all these case was N. meningitidis .

INTRODUCTION

Meningococcal disease causes life-threatening meningitis and sepsis conditions. In the case of meningitis, bacteria attack the lining between the brain and skull called the meninges. Infected fluid from the meninges then passes into the spinal cord, causing symptoms including stiff neck, fever and rashes. The meninges (and sometimes the brain itself) begin to swell, which affects the central nervous system. Even with antibiotics, approximately 1 in 10 victims of meningococcal meningitis will die; However, about as many survivors of the disease lose a limb or their hearing, or suffer permanent brain damage. **Centers for Disease Control and Prevention(1999).**The sepsis type of infection is much more deadly, and results in a severe blood poisoning called meningococcal sepsis that affects the entire body. In this case, bacterial toxins rupture blood vessels and can rapidly shut down vital organs. Within hours, patient's health can change from seemingly good to mortally ill. (Jeeri etal.,2007).

Patients suffering from meningococcal disease are treated with a large dose of antibiotic. The systemic antibiotic flowing through the bloodstream rapidly kills the bacteria but, as the bacteria are killed, even more toxin is released. It takes up to several days for the toxin to be neutralized from the body by using continuous liquid treatment and antibiotic therapy(Jeeri etal.,2008).

The identification of the pathogen from cerebrospinal fluid (CSF) usually takes 1 to 2 days by culture. Moreover, culture frequently remains negative, especially if the CSF is taken after initiation of antimicrobial therapy(**Hussein** etal.,2000)Since the outcome of infection depends highly on an early initiation of adequate therapy (Stevens etal.,2003), new rapid diagnostic methods are urgently needed (Segreti and Harris,1996)

Initial diagnosis of invasive meningococcal disease (IMD) is often based on clinical symptoms such as fever, vomiting, neck stiffness and skin rash. The diagnosis is confirmed by the isolation and biochemical identification of

N. meningitidis from cerebrospinal fluid (CSF), blood or other specimens. Blood and CSF cultures are often negative in patients with IMD, particularly if they have been treated with antibiotics before samples are collected for microbiological investigations. During the past decade, methods based on polymerase chain reaction (PCR) for detection of meningococcal DNA have provided valuable tools for diagnosis of IMD (**Markman,1993**). Increasing demand for more sensitive and rapid diagnostic procedures, such as fluorescence-based PCR has prompted the routine use of automated liquid handling systems in the clinical laboratory **(Smith et al.,2004).** Real-time PCR (RTPCR) methods have proved to be more sensitive than the equivalent gel-based system **(Livak etal.,1995)** .SYBR Green I is a dye specific for double-stranded DNA. During each phase of DNA synthesis, the SYBR Green I dye binds to amplified PCR products and the amplification is detected and quantified by the level of fluorescence. Specificity and sensitivity of amplification reactions detected with SYBR Green I dye can be greatly enhanced by combining amplification with a melting curve analysis. (**Tyagi, and Kramer, 1996)**

The aim of this study was to compare the conventional and RT-PCR to detect N.meningitidis in CSF samples over a two year period between 2014-2015

Methods

1- Sources of specimens

Samples from 100 individuals were There were 100 CSF samples tested with both Gram stain ,culture l and real-time PCR assays: In hospital, samples were collected by clinicians from patients in infectious diseases wards or intensive care units.

The suspected cases of acute bacterial meningitis were identified by a clinician, based on the following criteria: Acute onset of fever (usually > 38.5°C rectal or 38.0 °C axillary), headache and one of the following signs: neck stiffness, altered consciousness or other meningeal signs.

CSF samples were obtained via lumber puncture or from CSF shunts and were submitted to routine microbiology ,chemistry ,cell count and real-time PCR.

A-Gram stain :the specimens were centrifuge for 20 minutes at 2000rpm and performed gram stain procedure.
B-Bacterial culture: CSF specimens were inoculated on chocolate agar and blood agar .Both plates were inoculated on chocolate agar and blood agar .Both plates were incubated in a carbon dioxide enriched atmosphere at 35-37 C for up to 48 hours; plates were checked for growth after overnight incubation.

C-Isolation of DNA from patient samples

CSF specimens obtained from patients with signs and symptoms suggestive of IMD were examined for N. meningitidis DNA by the reference laboratory by both conventional and RTPCR. For isolation of target DNA from CSF, the sample was centrifuged at 1700g (Hettich, EBA 12) for 10min. An amount of 350µl of the supernatant was discarded and 150 µl of the precipitate was added to a mixture of 650 µl doubled distilled sterile water and 150 µl of Cheelex /Tween 80 buffer according to the method described by **Zambarti et al** The samples were heated at 100 C for 30 min and centrifuged again at 10,000g for 8 min. A volume of 150 µll of the supernatant was used for the PCR assay.

