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Research

Forensic Detection of Antibiotic Exposure: Utilizing Cefepime-Induced Growth Inhibition in *Bacillus subtilis* as a Novel Microbial Trace Marker"

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Abstract

Microbial communities are utilised as trace evidence in the expanding discipline of forensic microbiology for crime scene investigation, environmental exposure reconstruction, and postmortem interval estimation. However, the pervasive use of antibiotics in clinical and community settings can significantly alter these microbial signatures, complicating forensic interpretation. This study introduces the concept of **antibiotic footprints**-detectable phenotypic alterations in environmental bacteria following antibiotic exposure as a novel indirect marker for medicolegal investigations. Using *Bacillus subtilis*, a resilient, spore-forming bacterium ubiquitous in soil and clinical environments, we evaluated the effects of cefepime, a fourth-generation cephalosporin widely employed in healthcare. *B. subtilis* was isolated from hospital wastewater, confirmed via Gram staining and a panel of biochemical tests, and subjected to antibiotic sensitivity assays. Disk diffusion testing yielded a distinct 10 mm inhibition zone, while broth dilution assays demonstrated complete growth inhibition across a concentration range of 2-58 µg/mL. These reproducible and measurable changes constitute a clear antibiotic footprint. The results imply that some phenotypic changes in robust ambient bacteria caused by antibiotics may be used as forensic evidence. By integrating antimicrobial pharmacology and trace evidence analysis, this method can help recreate events involving suspected antibiotic contamination during hospital mortality, correctional settings, or ecological crime scenes.

Keywords:

Forensic microbiology; *Bacillus subtilis*; Cefepime; Antibiotic footprint; Microbial trace evidence; Medicolegal marker

1. Introduction

Microbial evidence has emerged as a powerful tool in forensic science, providing critical insights into postmortem intervals, geolocation, and event reconstruction [1,2]. Microorganisms recovered from soil, decomposing remains, dust, and clinical waste serve as durable biological trace evidence [3,4]. However, the integrity of such evidence is increasingly compromised by anthropogenic contaminants, particularly antibiotics, which are extensively used in healthcare, agriculture, and livestock farming [5,6]. The discharge of these antimicrobial agents into the environment via medical waste, pharmaceutical runoff, or improper disposal can suppress or permanently alter indigenous microbial populations [7]. From a forensic perspective, this suppression may erase vital microbial evidence, while the specific phenotypic changes induced in surviving bacteria could themselves hold evidentiary value.

Cefepime, a fourth-generation cephalosporin, exhibits broad-spectrum bactericidal activity by inhibiting bacterial cell wall synthesis through binding to penicillin-binding proteins (PBPs) [8]. Its extensive use in hospital settings, particularly for treating severe infections, makes it a relevant compound in investigations of hospital-acquired infections, medical negligence, or deaths occurring under clinical supervision [9]. *Bacillus subtilis*, a Gram-positive, endospore-forming bacterium, is ubiquitous in soil, dust, and clinical environments [10]. Its ecological resilience, predictable growth patterns, and non-pathogenic nature render it an ideal model organism for forensic microbiological studies [11].

We hypothesize that exposure to cefepime induces specific, measurable phenotypic changes in *B. subtilis* that can serve as a durable **antibiotic footprint**-a forensic marker indicative of prior antibiotic presence. This study aims to: (i) isolate and characterize *B. subtilis* from a forensic-relevant environment (hospital wastewater), (ii) quantify its sensitivity to cefepime using standardized microbiological assays, and (iii) conceptualize these antibiotic-induced alterations as a novel microbial marker for forensic reconstruction. By integrating principles of antimicrobial pharmacology with trace evidence analysis, this work seeks to expand the interpretive toolkit available to forensic investigators.

2. Materials and Methods

2.1. Sample Collection and Bacterial Isolation

Environmental wastewater samples were collected aseptically from liquid waste disposal sites of a tertiary-care hospital in Central India. Samples were transported in sterile containers and processed within two hours. A serial dilution series (10^{-1} to 10^{-9}) was prepared in sterile distilled water. Aliquots (100 µL) from dilutions 10^{-5} to 10^{-7} were spread onto pre-poured Nutrient Agar (NA) and Luria Agar (LA) plates. Plates were incubated aerobically at 30°C for 24 to 48 hours. Distinct, rough, off-white colonies were selected and repeatedly streaked on fresh media to obtain pure cultures.

2.2. Culture Media

Nutrient Agar (NA): Peptone (5 g/L), NaCl (5 g/L), beef extract (3 g/L), agar (15 g/L), pH adjusted to 7.4.
Luria Agar (LA): Tryptone (10 g/L), yeast extract (5.4 g/L), NaCl (0.5 g/L), agar (15-20 g/L), pH 6.8.
Media were autoclaved at 121°C for 15 minutes and poured under laminar airflow.

