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Article

Autoclaved But Not Safe: Persistence of bla_{CTX-M} and bla_{TEM} Genes Encoding Extended Spectrum β -Lactamase in Autoclaved Microbiological Waste Destined for Municipal Sewage Disposal in Mwanza, Tanzania

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Abstract

Background: Antimicrobial resistance (AMR) is a growing global public health threat, and effective treatment of microbiological waste is essential in preventing the spread of resistance genes into the environment. Autoclaving is commonly used in healthcare and laboratory settings to sterilize microbiological waste, ensuring the destruction of viable bacteria. However, the fate of antimicrobial resistance genes (ARGs), such as bla_{CTX-M} and bla_{TEM}, following autoclaving is not well understood. To fill this critical gap, this study determined the PCR stability of AMR encoding genes from autoclaved microbiological waste in Mwanza, Tanzania. **Methods:** A laboratory-based experimental study was conducted between May and August 2024 at the Catholic University of Health and Allied Sciences (CUHAS). Standard and resistant *E. coli* strains carrying bla_{CTX-M} and bla_{TEM} genes were subjected to autoclaving at 121°C and 0.1 MPa for varying duration. Bacterial viability was assessed through sub-culturing on MacConkey agar, while the presence of ARGs was evaluated using multiplex PCR. **Results:** Although autoclaving completely inactivated the bacteria, as indicated by the absence of growth on culture media, both bla_{CTX-M} and bla_{TEM} genes remained detectable even after 30 minutes of treatment. These findings highlight the persistence of ARGs in treated waste and call for a re-evaluation of current biosafety protocols.

Keywords: antimicrobial resistance; antimicrobial resistance genes; autoclaving; ESBL; bla_{CTX-M}; bla_{TEM}

1. Introduction

The spread and persistence of antimicrobial resistance (AMR) remains to be a global health threat, a review article on AMR estimated that approximately 10 million people will die annually due to AMR related cases by the year 2050 if this problem is not addressed[1,2]. The environment can be contaminated by pathogenic bacteria and AMR encoding genes from clinical waste turning it into a reservoir for future exogenous infections which significantly contributes to this problem[3,4].

Healthcare settings and teachings institutions that have a microbiology laboratory often produce infectious wastes ranging from infectious clinical samples to bacterial cultures. These wastes are often treated before disposal by autoclaving at 121°C and 0.1 MPa for 30 minutes which ensures the total killing of most bacterial pathogen, thereafter; the autoclaved waste is discarded into the municipal sanitary sewage system for further treatment and recycling[5–7]. Poor handling and disposal of microbiological wastes poses a risk of introducing AMR encoding genes and pathogens into the environment via the wastewater/sewage water system(s)[8,9].

Within the environment free naked AMR encoding genes from killed clinical isolates can be transferred to environmental bacteria via horizontal gene transfer (HGT) mechanisms such as conjugation, transformation, and transduction. The amplification of AMR in environmental bacteria niches intensifies the global burden of AMR, as it can be a reservoir for exogenous infections by multi drug resistant (MDR) bacteria which are often deadly and costly[10–12].

Autoclaving is a practice employed by microbiology laboratories and hospitals for the treatment of microbiological waste(s) before disposal. However, the stability status of AMR encoding genes (DNA fragments) carried by these pathogens is not well understood which poses a risk of introducing viable AMR genes from clinical pathogens into the environment which can later be assimilated by environmental bacteria leading to the propagation of AMR burden. This information is crucial to inform the effective public health interventions and environmental policies. Therefore, this study was designed to determine the stability/integrity status of AMR encoding genes following autoclaving of the microbiological waste in Mwanza, Tanzania.

2. Materials and Methods

Study Design, Duration and Setting

This was a laboratory based experimental study conducted between May and July of 2024 at the microbiology and immunology research laboratory of the Catholic University of Health and Allied Sciences (CUHAS).

Study Population and Data Collection

This study used fresh cultured wild type *E. coli* (ATCC 25922) control strains and in-house prepared extended-spectrum beta-lactamase (ESBL) producing *E. coli* carrying bla_{CTX-M} and bla_{TEM} genes which were available and stored at -80°C at the CUHAS microbiology laboratory. The *E. coli* samples were then aliquoted into 18 parts each (for *E. coli* ATCC 25922 and ESBL *E. coli*) using cryovial tubes before autoclaving. A research book was used to record experimental information such as temperature, autoclaving time and all other laboratory procedures.

