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RESEARCH ARTICLE
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REAL TIME AND RAPID DETECTION OF NDM-1 GENE IN BETA LACTAM RESISTANT BACTERIA USING ELECTROCHEMICAL ASSAY BASED APTAGENE NANORECEPTORS

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Abstract

Background: New Delhi Metallo-b-lactamase (NDM-1) is an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics

Methods: In this study, the target gene was NDM-1 of beta lactam resistant bacteria which isolated on Mueller Hinton Agar (MHA) and identified via DNA extraction and PCR amplification then compared with the electrochemical assay based aptamer nanoreceptors.

Results: The results were encouraged and indicate that specific DNA aptasensor identified signal based the aptamer NDM-1 gene binding and exhibited affinity for the target DNA single strand, resulting in the direct detection of the NDM-1 in complex clinical bacteria samples in compare with PCR amplification.

Conclusions: This novel electrochemical gene sensor method allows for the cost effective, rapid, specific, and more sensitive detection of the NDM-1.

Keywords: NDM-1 gene, aptamer binding, PCR amplification, aptasensor, single strand DNA.

1. Introduction

New Delhi Metallo-b-lactamase (NDM-1) is an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics [1]. The gene for NDM-1 is one member of a large gene family that encodes beta-lactamase enzymes called carbapenemases [2]. The over-expression of BLs (β-lactamases) which caused by among bacteria, resistance is able to inactivate and hydrolyze a vast number of β-lactam antibiotic [3]. United, I. A. (2019), listed that World Health Organization get more attention for antibiotic-resistance subject [4] which become life threatening for the human population [5]. The NDM-1 was first characterization of in 2009, and have 16 variants displaying comparable carbapenemase activities have been identified in this family. These variants differ from NDM-1 by one or two amino-acid substitutions [6]. Most NDM-positive isolates are multidrug resistant and Gram-negative bacilli producing NDM-type metallo-carbapenemases present a recognized threat to the healthcare system [7].Current study attention with (NDM-1) is a novel broad spectrum b-lactamase (class b

carbapenemase) with the ability to inactivate all b-lactam antibiotic [8]. New Delhi metallo-β-lactamases (NDMs) are considers new additions to the class of MBLs [9; 10]. A new era of antibiotic resistance emergence by novel plasmid-encoded MBL family that able to hydrolyze almost β-lactam antibiotics and rapid worldwide dissemination. Their highly efficient inactivation of the last-generation carbapenems, such as imipenem and meropenem. [11].

2. Materials and Methods

2.1 Study design and sample collection

A total of 30 cultured samples were collected from children patients in Basrah children's specialty hospital for a period from January to April 2021, and the range of age was from (1-15 years) ,18 were female and 12 were male .These samples includes 20 specimens outpatient were urine for culture , 8 specimens were wound swab , 2 samples were abscesses for culture and all the 10 specimen were inpatient .these collected specimens were processed in microbiology laboratory in Basrah children specialty hospital by used routine culture media including blood agar and MacConkey agar for urine sample and on blood agar, chocolate agar and MacConkey agar and incubation under routine condition aerobically $37^{O}C$ and CO_2 (5-10%) for 18-24 hr.

2.2 Full automated system Vitek 2 compact

After that will be identification growth of Gram negative bacteria on plate culture media and then antimicrobial susceptibility testing (AST) will be done by using the full automated system Vitek 2 compact (manufactured by bioMerieoux) ID/AST panel. That enables to determined ESBL (Extended spectrum beta lactamase) and CRE (Carbapenem resistant Enterobacteriaceae) by GN ID/AST N222 and AST N76 panel to detected NDM-1 producer depending on MICs for Carbapenem group (Imipenem ,Meropenem and Ertapenem according to the AST panel).

2.2.1 Molecular technique:

Total DNA was extracted from bacterial isolates by Favor Prep Genomic DNA Mini Kit (Special protocol for Gram negative bacteria) from Favorgen Biotech Corporation .NDM-1 gene based on a PCR molecular technique was detection by used the primers forward NDM-Fm (5-GGTTTGGCGATCTGGTTTTC-3, positions 133 to 153) and revers NDM-Rm (5-CGG AATGGCTCATCACGATC-3, positions 734 to 754), which amplified an internal fragment of 621 bp of the blaNDM-1 gene [12].Primers were designed according to the sequence of the NDM-1 gene in the GenBank database under accession no. FN392876.