2.3. Identification and Characterization

Colony Morphology: Observed for size, shape, color, elevation, and margin.
Gram Staining: Performed using crystal violet, iodine, ethanol decolorizer, and safranin counterstain. Smears were examined under oil immersion (1000× magnification).
Biochemical Tests: A standard panel was conducted:

Carbohydrate fermentation (lactose broth with phenol red indicator)

IMViC tests: Indole production (Tryptone broth with Kovac's reagent), Methyl Red (MR), Voges-Proskauer (VP) (using MR-VP broth), and Citrate utilization (Simmons' Citrate Agar).

2.4. Antibiotic Sensitivity Testing

Disk Diffusion Assay: A 0.5 McFarland standard suspension of the *B. subtilis* isolate was lawn-cultured on Mueller-Hinton Agar. A commercial cefepime disk (30 µg) was placed on the agar surface. Plates were incubated at 37°C for 18 to 24 hours, and the diameter of the inhibition zone was measured in millimeters.
Broth Dilution Method: A stock solution of cefepime (100 µg/mL) was prepared in sterile distilled water. A dilution series in nutrient broth yielded final antibiotic concentrations of 2-58 µg/mL (corresponding to 0.2-5.8 mL of stock in a 10 mL total volume). Each tube was inoculated with a standardized *B. subtilis* inoculum and incubated at 37°C for 24 hours. Growth was assessed visually and by measuring optical density at 600 nm.

3. Results

3.1.Isolation and Identification of *Bacillus subtilis*

The isolated strain formed large, rough, off-white colonies with irregular edges on both NA and LA, consistent with typical *B. subtilis* morphology. Microscopic examination of Gram-stained cells revealed Gram-positive, rod-shaped bacilli, often occurring in chains. Biochemical profiling conclusively identified the isolate as *Bacillus subtilis* (Table 1).

Table 1: Biochemical characterization of the isolated bacterium

Test	Observation	Result for *B. subtilis*
Gram stain	Purple rods	Positive
Carbohydrate fermentation	No acid production (red medium)	Negative
Indole production	No cherry-red ring	Negative
Methyl red	Yellow color (no change)	Negative
Voges-Proskauer	Red color development	Positive
Citrate utilization	Growth with blue color change	Positive

3.2. Antibiotic Sensitivity Profile

Disk Diffusion: Cefepime produced a clear, circular inhibition zone with a diameter of 10 mm, indicating susceptibility.

Broth Dilution: No visible turbidity was observed in any test tube across the entire concentration range (2-58 µg/mL), confirming complete growth inhibition of *B. subtilis* by cefepime at these concentrations.

4. Discussion

This study demonstrates that cefepime induces a pronounced and easily detectable phenotypic change, complete growth inhibition, in *B. subtilis*. This effect serves as a clear **antibiotic footprint**. The consistent results from both qualitative (disk diffusion) and semi-quantitative (broth dilution) methods underscore the reliability of this microbial response as an indicator of antibiotic exposure.

The forensic utility of this finding is multi-fold. First, *B. subtilis* is an environmental sentinel; its presence in soils, dust, and clinical settings is well-documented [12]. Its hardy endospores ensure long-term survival, making it a persistent component of the microbial trace evidence pool [13]. Second, the specific inhibition by cefepime is directly linked to the antibiotic's mechanism of action (cell wall synthesis inhibition), which is characteristic of β-lactam antibiotics [14]. In a forensic context, the recovery of environmental samples containing *B. subtilis* populations that show this specific growth suppression pattern could indirectly suggest recent contamination with a β-lactam antibiotic, with cefepime being a prime candidate in healthcare-associated scenarios.

This concept extends beyond mere presence or absence. The degree of inhibition (e.g., zone size, minimum inhibitory concentration) could potentially be correlated with antibiotic concentration or exposure time, offering a semi-quantitative dimension to scene reconstruction [15]. Furthermore, creating a database of footprint profiles for different antibiotics against a panel of common environmental bacteria could allow forensic experts to identify specific classes of antimicrobials present at a scene.

Limitations of this pilot study include its use of a single bacterial species and antibiotic under controlled laboratory conditions. Future research must validate this concept with complex environmental samples, mixed microbial communities, lower antibiotic concentrations, and a wider range of antimicrobial agents [16]. Molecular techniques, such as quantifying changes in gene expression or sporulation rates in response to antibiotics, could provide more sensitive and specific footprints [17].

5. Conclusion

The interaction between cefepime and *Bacillus subtilis* produces a distinct, measurable antibiotic footprint in the form of complete growth inhibition. This work provides proof-of-concept for using targeted microbiological assays to detect evidence of antibiotic exposure in forensic environments. By treating specific microbial responses as trace evidence, forensic investigators can gain an additional layer of interpretive power, particularly in complex cases involving healthcare settings, allegations of neglect, or unexplained deaths where antibiotic administration is a factor. Integrating forensic microbiology with antimicrobial pharmacology opens a promising frontier for medicolegal science.

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