Recovery of Bacteria Isolates, Autoclaving and PCR Amplification

E. coli ATCC 25922 and *E. coli* carrying bla_{CTX-M} and bla_{TEM} genes were retrieved out of -80°C, the vials were then left for 30 minutes at room temperature so as to allow thawing, thereafter; the isolates were sub-cultured onto MacConkey agar (OXOID, Hampshire, United Kingdom) supplemented with 2µg cefotaxime (MCA-C) for ESBL *E. coli* while the wild-type *E. coli* was sub-cultured onto plain MCA and incubated at 35–37°C for 18–24 hours to obtain pure fresh colonies.

A loopful (10µl) of fresh pure cultured *E. coli* ATCC 25922 and *E. coli* (carrying bla_{CTX-M} and bla_{TEM}) genes was obtained and transferred into two separate vials containing sterile normal saline to form a suspension (this process was repeated for 18 times for each isolate). Each aliquot was then subjected to autoclaving at a temperature and pressure settings of 121°C and 0.1 MPa (1 bar) respectively, for 5, 10, 15, 20, 25 and 30 minutes respectively using a commercial autoclave (YX-18LM-50A; Shanghai Boxun Medical Biological Instrument Corp., China). The experiment was repeated 3 times. Each autoclaved vial was then subjected to centrifugation at 13200 revolution per minute (rpm) for 10 minutes followed by supernatant retrieval for PCR analysis.

PCR Amplification and Detection of ESBL Genes

Multiplex PCR assay was used for detection of ESBL genes: bla_{CTX-M} and bla_{TEM}. Briefly, 2 µL of each DNA sample was amplified in a 25 µL reaction PCR Eppendorf tube containing HotStarTaq® DNA polymerase master mix (QIAGEN, Hilden, Germany) and each primer at a reaction concentration of 200nM, the primer sequence, melting temperature and amplicon size have been indicated in (Table 1) below. PCR reactions were carried out in thermal cycler machine (T100™,

BIO-RAD, Kaki-Bukit, Singapore) which was conditioned at: initial denaturation at 95 °C for 10 mins; 35 cycles of denaturation at 94 °C for 40 sec, annealing at 58 °C for 40 sec, and extension at 68 °C for 2 mins; and a final extension at 68 °C for 10 mins. PCR amplicons were separated by the electrophoresis on 1% agarose gel with SYBR Safe dye and visualized under UV light.

Table 1. Primer sequence, melting temperature (TM) and amplicon size of primers used.

Primer name	Sequence (5'-3'direction)	TM in °C	Amplicon size in bp	Reference
TEM-F	TCGCCGCATACACTATTCTCAGAATGA	53	445 kb	[13]
TEM-R	ACGCTCACCGGCTCCAGATTAT	52		
CTX-M-F	ATGTGCAGYACCAGTAARGTKATGGC	54	593 kb	[14]
CTX-M-R	TGGGTRAARTARGTSACCAGAAYCAGCGG	58		

Autoclaved wastes were left to cool at room temperature for 15-20 minutes followed by inoculation onto MCA which were incubated at 35-37°C for 18-24 hours, this was done to assess the post autoclave viability of bacteria which was confirmed by observing the growth of lactose fermenting colonies.

Quality Control

Autoclave tape (Alpine Dental Inc ,NY, USA) was used to control autoclave performance by observing the formation of black stripes on the tape after each session. For multiplex PCR assay, internal quality control was performed by running each DNA sample in duplicates and for external quality control, in-house prepared *E. coli* (carrying blaCTX-M and blaTEM genes) and DNA free water were used as a positive and negative control respectively.

3. Results

Viability and Stability Status of Bacteria and ARG's After Autoclaving

A total of 18 experiments were performed in this study to check the stability status of ARG's namely blaCTX-M and blaTEM harbored by *E. coli*. All autoclaved *E. coli* were killed as they did not grow after being subcultured onto MCA, however; blaCTX-M and blaTEM DNA fragments were still stable at the end of each autoclave session regardless of autoclaving time as they were amplified and detected by multiplex PCR, Table 2 & Figure 1 below.

Table 2. Viability status of bacteria and ARG's at different autoclave times.

