



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- β -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.*

INTRODUCTION

New Delhi Metallo- β -lactamase (NDM-1) is an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics¹. The gene for NDM-1 is one member of a large gene family that encodes beta-lactamase enzymes called carbapenemases². 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 antibiotic³.

United, I. A. (2019), listed that World Health Organization get more attention for antibiotic-resistance subject⁴ which become life threatening for the human population⁵. 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 substitutions⁶.

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 β -lactamase (class B carbapenemase) with the ability to inactivate all β -lactam antibiotic 8. New Delhi metallo- β -lactamases (NDMs) are considered 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.

MATERIALS AND METHODS

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 include 20 specimens outpatient were urine for culture, 8 specimens were wound swab, 2 samples were abscesses for culture and all the 10 specimens were inpatient. These collected specimens were processed in microbiology laboratory in Basrah children's 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°C and CO₂ (5-10%) for 18-24 hr.

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 bioMérieux) ID/AST panel. That enables to determine ESBL (Extended spectrum β -lactamase) and CRE (Carbapenem resistant Enterobacteriaceae) by GN ID/AST N222 and AST N76 panel to detect NDM-1 producer depending on MICs for Carbapenem group (Imipenem, Meropenem and Ertapenem according to the AST panel).

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 detected by using the primers forward NDM-Fm (5-GGTTTGGCGATCTGGTTTTC-3, positions 133 to 153) and reverse NDM-Rm (5-CGG AATGGCTCATCACGATC-3, positions 734 to 754), which amplified an internal fragment of 621 bp of the bla_{NDM-1} gene 12. Primers were designed according to the sequence of the NDM-1 gene in the GenBank database under accession no. FN392876.

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 100 mV/s recorded against Ag/AgCl (16 mm×1.5 mm straight line) pseudo-reference electrodes.

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), also 1,4-dithiothreitol (DTT). Hepes binding buffer (HBB) was prepared by dissolving 50 mM Hepes sodium salt, 3 mM MgCl₂, 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 Na₂HPO₄, 1.76 mM KH₂PO₄, 3 mM MgCl₂, 2.7 mM KCl, and 137 mM NaCl. The pH of the buffer was adjusted to 7.4. The addition of MgCl₂ 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 $-20\text{ }^\circ\text{C}$ in small aliquots in order to avoid repeated thaw-freeze cycles 13;14

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 $95\text{ }^\circ\text{C}$ followed by 1 min cooling at ($4\text{ }^\circ\text{C}$) 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.

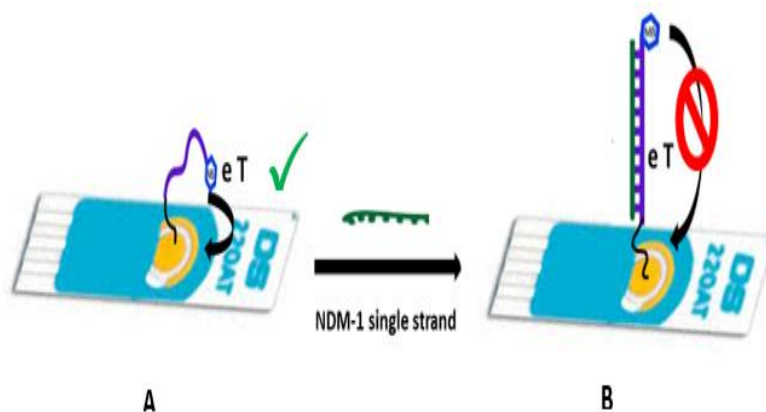


FIGURE 1: Schematic diagram of electrochemical aptasensor for NDM-1 gene detection using redox-labelled aptamer.