2.2.2 Electrochemical technique:

All CV electrochemical measurements were carried out on a DropSTAT4000P potentiostat instrument (from DropSens) controlled by Autolab software using DropSens screen printed gold electrodes (SPGEs). These electrodes have a conventional three electrode configuration with gold working electrode (4-mm diameter disk) and counter electrode (16 mm×1.5 mm curved line), and the potentials were ± 0.2 and ±0.5, at a scan rate of 100mV/s recorded against Ag/AgCl (16 mm×1.5 mm straight line) pseudo-reference electrodes.

2.3 Experimental Methodology

The aptamers and other chemicals which used in this work were modified with the thiol groups (SH) at the C3 termini or prime and purchased from Sangon-Biotech (China) and attached to provide strong and oriented binding of the aptamers to screen printed gold electrodes. The redox functional groups, e.g. methylene blue, were attached to C5 termini or prime in order to provide distinctive electrochemical properties, such as current peaks on CV characteristics associated with oxidation and reduction reactions. The other chemicals used (all from Sigma Aldrich) were Hepes and phosphate binding buffers (HBB and PBB), also1,4-dithiothreitol (DTT). Hepes binding buffer (HBB) was prepared by dissolving 50 mM Hepes sodium salt, 3 mM $MgCl_2$, 120 mM NaCl, and 5 mM KCl in

deionized Milli-Q water. The pH of the buffer was adjusted to 7.4. Similarly, phosphate binding buffer (PBB) was prepared by dissolving 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 3 mM $MgCl_2$, 2.7 mM KCl, and 137 mM NaCl. The pH of the buffer was adjusted to 7.4. The addition of $MgCl_2$ to the buffers was essential to preserve the aptamer single DNA strand from self-coiling. For long-term storage, the 100 µM solutions of received aptamer was prepared in sterilized deionized water and stored at -20 C° in small aliquots in order to avoid repeated thaw-freeze cycles [13;14]

2.3.1 Immobilization of aptamers

The aptamers were immobilized on gold surface via thiol groups on the 3'- termini in the following procedure. Stock solution of the required aptamer was diluted to 1 µM with HBB or PBB supplemented with 1 mM of 1,4-dithiothretiol (DTT) and 3 mM of $MgCl_2$. DTT causes the removal of the protecting group from the SH moiety and released the aptamers with free SH end groups that could then bind to the surface of screen printed gold electrode in the presence of Mg^{2+} aptamers are initially stretched. Before immobilization, the aptamers samples were activated by rapid (1 min) heating up to 95 °C followed by 1 min cooling at (4 °C) using a conventional thermocycler polymerase chain reaction unit (TECHNE PCR version TC-3000). Immobilization was carried out by casting aptamers solution onto the screen printed gold electrode surface; the samples were then incubated for 4 h at room temperature in a humidity chamber. The unreacted aptamers were removed from the electrode surface by several rinses with non-folding buffer (HBB), then the screen printed gold electrode with immobilized aptamers were kept in HBB to prevent aptamers from coiling [15].

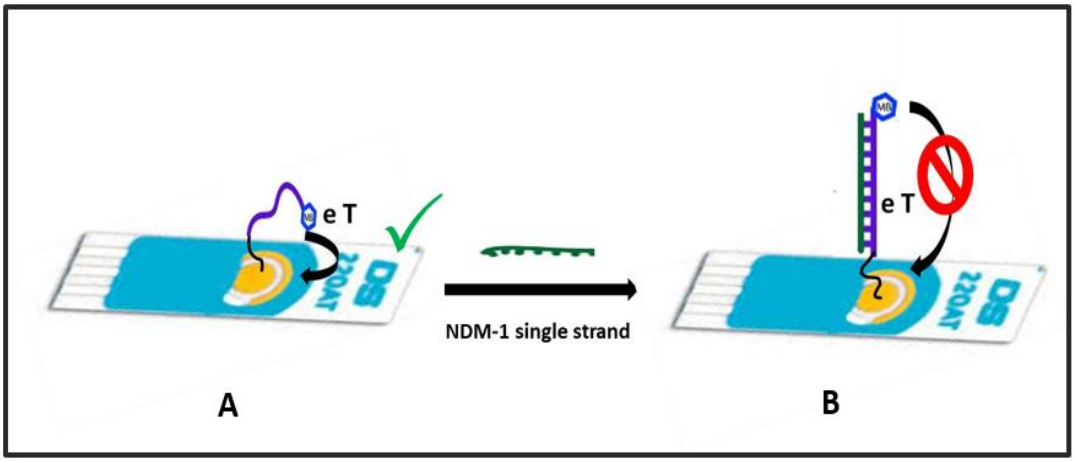

Figure (1): Schematic diagram of electrochemical aptasensor for NDM-1 gene detection using redox-labelled aptamer.