Autoclave time (minutes)	5'	10'	15'	20'	25'	30'
PCR detection of blaTEM	YES	YES	YES	YES	YES	YES
PCR detection of blaCTX-M	YES	YES	YES	YES	YES	YES
PCR detection of blaCTX-M & blaTEM from neg control	NO	NO	NO	NO	NO	NO

Growth of <i>E. coli</i> on MCA	NO	NO	NO	NO	NO	NO
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Figure 1. Image 1 Results of multiplex PCR amplification of genes blaCTX-M (593 bp) and blaTEM (445 bp) lanes; 1 to 18 = DNA samples of autoclaved *E. coli* resistant strains; L = 100 bp DNA ladder.

4. Discussion

This study utilized multiplex PCR to assess the integrity of DNA fragments of blaCTX-M and blaTEM genes after being autoclaved at different exposure times. The findings of this study showed that the DNA fragments of blaCTX-M and blaTEM genes maintained their integrity despite being exposed to prolonged autoclaving time confirming that ARG's are not completely destroyed following autoclaving. The findings are of critical concern about the potential risk of contaminating the environment once the waste is discarded into municipal sewage systems and therefore acting as an ARG's hotspot[11]. Our study findings are in agreement with those by Franco et al, 2020 who documented the persistence of DNA fragments from cell cultures despite being autoclaved for up to 30 minutes at 121°C and at a pressure of 1.1 atm[15].

Autoclaving is widely regarded as a robust method for sterilizing microbiological waste [16], this was evident in this study where all *E. coli* were completely killed after being autoclaved. However, its effectiveness in degrading genetic material, such as plasmid-encoded ARG's, has not been well-documented. This study showed that although autoclaving killed all bacterial cells, residual genetic material, including blaCTX-M and blaTEM, were still detected in the treated microbiological waste. Their persistence in sewage waste streams poses a risk for horizontal gene transfer (HGT) to other bacterial populations present in municipal sewage systems and in turn escalates the burden of AMR in the environment[11,17].

This study highlights the importance of re-evaluating current bio-safety protocols, especially in laboratories and healthcare settings where large volumes of microbiological waste are generated. The persistence of ARGs like blaCTX-M and blaTEM in autoclaved waste presents potential public health risk if such waste is not appropriately treated before being released into municipal sewage systems. Future research should focus on assessing the efficacy of alternative or additional treatments for

complete eradication of ARGs in autoclaved waste and evaluating the environmental impact of these genes in Wastewater Treatment Plants (WWTPs) and downstream the ecosystems.

This study did not assess if the plasmids remain intact following autoclaving, this can be an area of focus for future studies so as to unmask the true burden and potential risk of ARG's persistence in the microbiological waste.

5. Conclusions

Autoclaving achieved total killing of bacteria, however, failed to destroy the stability/integrity of ARG's DNA fragments in the microbiological waste. We recommend enhancement of waste treatment strategies that go beyond microbial inactivation so as to address the persistence of ARGs in autoclaved waste so as to mitigate their contribution to the global AMR crisis.

Supplementary Materials: The following supporting information can be downloaded at website of this paper posted on Preprints.org, Figure 1: Results of multiplex PCR amplification of genes blaCTX-M (593 bp) and blaTEM (445 bp); Table S1: Viability status of bacteria and ARG's at different autoclave times.

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Informed Consent Statement: Not applicable

Data Availability Statement: All data generated or analyzed during this study are fully included in this manuscript. No additional datasets were generated or analyzed beyond what is presented herein.

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Conflicts of Interest: The authors declare that they had no competing interests.

Abbreviations

The following abbreviations are used in this manuscript:

AMR	Antimicrobial Resistance
ARGs	Antimicrobial Resistance Genes
CTX-M	Cefotaxime-Munich
CUHAS	Catholic University of Health and Allied Sciences
DNA	Deoxyribonucleic Acid
ESBL	Extended Spectrum Beta Lactamase
HGT	Horizontal Gene Transfer
MCA	MacConkey Agar
MCA-C	MacConkey Agar-Cefotaxime
MDR	Multi Drug Resistance
PCR	Polymerase Chain Reaction
RPM	Revolutions per minute
SHV	Sulphydryl variable
TEM	Temoneira
WHO	World Health Organization

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