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Investigation of Impact of Antibiotics on Bacterial Strains Identified in Spring Water Located in Massa City, Libya

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Abstract

The emergence of antibiotic-resistant bacteria in drinking water sources poses a significant public health threat. This study examines the presence of antibiotic-resistant bacteria in four springs in Massa, Libya, and explores the impact of seasonal changes on antibiotic sensitivity. Water samples were collected from each spring during the autumn and winter of 2023, and antibiotic susceptibility tests were performed using different classes of antibiotics. The results revealed the presence of antibiotic-resistant strains of *Escherichia coli*, with significant interactions between seasonal changes, bacterial species, and antibiotic types. The study highlights the complex interplay between these factors, underscoring the need for a multifaceted approach to address antibiotic resistance in drinking water sources. The findings contribute to a deeper understanding of the dynamics influencing antibiotic resistance and provide valuable insights for the development of targeted strategies to mitigate this public health challenge.

Keywords: Spring Water, Antibiotic Resistance, Bacteria, Pathogens

INTRODUCTION

The emergence of antibiotic-resistant bacteria in sources of drinking water is an escalating global issue. Research conducted by (Coleman et al., 2013) revealed that private drinking water sources in Canada were found to be contaminated with antimicrobial-resistant strains of *Escherichia coli*, which underscores the potential dangers posed by waterborne pathogens. Furthermore, a study by (Babalola et al., 2021) examined bacterial isolates from household water distribution tanks in Ado-Ekiti, specifically analyzing patterns of antibiotic resistance and the profiles of plasmids present in these isolates.

In a related investigation, (Kothari et al., 2023) assessed the interplay between antibiotics and heavy metal arsenic, focusing on the horizontal gene transfer from multidrug-resistant clinical strains to antibiotic-sensitive environmental strains. This research provides valuable insights into the mechanisms that facilitate the dissemination of antibiotic resistance. The significance of maintaining low bacterial concentrations in drinking water systems is emphasized by the US EPA's National Primary Drinking Water Regulations, which aim to safeguard water quality (US EPA, 2024). Coliform bacteria, often utilized as indicators of water quality, exhibit behaviors akin to pathogens and are influenced by environmental conditions and treatment processes (McDonald, 2023).

The detection of multiple antibiotic-resistant bacteria in drinking water has raised significant concerns regarding their prevalence and the effectiveness of removal strategies within water systems (Sanganyado & Gwenzi, 2019). The improper use of antibiotics is a contributing factor to the rise of antibiotic-resistant bacteria, highlighting the necessity for responsible antibiotic usage and the avoidance of unnecessary prescriptions (Reitter et al., 2021). Reports of antibiotic-resistant bacteria and resistance genes in drinking water distribution systems further emphasize the urgent need for effective monitoring and mitigation strategies to address this public health challenge (Gu et al., 2022; Tiwari et al., 2022).

Recent investigations have focused on the seasonal variations in the quality of drinking water, particularly concerning bacterial populations. A study conducted by (Siedlecka et al., 2020) examined the occurrence of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) within a drinking water distribution system (DWDS) across different seasons, specifically summer and winter. This research was carried out in Wrocław, Poland, utilizing a full-scale DWDS that received water from two distinct water treatment plants (WTPs). The findings underscored significant spatiotemporal fluctuations in both antibiotic resistance and the composition of bacterial communities present in the DWDS.

Coliform bacteria, which are typically found in the intestines of humans and animals, have been observed to thrive in drinking water reservoirs, with concentrations exceeding 10,000 per 100 ml during certain seasonal periods (Wisconsin Department of Health Services, 2024). Research by (Reitter et al., 2021) identified the genera Lelliottia and Enterobacter as key players in the seasonal variations of coliform bacteria populations. Additionally, elevated turbidity levels in drinking water have been linked to a rise in pathogenic microorganisms, including viruses, parasites, and specific bacterial strains (US EPA, 2024; McDonald, 2023).