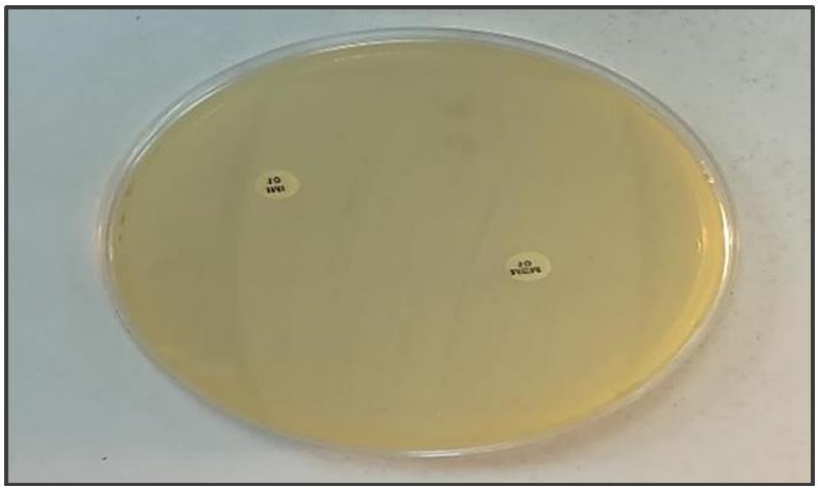

Figure (2): NDM-1 isolate cultured on Mueller Hinton Agar (MHA).

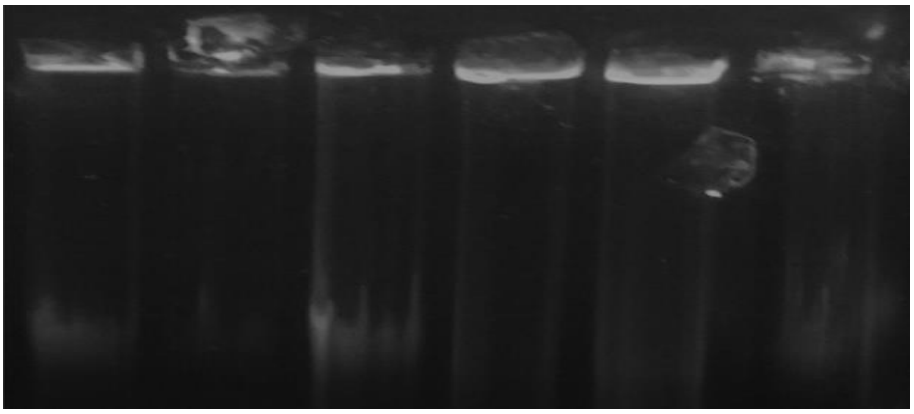

Figure (3): Agarose gel (1.5 %gm and 60V,2MA) electrophoresis patterns show DNA bands of bacterial DNA extraction (1-6) extracted beta-lactam resistant bacteria.

