Title: Gene expression in bacterial pathogen against antibiotics

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Abstract: Carbapenem resistance among Enterobacteriaceae has emerged as a significant global antibiotic resistance issue. Identifying the resistance mechanisms in these clinically significant isolates is crucial for infection control and public health strategies. This study examined blood samples to detect the presence of the NDM-1 gene and qualitatively assessed its expression using real-time PCR. Our findings revealed that one sample tested positive for NDM-1, while another yielded inconclusive results. We discuss the implications of these findings and the study's limitations.

Keywords: NDM-1, Antibiotics, Real-time PCR, Antibiotic resistance

Objectives:

- Culture blood samples on MacConkey agar plates incubate at 37°C for 24 hours.
- After incubation, observe the plates and further analyze isolated colonies to identify the bacteria or pathogens present.
- Amplify antimicrobial resistance genes using specific primers through PCR.
- Perform real-time PCR following established laboratory protocols to detect gene presence.

Introduction:

Antibiotics combat bacterial infections by killing bacteria (bactericidal) or inhibiting their growth and reproduction (bacteriostatic). Typically derived from bacteria or fungi, these agents have been employed for over 70 years to treat infectious diseases, significantly reducing illness and mortality since the 1940s. However, extensive and prolonged use has led to adaptations in targeted infectious organisms, diminishing antibiotic efficacy.

In the United States, at least 2 million individuals annually contract infections from antibiotic-resistant bacteria, with at least 23,000 fatalities directly attributed to these infections. The surge in antibiotic use is partly due to overprescription and excessive consumption.

Antibiotics can penetrate bacterial outer membranes, with the lipid and protein composition influencing bacterial sensitivity. Bacteria may also regulate chemical entry and expulsion, reducing antibiotic permeability and contributing to resistance.

Gram-positive pathogens are major culprits in healthcare-associated and community-acquired bacterial infections. Community-acquired infections occur outside hospitals or are diagnosed

within 48 hours of admission without prior encounters. Factors such as population density, an increasing elderly population, more immunocompromised individuals, frequent travel, antibiotic sales, and widespread broad-spectrum antibiotic use contribute to resistance development in communities.

Healthcare-associated infections (HAIs), or nosocomial infections, arise during patient care in hospitals or other healthcare facilities. Antibiotic use in any context promotes resistance; susceptible bacteria are eliminated, allowing resistant strains to survive and proliferate. Consequently, antibiotic-resistant pathogens emerge in settings with heavy antibiotic use, like hospitals.

The misuse of antibiotics leads to the emergence of superbugs. Excessive or inappropriate antibiotic use, including self-prescription, overexposes bacteria, prompting mutations for survival. Methicillin-resistant Staphylococcus aureus (MRSA) is notably significant as a Gram-positive, multi-resistant pathogen, being a leading cause of healthcare-associated infections globally and an emerging cause of community-acquired infections.

In India, 10-20% of hospitalized patients acquire nosocomial infections, with intensive care units (ICUs) being primary sites, accounting for 25% of all hospital infections. Staphylococcus aureus is the most common cause in clinical settings. Over 24% of patients with healthcare-associated sepsis and 52.3% of those in ICUs die annually.

The risk of acquiring HAIs in India is up to 20 times higher than in other countries. Surveillance data from 40 Indian hospitals between 2004 and 2013 reported the prevalence of catheter-associated urinary tract infections (CAUTI) and central line-associated bloodstream infections (CLABSI).

The NDM-1 gene significantly contributes to bacterial antibiotic resistance. NDM-1 (New Delhi metallo-beta-lactamase-1) encodes an enzyme that deactivates beta-lactam antibiotics, including penicillins, cephalosporins, and carbapenems—some of the most effective antibiotics for bacterial infections. Bacteria harboring the NDM-1 gene become resistant to a broad spectrum of beta-lactam antibiotics, posing a serious public health threat. Untreatable common infections can lead to increased complications, prolonged hospital stays, and higher mortality rates. Current countermeasures include surveillance, patient isolation, and stringent hospital hygiene practices to prevent spread. The emergence of NDM-1 underscores the urgent need for global collaboration to address antibiotic resistance and develop effective treatments.