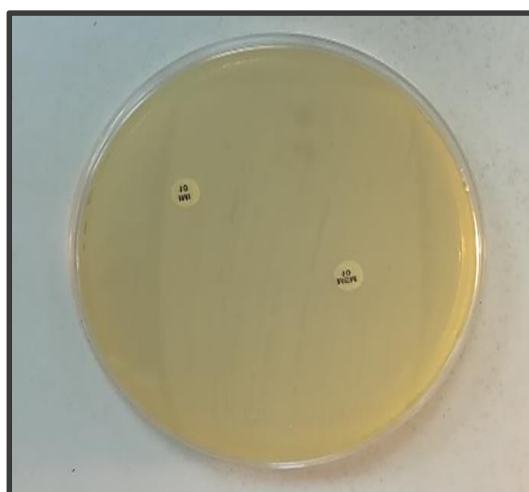


FIGURE 2: NDM-1 isolate cultured on Mueller Hinton Agar (MHA).

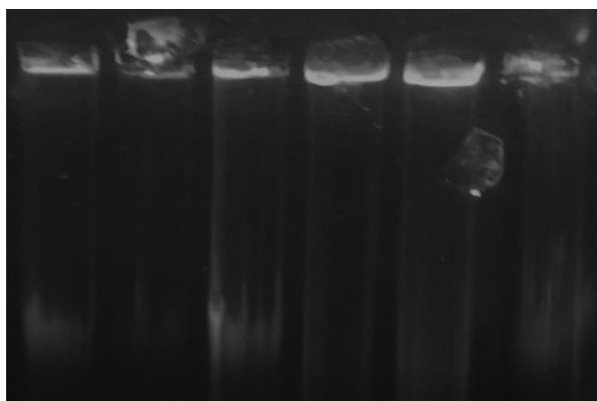


FIGURE 3: Agarose gel (1.5 % gm and 60V,2MA) electrophoresis patterns show DNA bands of bacterial DNA extraction (1-6) extracted beta-lactam resistant bacteria.

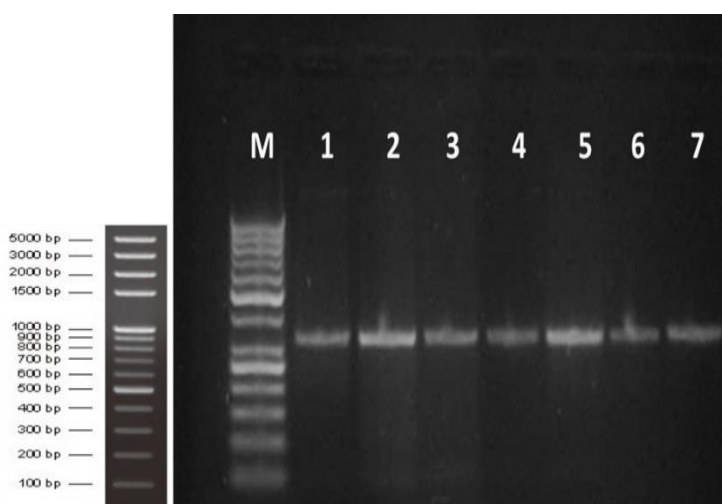


FIGURE 4: Agarose gel (2% gm and 60V,2MA) electrophoresis patterns show PCR amplified products of NDM-1 gene, Lane M: 5000bp DNA ladder, lanes 1-7: NDM-1 gene bands of beta-lactam resistant bacteria 625 bp.