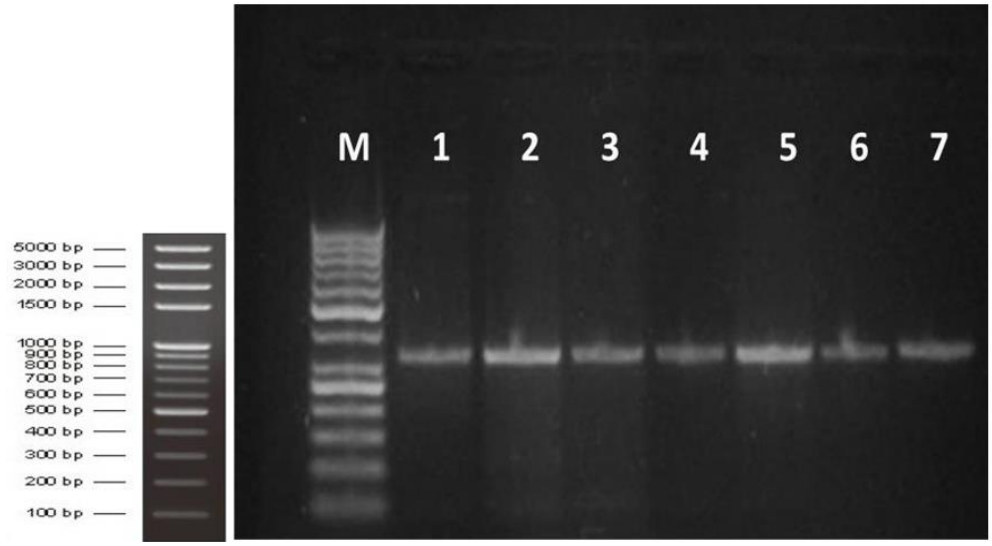

Figure (4): Agarose gel (2% gm and 60V,2MA) electrophoresis patterns show PCR amplified products of

NDM-1 gene, Lane M: 5000bp DNA ladder, lanes 1-7: NDM-1 gene bands of beta-lactam resistant bacteria 625 bp.

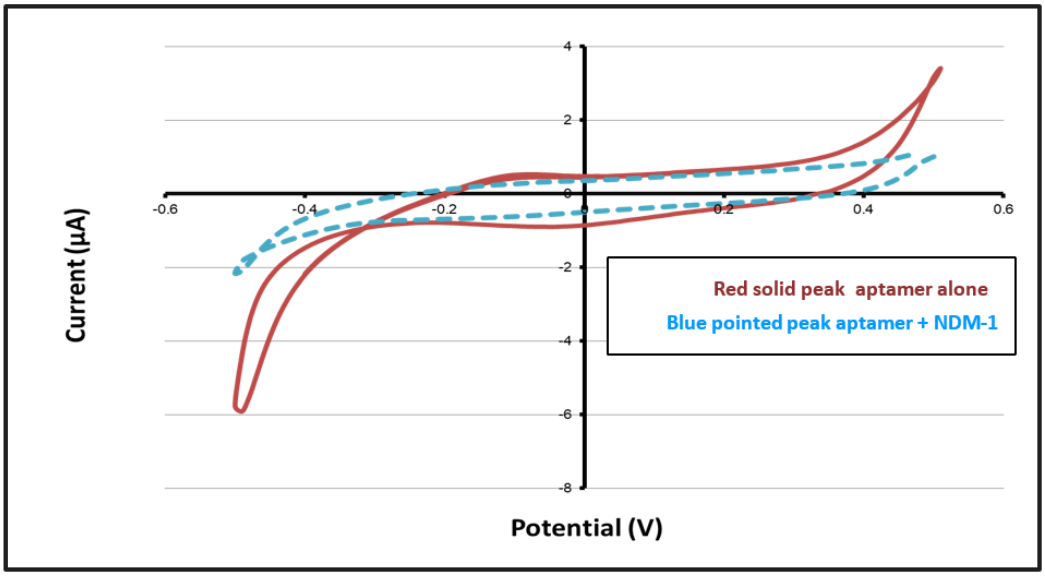

Figure (5): Cyclic voltammograms of screen printed gold electrodes with immobilized aptamer.

