Molecular Detection of Antibiotic-Resistant Genes in Pseudomonas aeruginosa from Nonclinical Environment: Public Health Implications in Mthatha, Eastern Cape Province, South Africa

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Evaluation of resistant profiles and detection of antimicrobial-resistant genes of bacterial pathogens in the nonclinical milieu is imperative to assess the probable risk of dissemination of resistant genes in the environment. This paper sought to identify antibiotic-resistant genes in Pseudomonas aeruginosa from nonclinical sources in Mthatha, Eastern Cape, and evaluate its public health implications. Samples collected from abattoir wastewater and aquatic environment were processed by membrane filtration and cultured on CHROMagar™ Pseudomonas medium. Species identification was performed by autoSCAN-4 (Dade Behring Inc., IL). Molecular characterization of the isolates was confirmed using real-time polymerase chain reaction (rPCR) and selected isolates were further screened for the possibility of harboring antimicrobial resistance genes. Fifty-one Pseudomonas species were recovered from abattoir wastewater and surface water samples, out of which thirty-six strains were Pseudomonas aeruginosa (70.6%). The P. aeruginosa isolates demonstrated resistance to aztreonam (86.1%), ceftazidime (63.9%), piperacillin (58.3%), cefepime (55.6%), imipenem (50%), piperacillin/tazobactam (47.2%), meropenem (41.7%), and levofloxacin (30.6%). Twenty out of thirty-six P. aeruginosa displayed multidrug resistance profiles and were classified as multidrug-resistant (MDR) (55.6%). Most of the bacterial isolates exhibited a high Multiple Antibiotic Resistance (MAR) Index ranging from 0.08 to 0.69 with a mean MAR index of 0.38. In the rPCR analysis of fifteen P. aeruginosa isolates, 14 isolates (93.3%) were detected harboring blaSHV, six isolates (40%) harbored blaTEM, and three isolates (20%) harbored blaCTX-M, being the least occurring ESBL. Results of the current study revealed that P. aeruginosa isolates recovered from nonclinical milieu are resistant to frontline clinically relevant antipseudomonal drugs. This is concerning as it poses a risk to the environment and constitutes a public health threat. Given the public health relevance, the paper recommends monitoring of multidrug-resistant pathogens in effluent environments.

1. Introduction

Antimicrobial resistance (AMR) is a public health crisis in both human and veterinary medicine [1, 2]. The irrational use of antibiotics in both human medicine and animal production for growth-promoting purposes, metaphylaxis, and prophylaxis has fueled the proliferation and spread of antibiotic-resistant bacteria and resistance genes resulting in aggravated public health and environmental risks [3–5]. The threat posed by AMR to human health is particularly concerning in low- to middle-income countries (LMICs). This is due to the higher possibility of community-acquired
resistant infections, the high transmissible disease burden in the general populace, and poor access to health services [6], thereby leading to increased morbidity, prolonged hospitalization, and increased healthcare costs, thus exerting an economic burden on family units and the society [7].

*Pseudomonas aeruginosa* (P. aeruginosa), an environmental bacterium, can be found in various terrestrial and aquatic habitats. This is due to its extensive metabolic versatility that enhances its distribution, proliferation, and survival despite adverse physical and chemical conditions, thereby enhancing its ecological success and potential threat to public health [8, 9]. *P. aeruginosa* is an opportunistic human pathogen competent for a wide array of infections including respiratory tract, blood, urinary tract, and skin infections [10]. This competence for infections and ability for antibiotic resistance has made the organism to be recognized as a threat to public health [9, 10]. *P. aeruginosa* make use of both intrinsic and acquired resistance mechanisms. The acquired resistance mechanism is facilitated by mobile genetic elements through horizontal gene transfer (HGT). This poses a greater risk to human health because of the ease of expression and dissemination [11].

Antibiotic usage in the agricultural sector has compounded the spread of resistance in the human community due to the environmental dissemination of transferable resistance genes [12]. Abattoir wastes have the ability to contaminate both surface and groundwater. Discharge from abattoir effluent contaminates the environment by introducing pathogens that can affect land and water qualities, thus endangering human, animal, and aquatic ecosystem’s health and constituting a menace to human health and environmental safety [13]. The possibility of pathogens from abattoir effluent and animal waste reaching or discharging into water bodies and developing resistance to antibiotics in human infection is a concern. This is because these infections are usually difficult to treat and often result in morbidity and mortality especially in the most vulnerable members of the community [14].