The detection of coliform bacteria in drinking water serves as an indicator of potential contamination risks, which may include the presence of more harmful pathogens (Michigan Department of Health and Human Services, 2024; Wisconsin Department of Health Services, 2024). Understanding the effects of seasonal changes, rainfall, and other environmental influences on bacterial presence and diversity in drinking water is crucial for maintaining water quality and safeguarding public health (MacDonald, 2016). This knowledge is essential for developing effective strategies to monitor and manage drinking water systems in the face of changing environmental conditions.

The emergence of antibiotic-resistant bacteria in sources of drinking water represents a critical public health challenge. It is essential to comprehend the pathways through which antibiotic resistance disseminates, to conduct thorough assessments of water quality, and to advocate for the judicious use of antibiotics as fundamental measures to tackle this problem. There is a pressing need for additional research to investigate the occurrence of antibiotic-resistant bacteria across various water sources and to evaluate the efficacy of strategies aimed at reducing their prevalence. Moreover, further studies are required to gain insights into the factors influencing seasonal variations in water quality and to formulate effective approaches for monitoring and controlling bacterial contamination in drinking water supplies.

In the current study, we aim to explore and assess the presence of antibiotic-resistant bacteria within the environmental context of four springs located in the Massa region. This study seeks to elucidate the impact of antibiotics on *Escherichia coli* and various other bacterial species.

MATERIALS AND METHODS

Study Area

Massa is a small city situated in eastern Libya, approximately 10 kilometers to the west of Beida. The Green Mountain region, known for its elevated terrain and forested landscapes, is located in northeastern Libya. This area is distinguished by its significant elevation compared to much of the country, as well as its notably high levels of precipitation. Furthermore, the fertile soil found in this region is conducive to agricultural activities, enhancing its value for farming endeavors.

Collecting of samples

Water samples were collected from four distinct springs, with three replicate samples gathered from each site during the autumn and winter seasons of 2023 in Massa city. In total, twelve samples were obtained and analyzed. The samples were stored in plastic containers with ice packs to maintain a cool temperature during transport, ensuring they did not freeze. Following collection, the samples were delivered to the microbiology laboratory for further analysis.

Growth media

The study employed plate count agar as the medium for culturing bacterial cells. To identify coliform bacteria present in water samples, lactose broth was utilized. For the isolation, enumeration, and differentiation of Enterobacteriaceae, Eosin Methylene Blue (EMB) agar was implemented. Additionally, Salmonella Shigella agar (SS agar) was used specifically for the isolation of S*almonella* and *Shigella* species. Finally, ECD agar served as the medium for isolating *E. coli.*

Heterotrophic plate count (HPC)

The standard plate count method, a foundational technique in microbiology for quantifying microorganisms, remains one of the most prevalent approaches utilized in the field. Another significant method for assessing the bacteriological quality of drinking water is the heterotrophic plate count (HPC) test. In this procedure, water samples are serially diluted in normal saline (8.5 g/l NaCl solution) up to 10-9 by serial dilution method. Subsequently, 0.1 ml from each dilution is spread onto nutrient agar plates, with three replicates prepared for each dilution. The plates are then incubated at 37 °C for a duration of 24 to 48 hours, after which the average number of colonies is determined and expressed as colony-forming units (CFU) per 100 µl.

Enumeration of bacteria most probable number (MPN)

The Most Probable Number (MPN) counts are regarded as statistically reliable estimates derived from the cultivation of several sample volumes, typically five, or their respective dilutions. The MPN methodology was employed to quantify coliform and fecal coliform bacteria through a series of tests. In the presumptive test phase, 10 ml, 1 ml, and 0.1 ml (from a 1:10 dilution) of water samples were inoculated into three sets of sterilized test tubes. Each set comprised five test tubes containing 9 ml of lactose broth and an inverted Durham tube, followed by incubation at 37°C for a duration of 24 to 48 hours. After the initial 24-hour incubation, the test tubes were assessed for gas production, as coliform bacteria generate gas from lactose, which becomes trapped in the inverted Durham tube. The number of positive tubes exhibiting gas production was then counted, allowing for the determination of MPN using a standard reference table.