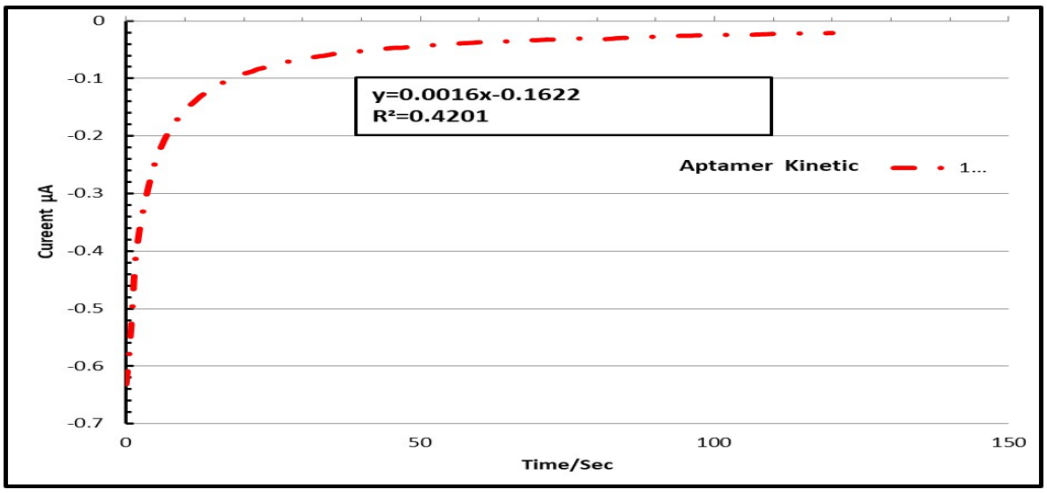

Figure (6): Typical kinetic of aptamer and NDM-1 binding which corresponding to aptamer affinity.

3 Results:

According to the table (1) which shows the results of NDM-1 isolates were cultured on Mueller Hinton Agar (MHA) at 35^0C ± 2^0C incubation for 16-18 hours, 0.5 McFarland standard and both antibiotic disc Imipenem (10µg) IMP and Meropenem (10µg) MEM which applied on MHA plate gave resistant result that means (Carbapenemase enzyme producer from Beta- Lactams Resistance Bacteria) depending to Clinical and Laboratory Standards Institute (CLSI) which updated in 2021 from WHO. The observed results in table 1 has most common samples which was urine about 20 specimen , 8 specimens was wound swab and 2 specimens was abscesses .and most the isolates were detected as Gram negative bacilli (*E.coli* about 21 isolate , *Klebsiella pneumonia* 6 isolate , Proteus mirabilis 2 isolate and *Acinetobacter baumannii* 1 isolate . ESBL +ve isolates were reach to 24 isolates about 16 isolate were *E.coli* , 3 isolates were *Klebsiella pneumonia* , 2 isolates were *Proteus mirabilis* and one isolate was *Acinetobacter baumannii*. While CRE +ve represent in four isolates only 3 isolates from these in *Klebsiella pneumonia* and one isolate was found in *Acinetobacter baumannii* . This study showed high resistance to cephalosporins CAZ,CTX and CRO but low resistance to carbapenems IPM and MEM. The isolates number (7,14, 29,30) were have positive results for both extended spectrum beta lactamase (ESBL) and Carbapenem resistant Enterobacteriaceae CRE.

Table 1: show characterization of samples and most common important antibiotic ,ESBL and CRE assessment by Vitek 2 compact

No	Sample	Ward	Isolate	ESBL/CPE	CAZ	CRO	CTX	IPM	MEM
1	Urine	O.P	*E.coli*	+Ve/ -Ve	R	R	R	S	S
2	Urine	O.P	*E.coli*	+Ve/ -Ve	R	R	R	S	S
3	Urine	O.P	*Klebsiella pneumonia*	-Ve/ -Ve	S	S	S	S	S
4	Urine	O.P	*E.coli*	+Ve/ -Ve	R	R	R	S	S
5- a	Urine	O.P	*E.coli*	+Ve/ -Ve	R	R	R	S	S
5- b	Urine	O.P	*Proteus mirabilis*	+Ve/ -Ve	R	R	R	R	S
6	Urine	O.P	*Klebsiella pneumonia*	-Ve/ -Ve	R	R	R	S	S
7	Wound Swab	ICU	*Klebsiella pneumonia*	+Ve / +Ve	R	R	R	R	R
8	Urine	O.P	*E.coli*	+Ve/ -Ve	R	R	R	S	S
9	Urine	O.P	*E.coli*	-Ve / -Ve	R	R	R	S	S
10	Urine	ICU	*E.coli*	+Ve/ -Ve	R	R	R	S	S
11	Urine	O.P	*E.coli*	+Ve/ -Ve	R	R	R	S	S
12	Urine	O.P	*E.coli*	+Ve/ -Ve	R	R	R	S	S