While NDM-1 confers resistance to many antibiotics, some classes, such as tigecycline and colistin, may remain effective against NDM-1-positive bacteria.

This study aims to compare mortality outcomes and antibiotic susceptibility in NDM-1-producing bacterial isolates from patients with bloodstream infections in an ICU in India and to evaluate different laboratory methods for their detection.

Methodology:

2.1. Sample Collection and Preparation

- Sample Collection: Aseptically collected blood samples from 20 patients with suspected bloodstream infections.
- Sample Processing: Cultured blood samples on MacConkey agar plates, incubated at 37°C for 24 hours to isolate bacterial colonies (Figure I).

2.2. DNA Extraction (TRUPCR® Viral RNA Extraction Kit)

- Sample Lysis:
  - Combine 25 µl of Proteinase K and up to 200 µl of the isolated bacterial colonies in liquid media in a 1.5 ml microcentrifuge tube.
  - Add 300 µl of Lysis Buffer BAV1 (with carrier RNA) to the tube.
  - Vortex vigorously for 10-20 seconds to mix thoroughly.
  - Incubate at 56°C for 15 minutes to lyse cells and release DNA.
- **Adjust DNA Binding Conditions:**

DNA Extraction and Purification:

1. **Ethanol Addition and Incubation:**
   - Add 250 µl of ethanol (96–100%) to the lysed sample and vortex to mix.
   - Incubate the mixture for 5 minutes at room temperature to facilitate DNA binding to the silica membrane.
2. **DNA Binding and Washing:**
   - Spin Column Setup: Place a spin column into a 2 ml collection tube.
   - Load Lysate: Transfer the entire lysate into the spin column.
   - Centrifugation: Centrifuge at 10,000 rpm for 1 minute to allow DNA binding.
   - Wash Steps:
     - Add 500 µl of Buffer BAW1, centrifuge at 10,000 rpm for 1 minute, and discard the flow-through.
     - Add 500 µl of Buffer BAW2, repeat centrifugation, and discard the flow-through.
     - Add 500 µl of ethanol (96–100%), centrifuge, and discard the flow-through.
3. **Drying and Elution:**
   - Centrifuge the spin column in a new collection tube at 12,000 rpm for 3 minutes to remove residual ethanol.
   - Place the spin column into a 1.5 ml microcentrifuge tube, add 40 µl of Elution Buffer BRE, incubate for 1 minute, and centrifuge at 10,000 rpm for 1 minute to elute the purified DNA.

Detection of NDM-1 Gene via Real-Time PCR:

- **PCR Amplification:**
  - Use specific primers targeting the NDM-1 gene:
    - Forward Primer: 5'-TAGTGCTCAGGTCGGCATC-3'
    - Reverse Primer: 5'-CTTCCAACGGTTTGATCGTC-3'
  - Prepare a 25 µl reaction mixture containing primers, SYBR Green, genomic DNA, and water.
  - Perform PCR with the following cycling conditions:

- Initial denaturation at 95°C for 3 minutes.
- 40 cycles of:
  - Denaturation at 95°C for 10 seconds.
  - Annealing at 61.6°C for 30 seconds.
  - Extension at 65°C for 0.5 seconds.
- Final step at 95°C for 5 seconds.

○ Measure fluorescence during the extension phase and analyze data using BIORAD CFX Maestro software.

Data Analysis:

- Determine the cycle threshold (Ct) value, which indicates the cycle number at which fluorescence exceeds the threshold, reflecting the presence of the target gene.

Results:

- The DNA extraction yielded a concentration of 100 ng/µl with a λ260/280 ratio of 1.77, indicating good purity.

This protocol effectively combines silica membrane-based DNA extraction with real-time PCR to detect the NDM-1 gene, facilitating the identification of antibiotic resistance in bacterial samples.