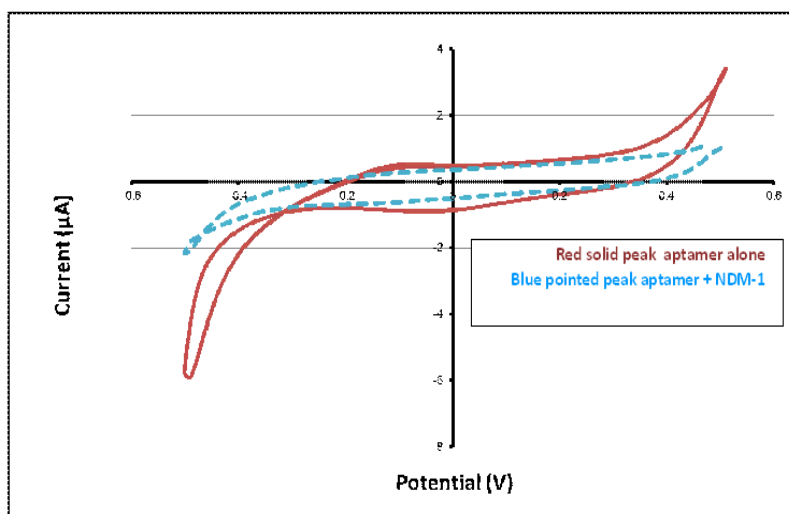


FIGURE 5: Cyclic voltammograms of screen printed gold electrodes with immobilized aptamer.

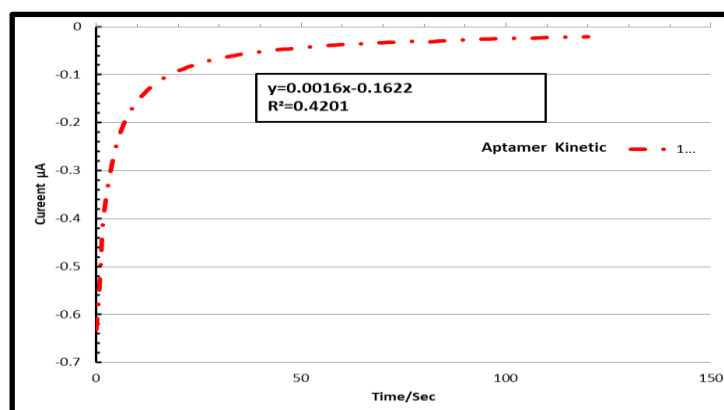


FIGURE 6: Typical kinetic of aptamer and NDM-1 binding which corresponding to aptamer affinity.

RESULTS

According to the table (1) which shows the results of NDM-1 isolates were cultured on Mueller Hinton Agar (MHA) at $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ 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	<i>E.coli</i>	+Ve/ -Ve	R	R	R	S	S
2	Urine	O.P	<i>E.coli</i>	+Ve/ -Ve	R	R	R	S	S
3	Urine	O.P	<i>Klebsiella pneumonia</i>	-Ve/ -Ve	S	S	S	S	S
4	Urine	O.P	<i>E.coli</i>	+Ve/ -Ve	R	R	R	S	S
5- a	Urine	O.P	<i>E.coli</i>	+Ve/ -Ve	R	R	R	S	S
5- b	Urine	O.P	<i>Proteus mirabilis</i>	+Ve/ -Ve	R	R	R	R	S
6	Urine	O.P	<i>Klebsiella pneumonia</i>	-Ve/ -Ve	R	R	R	S	S
7	Wound Swab	ICU	<i>Klebsiella pneumonia</i>	+Ve / +Ve	R	R	R	R	R
8	Urine	O.P	<i>E.coli</i>	+Ve/ -Ve	R	R	R	S	S
9	Urine	O.P	<i>E.coli</i>	-Ve / -Ve	R	R	R	S	S

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

The results of all isolates have been presented in Table (1). The identification was with similarity $\geq 99\%$ between both approaches i.e the PCR amplification and electrochemical aptagene assay^{16,17}.

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 field²⁰. Therefore, the aim of the present study was to detect the NDM-1 of the beta – lactam resistant isolates using electrochemical aptasensor²¹.

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 expression²².

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.

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

The study is self-funded. 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.