13	**Wound Swab**	**ICU**	***E.coli***	**+Ve/ -Ve**	**R**	**R**	**R**	**S**	**S**
14	**Wound Swab**	**ICU**	***Klebsiella pneumonia***	**+Ve / +Ve**	**R**	**R**	**R**	**R**	**R**
15	**Urine**	**O.P**	***Klebsiella pneumonia***	**+Ve/ -Ve**	**R**	**R**	**R**	**I**	**S**
16	**Urine**	**O.P**	***E.coli***	**-Ve/ -Ve**	**S**	**S**	**S**	**S**	**S**
17	**Ear Swab**	**O.P**	***E.coli***	**-Ve / -Ve**	**R**	**R**	**R**	**S**	**S**
18	**Urine**	**O.P**	***E.coli***	**+Ve/ -Ve**	**R**	**R**	**R**	**S**	**S**
19	**Urine**	**O.P**	***E.coli***	**+Ve/ -Ve**	**R**	**R**	**R**	**S**	**S**
20	**Urine**	**O.P**	***E.coli***	**-Ve/ -Ve**	**S**	**S**	**S**	**S**	**S**
22	**Abscess**	**C**	***Proteus mirabilis***	**-Ve/ -Ve**	**R**	**R**	**R**	**S**	**S**
23	**Abscess**	**C**	***E.coli***	**+Ve/ -Ve**	**R**	**R**	**R**	**S**	**S**
24	**Urine**	**O.P**	***E.coli***	**+Ve/ -Ve**	**R**	**R**	**R**	**S**	**S**
25	**Wound Swab**	**C**	***E.coli***	**-Ve/ -Ve**	**S**	**S**	**S**	**S**	**S**
26	**Wound Swab**	**ICU**	***E.coli***	**+Ve/ -Ve**	**R**	**R**	**R**	**S**	**S**
27	**Urine**	**O.P**	***E.coli***	**+Ve/ -Ve**	**R**	**R**	**R**	**S**	**S**
28	**Urine**	**O.P**	***E.coli***	**+Ve/ -Ve**	**R**	**R**	**R**	**S**	**S**
29	**Wound Swab**	**ICU**	***Acinetobacter baumannii***	**+Ve / +Ve**	**R**	**R**	**R**	**R**	**R**
30	**Wound Swab**	**ICU**	***Klebsiella pneumonia***	**+Ve / +Ve**	**R**	**R**	**R**	**R**	**R**

The results of all isolates have been presented in Table (1).The identification was with similarity ≥99% between both approaches i.e the PCR amplification and electrochemical apat-gene assay [16;17].

4 Discussion

The current study focused on amplify the NDM-1 gene of multi drug resistance bacteria of beta-lactam which were collected from the area of study as mentioned in the methodology. Thirty isolates were identified according to the size of PCR products. In the meantime the comparative results with the apta-gene assay based on the ss DNA aptamer give the same outcome within short time and accurate results.

The present study approves the results of the previous studies which showed the same domains of this assay such as [18; 19]. Also the current results are in agreement with many different studies in this field [20] .Therefore, the aim of the present study was to detect the NDM-1 of the beta – lactam resistant isolates using electrochemical aptasensor [21].

5 Conclusions

Here, we describe and fabricate the modified an electrochemical aptasensor based NDM-1 gene detection test, including the design of NDM-1 specific nucleotides of DNA nucleic acid probes and assay specificity optimization through test the real-time kinetic of aptamer binding via C.V measurements. We identified all the isolates in this study although the low number of samples, restricted area of study and lack of gene expression [22].

Abbreviations

NDM- [1] : New Delhi Metallo-b-lactamase; AST: Antimicrobial Susceptibility Testing; ESBL: Extended spectrum beta lactamase; SPGEs: screen printed gold electrodes; SH: thiol groups; CV: cyclic voltammogram; HBB: Hepes binding buffer; PBB: phosphate binding buffers; DTT: 1,4-dithiothreitol; MHA: Mueller Hinton Agar; WHO: World Health Organization; PCR: Polymerase Chain Reaction.

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Ethics approval and consent to participate
We have obtained approval to conduct our study from the ethics committee of Basrah Children Specialty Hospital-Microbiology laboratory and University of Basrah, College of Medicine, all patients' parents provided written informed consent.

Consent for publication
Not applicable because this manuscript does not contain any individual personal data.

Availability of data and materials
In this study, all the data and materials are included. If more information is needed, please contact the author for data requests

Competing interests
The authors declare no competing interest

Funding
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Authors' contributions
HF, participated in the design of the study. The samples collection and interpretation were done by HS and MM. The manuscript preparation was conducted by AA. HF and HS participated in the data editing. The authors read and approved the final manuscript.

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