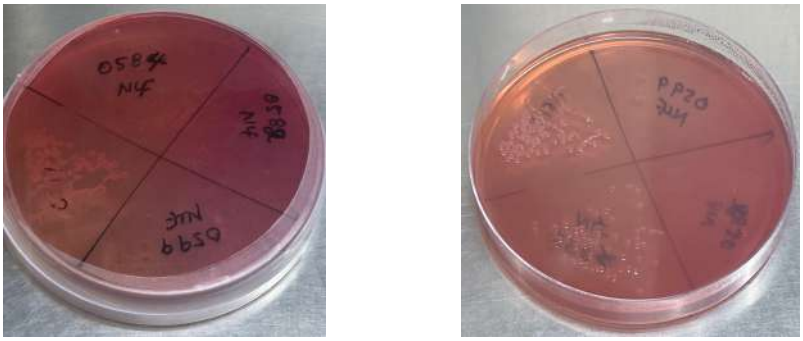

Figure I: Blood samples cultured on McConkey agar plates and incubated at 37°C for 24 hours to isolate bacterial colonies.

Gene sequence of NDM1 (from NCBI database MW464182.1)
tcagcgcagcttgtcggccatgcgggccgtatgagtgattgcggcgcggctatcgggggcggaatggctcatcacgatcatgctggcc
ttggggaacgccgcaccaaacgcgcgcgctgacgcggcgtagtgctcagtgtcggcatcaccgagattgccgagcgacttggcctt
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tttgctggccaatcgtcgggcggatttcaccgggcatgcacccgctcagcatcaatgcagcggctaatgcggtgctcagcttcgcgacc
gggtgcataatattgggcaattccat

Left primer sequence: tagtgctcagtgtcggcatc

Right primer sequence: cttccaacggtttgatcgtc

Qualitative analysis:

Here (pathogen diagnostics) RT PCR was used to detect the presence of the NDM1 nucleic acid template extracted from bacterial colonies from culturing infected patients' blood samples. This technique is referred to as qualitative PCR.
In the present investigation, 3 samples were studied, including a negative control. One of the samples was positive while the other did not peak as expected (Figure II). The possible conclusion was that the DNA might have degraded and thus this sample result was indeterminate/inconclusive.