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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REFERENCES

1. Venkata K C, Ellebrecht, N M, Tripathi SK. Efforts towards the inhibitor design for New Delhi metallo-beta-lactamase (NDM-1). European journal of medicinal chemistry. 2021; 225, 113747.
2. Heinz E, Ejaz H, Bartholdson Scott J, Wang N, Gujran S, Pickard D, Strugnell, R A. Resistance mechanisms and population structure of highly drug resistant Klebsiella in Pakistan during the introduction of the carbapenemase NDM-1. Scientific reports. 2019; 9(1):1-13.
3. United, I A C G. No time to wait—securing the future from drug-resistant infections. Report to the Secretary General of the Nations. 2019.
4. D'Andrea M M, Fraziano M, Thaller M C, Rossolini G M. The urgent need for novel antimicrobial agents and strategies to fight antibiotic resistance. Antibiotics. 2019; 8(4): 254.
5. Frère J M. Beta-lactamases and bacterial resistance to antibiotics. Mol. Microbiol. 1995; 16: 385–395. [CrossRef] [PubMed]
6. Taubes G. The bacteria fight back. Science. 2008; 321: 356–361. [CrossRef] [PubMed].
7. Yong D, Toleman MA, Giske CG. Characterization of a new metallo-beta-lactamase gene, bla_{NdM-1}, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from india. antimicrob agents Chemother. 2009; 53: 5046-5054.
8. Groundwater P W, et al. New Delhi metallo-beta-lactamase-1: structure, inhibitors and detection of producers. Future Med. Chem. 2016; 8: 993–1012.

9. Khan A U, Maryam L, Zarrilli R. Structure, genetics and worldwide spread of New Delhi metallo-beta-lactamase (NDM): a threat to public health. *BMC Microbiol.* 2017;17: 101.
10. Yong D. et al. Characterization of a new metallo-beta-lactamase gene, bla (NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob. Agents Chemother.* 2009;53: 5046–5054.
11. Nordmann P, Poirel L, Carre'r A, et al. How to detect NDM-1 producers. *J Clin Microbiol* 2011; 49: 718–21.
12. Shenoy KA, Jyothi EK, Ravikumar R. Phenotypic identification & molecular detection of bla (ndm-1) gene in multidrug resistant Gram-negative bacilli in a tertiary care centre. *Indian J Med Res.* 2014 Apr;139(4):625-31. PMID: 24927351; PMCID: PMC4078503.
13. Nabok A, Abu-Ali H, Takita S, Smith D P. Electrochemical detection of prostate cancer biomarker PCA3 using specific rna-based aptamer labelled with ferrocene. *Chemosensors.*2021; 9(4): 59.
14. Abu-Ali H, Ozkaya C, Davis F, Walch N, Nabok A. Electrochemical aptasensor for detection of opamine. *Chemosensors.* 20208;2: 28.
15. Abu-Ali H, Nabok A, Smith T J. Development of novel and highly specific ssDNA-aptamer-based electrochemical biosensor for rapid detection of mercury (II) and lead (II) ions in water. *Chemosensors.* 2019;7(2):27.
16. Lai Y H, Sun S C, Chuang M C. Biosensors with built-in biomolecular logic gates for practical applications. *Biosensors.* 2014;4(3):273-300.
17. Vsxcz S, Punjabi K, Ahamad N, Singh S, Bendale P, Banerjee R. Nanotechnology Approaches for Rapid Detection and Theranostics of Antimicrobial Resistant Bacterial Infections. *ACS Biomaterials Science & Engineering.*2022; 8:(6), 2232-2257.
18. Khobragade S. Bacterial detection using an anharmonic acoustic aptasensor (Doctoral dissertation, Loughborough University); 2018 .
19. Bruno, J G. Predicting the uncertain future of aptamer-based diagnostics and therapeutics. *Molecules.*2015;20(4): 6866-6887.
20. Courbet A. Engineering next generation diagnostics through synthetic biology (Doctoral dissertation, Universite Montpellier); 2015.
21. Hendriksen R S, Vieira A R, Karlsmose S, Lo Fo Wong D M, Jensen A B, Wegener H C, Aarestrup F M. Global monitoring of Salmonella serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: results of quality assured laboratories from 2001 to 2007. *Foodborne pathogens and disease.* 2011; 8(8): 887-900.
22. Courbet A. *Engineering next generation diagnostics through synthetic biology* (Doctoral dissertation, Universite Montpellier); 2015.