In the confirmed test phase, 10 µl samples were taken from the positive presumptive tests and streaked onto Eosin Methylene Blue (EMB) agar plates, which were subsequently incubated at 37°C for 24 to 48 hours. For the completed test, lactose broth was inoculated with the positive confirmed test samples and incubated at 44.5°C for 24 to 48 hours. After this incubation period, the test tubes were again examined for gas production, and the positive tubes were counted to ascertain the MPN from the standard table. Additionally, 10 µl samples from the positive completed tests were streaked onto EMB plates and incubated at 37°C for 24 to 48 hours. To confirm the identity of the bacteria, differential staining, specifically Gram staining, was performed to verify that the isolates were gram-negative and rod-shaped.

The identification of bacterial isolates was conducted using the Phoenix TM identification panel system, which allows for species-level identification. All procedures for setting up the panels were strictly adhered to according to the manufacturer's guidelines, as outlined in the works of (Salomon et al., 1999). This systematic approach ensures accurate identification and characterization of the bacterial isolates, contributing to a better understanding of the microbial composition in the tested samples (Salomon et al., 1999).

The primary microorganisms present in drinking water samples were characterized through a series of biochemical assays. Isolates exhibiting distinct morphological colony types were carefully selected from plate count agar and subsequently subcultured. These cultures were preserved in a refrigerator at 4°C until they were required for additional testing. The biochemical assays performed included Gram staining, catalase testing, and oxidase testing (Salomon et al., 1999).

For bacterial isolation, a loopful of enriched media was inoculated onto various culture media, such as MacConkey and Eosin-Methylene Blue agar, to isolate *E. coli*, *Klebsiella*, *Enterobacter*, *Hafnia*, *Morganella*, and *Citrobacter*. Additionally, *Salmonella Shigella* agar was employed for the isolation of *Salmonella* and *Shigella* species, while Thiosulfate Citrate Bile Salt agar was utilized for isolating *Vibrio* species (Murray et al., 2006).

The Antibiotic susceptibility testing

Antibiotic susceptibility (AST) is performed using different classes of antibiotics: Ampicillin (30 micrograms), Amoxicillin (30 micrograms), Penicillin (30 micrograms), Colexilin (10 micrograms). Prepare the Muller Hinton agar medium, sterilize it, and pour it in sterile conditions. Leave it to solidify for 1 minute until the activity of the bacteria is stimulated, or prepare it in water 15 - Prepare a bacterial suspension on the nutrient broth tube and incubate it for 2 hours in lukewarm or saline water 0.9%. Using a cotton swab, immerse it through the tube inoculated with the bacterial suspension and wipe the entire plate clockwise. Place the tablets to be the ability of the antibiotic to inhibit bacteria on the surface of the plate is determined it is done by Measuring the distance in millimeters (mm) between the boundaries of the growing colony around the antibiotic-soaked disk.

The antibiotics are selected based on their resistance to bacterial strains. The antibiotic susceptibility of *E.coli* growth at 44.5°C will be determined by the disk diffusion method (JC & Turck, 1966). Four antibiotic susceptibility test disks will be obtained penicillin 10 units, Amoxicillin 25 µg , streptomycin 25 µg, tetracycline 50 µg. 0.1 ml of fresh culture will be spread on Muller medium plates using a sterile glass grater and then allowed to dry for 10 minutes. The four antimicrobial disks will be placed on the bacterial film (one in each quadrant of the plate). Three plates will be used for the test and a plate without any antibiotics will be used as a control. The plates will be incubated (not inverted) at 25°C overnight. The zone of inhibition for each antibiotic will be measure and the average calculated.

The data collected throughout the research was meticulously input into the R file for statistical analysis. Subsequently, the analysis of variance (ANOVA) was employed to examine the influence of different variables and their interactions on the measured inhibition zones in millimeters, which serve as indicators of antibiotic sensitivity and facilitate the identification of the most effective antbiotic.

RESULTS

E. coli and coliform bacteria are frequently employed as indicator organisms to assess fecal contamination in water sources. Nevertheless, the mere absence of these bacteria does not guarantee that the treated water is devoid of pathogens.