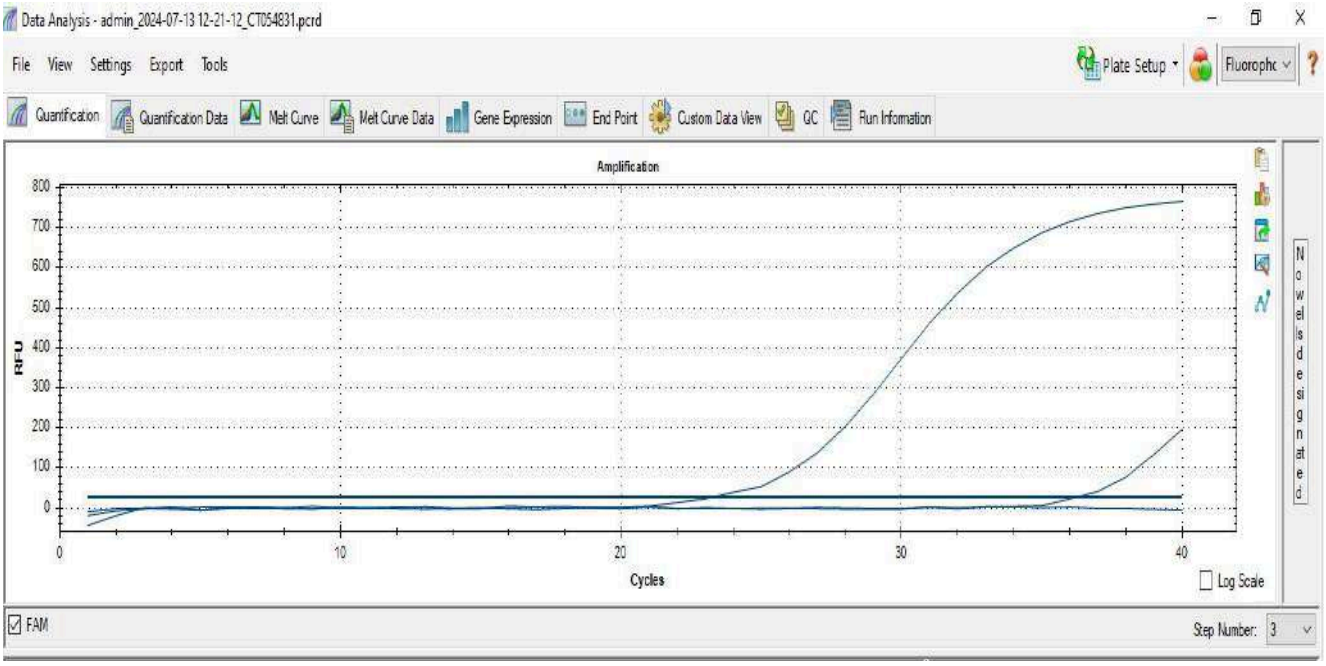

Figure II: The figure illustrates the Results of the qRT-PCR assay. On the y-axis is the relative Fluorescence Unit (RFU); on the x-axis: is the threshold cycle (CT).

4. Discussion:

The present study aimed to investigate the presence and expression of the *NDM1* gene in bacterial colonies isolated from a patient's blood culture. The NDM1 is a carbapenemase gene that causes resistance against a specific class of antibiotics called carbapenem antibiotics. These antibiotics are the last resort against bacterial infections.

The detection of NDM1 in one of the samples was concerning. NDM1 is an important antibiotic resistance marker. Immediate infection control measures are required to prevent the spread of the NDM1-producing organism like contact prevention, hand hygiene, and vicinity cleaning. Also, there is a need to identify potential sources of the NDM1-producing organism in this case and trace individuals who might have been exposed to prevent further transmission. Additionally, alternative treatment options need to be explored including the selection of optimal antibiotic therapy (combinatorial therapy).

The RT-PCR results for the second sample were inconclusive. The absence of a clear peak suggested that the DNA might have degraded. The possible reasons for this could be errors in sample collection, storage, and/or RNA extraction methods. Further investigation is required to determine the presence or absence of NDM1 in this sample. Additional techniques could be used to confirm the presence or absence of NDM1 in the indeterminate sample (e.g., conventional PCR, and whole-genome sequencing).

Lastly, the negative control successfully validated the RT-PCR assay, ensuring that the positive result was not due to contamination.

This study, however, had certain limitations:
1. Small sample size: Due to this the study cannot determine the sensitivity of the test.

2. The inconclusive result for one of the samples highlighted the need for effective and sensitive methods to detect *NDM1*.

In conclusion, the detection of *NDM1* in one of the blood culture samples was a significant finding, highlighting the need for effective measures for infection control. We faced some challenges during the study such as DNA degradation, which underlines the importance of robust and accurate detection techniques for identifying antibiotic-resistant organisms. Continued research and development of novel therapeutic approaches are essential to address the growing threat of NDM1-mediated infections.

Future implications:
To understand the prevalence of NDM1, larger-scale studies involving a diverse patient population are required. Whole-genome sequencing could provide a more detailed analysis of the bacterial isolates, along with the identification of other genes responsible for resistance development in bacteria. Furthermore, investigating the mechanisms of *NDM1* gene dissemination and the development of novel antimicrobial strategies is required for inhibiting antibiotic resistance.

Acknowledgment: I sincerely thank the mentors at the laboratory, my teachers, and my parents who supported me in performing and writing this research work.

Statement of informed consent: The identity of patients was kept strictly confidential. The author being a minor was not allowed to handle the patient's sample in the laboratory. A consent form was signed by the author and the guardian before performing experiments in the lab. The author participated in a 2-day induction program including topics related to "Biohazards and Biosafety" and "Good lab Practices" before starting their work in the lab.

Conflict of interest: The author declares no conflict of interest.

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