To identify N. meningitidis, primers amplifying specifically a 73 bp long region of the conserved regulatory gene Sod C was used (**Taha ,2000).** The following primers were used: forward, 5-TACCCATGGCAAGATGATGC -3; reverse, 5- TTGTTGCTGTGCCATCATGC -3The PCR mixture (50 µl) contained the following components: 3 µllMgCl (20 mM); 5 µl of the PCR buffer composed of Tris–HCl (750 mM), (NH4) 2SO4 (200 mM) and Tween 20 (0.1%, v/v); 0.5 µl l of each deoxynucleotide triphoshate (200 µ M); 0.5 µl of the corresponding oligonucleotides 100 µl; 0.2 µl of Taq polymerase (5 U/ µl (AB gene); 10 µlof template DNA.After an initial denaturation for 5 minute at 95 c, the PCR were performed in a PCR thermocycler at 39 repetitions of the cycle:25 s at 55 C° and 1 minute at 72 C° .

The amplicons were detected following electrophoresis in 2% agarose gel and were visualized with a UV transilluminator following staining with ethidium bromide. Negative controls consisting of distilled water were used in each assay. Positive controls included standard strains used for species identification.

LightCycler amplification protocol

To improve sensitivity and detection time for clinical applications, the Sod C gene was amplified by a RTPCR protocol with the DNA Master SYBR Green I kit (Roche Diagnostics) in a LightCycler instrument (Roche Diagnostics). SYBR Green fluorescence melting curve analysis was used for detection and identification of amplicons. In this system, all reactions were carried out in glass capillaries with a total volume of 20 µl. The reaction mixture consisted of 2 µl (10×) of Hot-Faststart DNA Master SYBR Green I dye (Roche Diagnostics), $MgCl_2$ (2.5 mM). Both primers were added to a final concentration of 0.5 µM with 10 µl of the target DNA sample. The final reaction volume was adjusted with sterile PCR grade water. Negative controls consisting of PCR grade water instead of the target DNA (5 ng per reaction) were used in each assay as well as positive controls of standard strains used for species identification.

Cycling conditions included: an initial Hot start Taq activation step at 95 °C for 10 min; a denaturation step at 95 °C for 5 s; an annealing step at 63 °C for 5 s; an extension step at 72 °C for 10 s. Touch-down PCR conditions were used for the initial annealing temperature of 63 °C for 5 s with a decreasing temperature step of 0.5 °C s^{+1} at each cycle for the first seven cycles until the final annealing temperature reached 60 °C. The following 33 cycles were carried out under following conditions: 95 °C for 5 s; 60 °C for 5 s and 72 °C for 10 s.

The detection of the amplicons was verified using the melting curve analysis feature of the LightCycler instrument. Briefly, following the last amplification cycle, the internal temperature of the LightCycler was rapidly increased to 95 °C then decreased to 65 °C for 15 s, followed by a slow increase to 95 °C at a rate of 0.05 °C s^{+1} The fluorescence was monitored continuously. The specific melting temperature (T_m) was recorded for each amplified sample.

Results

Gram stain and bacteria culture for all samples were positive in 5 case (5%) including two bacterial genus Listeria monocytogenes in four case (80%) .This prevalence is higher than what was detected by Boving etal.,(2009) (1.8%) , **Chiba etal**.(2009)(0.8%) and **Yahia etal.,(2014)(**11.7%).So in this study Staphylococcus aureus was detected in one case (20%) while in RT-PCR (Figure 1) based on SYBR Green for detection of SodC gene which is specific gene to diagnosis of N. meningitidis were positive in 42 case (42%).Table (1). this result similar to that was found by **Yahia et al., (2014)** who found (45.5%) of acute bacterial meningitis were diagnosed by RT-PCR method .

Table (1)Show performance characteristics of gram stain, culture and RT-PCR.

Laboratory methods	**No. of positive (Total 100)**
Gram stain	5%
L. monocytogenes	4 (80%)
S. aureus	1 (20%)
Culture method	5 (5%)
L. monocytogenes	4 (80%)
S. aureus	1 (20%)
RT-PCR	42 (42%)
N. meningitidis	42 (42%)

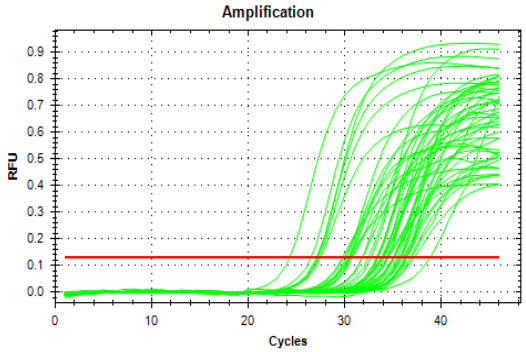

Figure (1) Amplification plot in Real-Time PCR for Sodc gene specific for Neisseria meningitis

So ,CT value in positive samples was ranged between (26.63-37) figure (2).this result similar to that was found by Kesanopoulos et al.,(2004) who referred that CT value for Sod C gene in Neisseira meningitidis was ranged between (26-37)