The initial analysis of the comprehensive model, which encompassed all relevant factors, revealed that isolation did not emerge as a significant variable (F=0.563, p-value=0.57). Consequently, this factor was entirely excluded from the model, leading to its reconstruction without isolation.

The relationships depicted in Figure (1) are effectively illustrated through grouped box plots, which categorize the data into five distinct groups based on the types of antibiotics. Each group encompasses various bacterial species while simultaneously accounting for seasonal variations. These elements collectively exert a significant influence on antibiotic sensitivity, which is quantitatively represented by the inhibition zones measured in millimeters.

The revised model subsequently highlighted the pronounced influence of the remaining factors, which included seasonal variations (F=6.87, p-value=0.009), bacterial species (F=95.370, p-value < 2e-16), and the specific types of antibiotics administered (F=11.69, p-value=4.11e-09) that were highly significant factors in the model.

Furthermore, the analysis underscored the significant interactions between seasons coliform and fecal coliform and each bacterial species (F=24.1, p-value=1.25e-06), as well as between seasons and the types of antibiotics used (F=7.99, p-value=2.89e-06). These findings indicate that the effects of seasonal changes are not uniform across different bacterial species or antibiotic types, suggesting a complex interplay that warrants further investigation. Additionally, the interaction between bacterial species and antibiotic types also demonstrated a notable effect (F=19.11, p-value=1.02e-14), reinforcing the importance of considering these variables in tandem.

The results further illustrated the significance of interactions among all variables collectively (F=7.66, p-value=5.28e-06), indicating that the relationships between the factors are not merely additive but rather synergistic. This complexity highlights the necessity for a multifaceted approach to understanding the dynamics at play in the study. Overall, the findings emphasize the critical role of both individual factors and their interactions in shaping the outcomes of the research, thereby providing a more nuanced understanding of the underlying mechanisms involved.

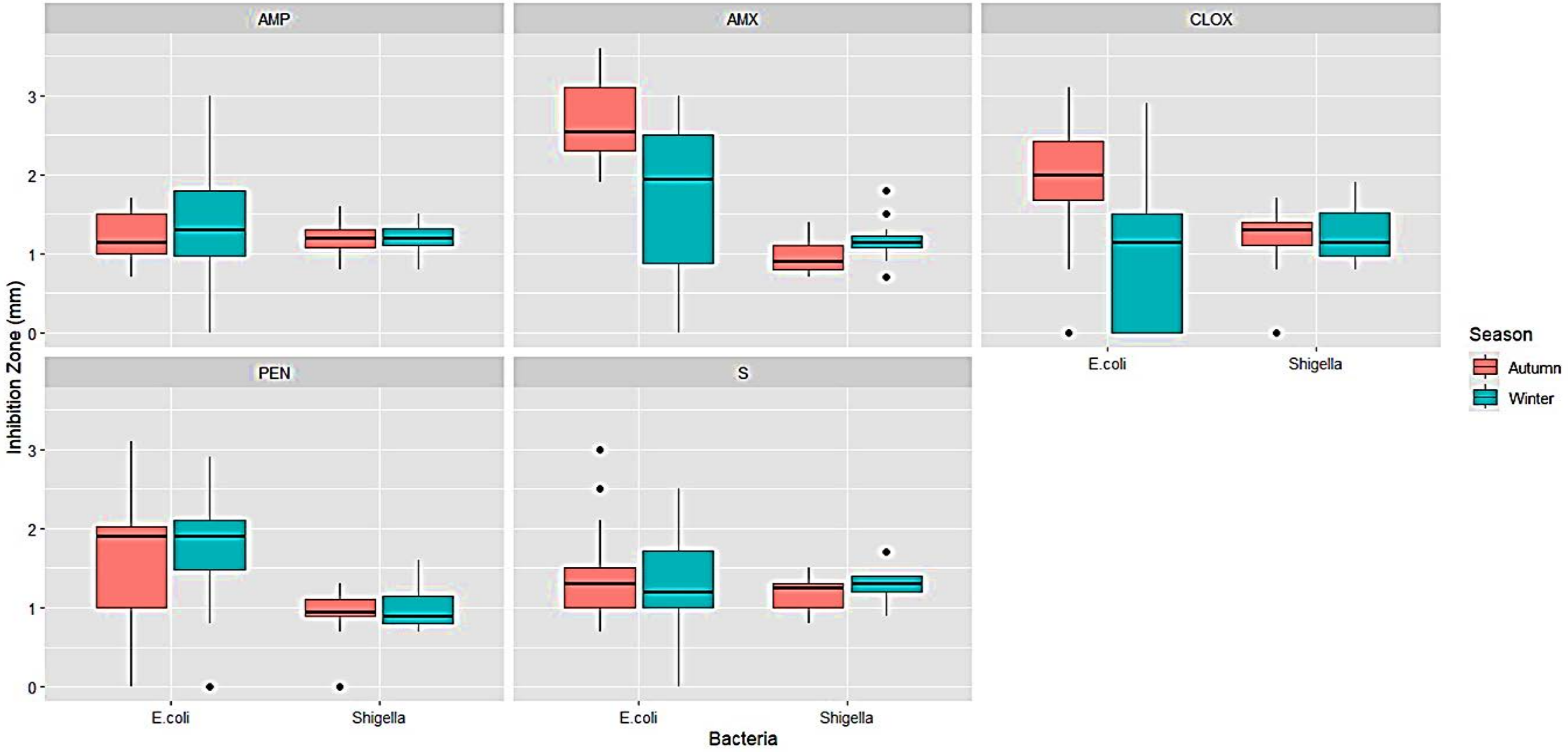

Figure (1).. Illustrates grouped box plots, which organize the data into five separate categories corresponding to different types of antibiotics. Each category includes a range of bacterial species and also considers seasonal fluctuations. Together, these factors play a crucial role in determining antibiotic sensitivity, which is quantitatively expressed through the measurement of inhibition zones in millimeters.

DISCUSSION

The extensive model examined the intricate relationships among antibiotics, various bacterial species, seasonal fluctuations, and the sensitivity of bacteria to antibiotics. The preliminary analysis indicated that isolation did not play a significant role, resulting in its removal from the model. The updated model underscored the substantial impact of seasonal changes, bacterial species, and the types of antibiotics on their sensitivity.

Noteworthy interactions were identified between the seasons and bacterial species, the seasons and antibiotic types, as well as between bacterial species and antibiotic types. These observations point to a complex interrelationship among these factors, suggesting that their influences are not merely additive but rather synergistic.

The findings of the research indicated the existence of antibiotic-resistant strains of E. coli in the drinking water sources of Massa, paralleling similar findings reported in Canada by Coleman et al. The data aligned with the observed seasonal variations in bacterial proliferation, which was notably higher during the summer months compared to winter. However, the differences in bacterial sensitivity to antibiotics across seasons were not as pronounced as those documented in the study by (Siedlecka et al., 2020)., which highlighted significant variations in antibiotic efficacy on bacteria within the drinking water distribution system (DWDS) during different seasons, particularly between summer and winter in Wrocław, Poland.

This study contributes to a deeper understanding of the complex interactions influencing antibiotic resistance, particularly in the context of environmental factors such as seasonal changes. The results underscore the necessity for a comprehensive approach to address the multifaceted nature of antibiotic sensitivity and resistance. By elucidating these dynamics, the research provides valuable insights that can inform the development of targeted strategies aimed at mitigating antibiotic resistance in various ecological contexts.

CONCLUSION

The results highlight the existence of antibiotic-resistant E. coli strains in drinking water sources, with seasonal variations in bacterial proliferation and antibiotic sensitivity. This research underscores the need for a multifaceted approach to understanding the complex dynamics of antibiotic resistance, emphasizing the importance of considering environmental factors, bacterial species, and antibiotic types in tandem. The findings provide valuable insights for the development of targeted strategies to mitigate antibiotic resistance in various ecological contexts.

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