Well	Fluor	Target	Sample	Cq
H06	SYBR	sodC gene	N.menengitis	37.00
A05	SYBR	sodC gene	N.menengitis	36.98
B02	SYBR	sodC gene	N.menengitis	36.89
G04	SYBR	sodC gene	N.menengitis	36.19
C06	SYBR	sodC gene	N.menengitis	36.16
C02	SYBR	sodC gene	N.menengitis	36.12
G06	SYBR	sodC gene	N.menengitis	35.98
F04	SYBR	sodC gene	N.menengitis	35.90
F05	SYBR	sodC gene	N.menengitis	35.75
H01	SYBR	sodC gene	N.menengitis	35.25
B05	SYBR	sodC gene	N.menengitis	35.24
B01	SYBR	sodC gene	N.menengitis	35.15
C01	SYBR	sodC gene	N.menengitis	34.80
H04	SYBR	sodC gene	N.menengitis	34.18
H03	SYBR	sodC gene	N.menengitis	33.98
G03	SYBR	sodC gene	N.menengitis	33.75
H02	SYBR	sodC gene	N.menengitis	33.38
B04	SYBR	sodC gene	N.menengitis	32.78
D06	SYBR	sodC gene	N.menengitis	30.65
D04	SYBR	sodC gene	N.menengitis	29.84
D03	SYBR	sodC gene	N.menengitis	29.47
D05	SYBR	sodC gene	N.menengitis	29.45
D02	SYBR	sodC gene	N.menengitis	28.80
D01	SYBR	sodC gene	N.menengitis	26.63
A06	SYBR	sodC gene	N.menengitis	N/A
H05	SYBR	sodC gene	N.menengitis	N/A

Figure (2) Threshold cycle number (CT) Value by using SYBER Green Dye for sod C gene specific for Neisseria meningitis in CSF samples. (N/A)=Negative Results

Disscussion :

Bacterial meningitidis is alife – threatening disease . However it is proven that early diagnosis improves clinical out com **(Arosso et al., 2008)** .

Meningococcal infection requires a fast and accurate diagnostic method in order to correctly initiate the antibiotic therapy**(Nemescu et al.,2015)**

So rapid confirmation of the disease is required not only for patient treatment and case contact chemoprophylaxis but also for contact management, out break recognition and detailed epidemiological surveillance of these infecting **(Kesanopoulos et al., 2004)** .

Although bacterial culture is considered to be the standard method, The negative effect of prior antimicrobial drug usage on its sensitivity makes necessary to search for non culture techniques for diagnosis , among non- culture diagnostic tests , PCR and RT-PCR is the most accurate and reliable method , especially among patients with ahistory of antimicrobial drug use before spinal tap **(Ceyhan et al., 2008)** .

So another limitation of conventional methods is the relatively long time required to get the results in case of cultures. therefore the introduction of RT-PCR has significantly improved the confirmation rate.(**Nemescu et al.,2015)**

In the present study, among the bacterial culture and gram stain positive cases L. monocytogenes was detected,this result disagree with result that was found by **Yahia et al., (2014)** who referred to L. monocytognes were not isolated by bacterial culture the second agent detected by bacterial culture and gram stain was Staph. aureus in 1 cases (20%) , this rate of isolation of this bacteria may be consistent with the presence of trauma or neurosurgical implanted devices .

The low rate of positive bacterial culture and gram stain may be due to the preceding antibiotic therapy of the patients before lumber punctures .

N. meningitidis was the most common cause of acute bacterial meningitis , but in this study this bacteria was not isolated by bacterial culture or gram stain which is similar to that was found by **Yahi et al., (2014**) ; **Chakrabartie et al., (2009)** . The light cycler system was effective for detecting N. meningitidis SodC gene in 42 case (42%) . This result agreement with results of **Kesanopoulos et al., (2005)** .

Real-time PCR is a widely used molecular method for detection of Neisseria meningitidis (Nm). Several RT-PCR assays for Nm target the capsule transport gene, ctrA. However, over 16% of meningococcal carriage isolates lack ctrA, rendering this target gene ineffective at identification of this sub-population of meningococcal isolates. The Cu-Zn superoxide dismutase gene, sodC, is found in Nm but not in other Neisseria species. This sodC RT-PCR assay is a highly sensitive and specific method for detection of Nm, especially in carriage studies where many meningococcal isolates lack capsule genes.(Dolan etal.,2011)

However , the above information denotes that the gram stain and bacterial culture can not be a considered as a method for early and exact diagnosis of meningitis .

In conclusion, RT-PCR was an effective system which produced rapid and accurate detection of N. meningitidis DNA in CSF further refinements in this technique may make it useful for the diagnosis of other bacteria especially when results of CSF gram stain and bacterial culture are negative and when patients had received antibiotics before the lumbar puncture was done .

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