Several studies have investigated the prevalence and detection of extended-spectrum β-lactamase- (ESBL-) and metallo β-lactamase- (MBL-) producing *P. aeruginosa* isolated from clinical samples [15, 16], but there is a scarcity of data on the occurrence in nonclinical samples. The need to identify and monitor antibiotic-resistant genes in water bodies and wastewater is necessary to assess their potential risk to human health. The present study investigated the prevalence of antimicrobial resistance and antibiotic-resistant genes in nonclinical strains of *Pseudomonas aeruginosa* in Mthatha, Eastern Cape, and highlighted its public health implications.

### 2. Materials and Methods

#### 2.1. Study Design and Setting. A cross-sectional study was conducted within the period of January to June 2019. The study sites were Umzikantu Red Meat Abattoir, Zimbane Mthatha, Mthatha River, and Mthatha Dam. Umzikantu Red Meat Abattoir is a high-throughput abattoir located in Zimbane location in Mthatha, Eastern Cape. It is the only operational red meat abattoir serving Mthatha and its environs in the OR Tambo District Municipality. It is certified and has the capacity to slaughter 50 units of animals on a daily basis. One unit equals one cow/ox or two calves or six sheep or four pigs. The abattoir is open to the public and offers slaughter and cutting services at an affordable price. It also doubles as a wholesaler supplying meat to butcheries, supermarkets, and hospitals.

Mthatha Dam (31°33′2″S 28°44′24″E Coordinates) is an earth-fill type dam on the Mthatha River, located close to Mthatha Town, in the OR Tambo District Municipality of the Eastern Cape. This dam was built in 1977 to serve municipal and industrial purposes. The Department of Water and Sanitation oversees the affairs of the dam. The catchment area of the dam is 886 km² with a surface area measuring 25.42 km². It has a height of 38 m with a length measuring 620 m. The reservoir capacity of the dam is 253,674,000 m³.

#### 2.2. Sample Collection. Abattoir wastewater samples: using standard methods for the examination of water and wastewater [17], 100 mL of abattoir effluents was taken from two sampling points into sterile bottles appropriately labeled. All samples were stored in cooler boxes for transportation to the Medical Microbiology laboratory at Walter Sisulu University Mthatha, for further analyses, within 4 h of sample collection.

Aquatic environment samples: water samples from the Mthatha Dam were collected aseptically in sterile 100 mL Duran Schott glass bottles from different sampling points by directly dipping the bottles about 20 cm below the surface of the water. After collection, the samples were stored in iced cooler boxes, transported to the laboratory, and kept at about 4°C until analyzed.

#### 2.3. Bacteriological Analysis. The membrane filtration method was used for isolation according to standard methods [17]. For all samples, three volumes of 100 mL were filtered [10] through a 0.45 μm pore sized gridded membrane filter (Whatman Laboratory Division, Maidstone, England) using a water pump (model Sartorius 16824). Filters were removed and aseptically placed on CHROMagarTM *Pseudomonas* (CHROMagarTM; Paris, France) agar plates ensuring that no air bubbles were trapped. All media were prepared according to the manufacturers’ instructions (CHROMagarTM; Paris, France). Each sample was analyzed in triplicate. The plates were incubated aerobically at 37°C for 24–48 hours. Blue colonies which were characteristics of *Pseudomonas* spp. were subcultured to obtain pure cultures.

#### 2.4. Characterization of *Pseudomonas aeruginosa*. Blue colonies typical of *Pseudomonas* species on the chromogenic medium were subcultured on both Cetrimide agar and CHROMagar to get pure colonies. The characteristic grape-like odor was a useful marker of identification. Phenotypic tests such as Gram stain, oxidase test, and catalase test were performed [18]. Species identification was carried out using...
Gram-negative ID type 2 panels (Beckman Coulter, Inc., USA) of MicroScan autoScan-4 automated system (Dade Behring Inc., Deerfield, IL). Growth at 42°C [18] in an aerobic incubator was also used to confirm the identity of the P. aeruginosa isolates. All of the strains were stored at −80°C in 15% glycerol until further use.

2.5. Molecular Confirmation of Strains by rPCR. DNA Extraction: DNA was extracted from overnight colonies of a bacterial culture grown on Cetrimide agar. This was resuspended in Roche MagNA Pure Bacteria Lysis Buffer, vortexed briefly, heated at 95°C for 10 minutes, and pelleted by centrifugation at 13000 g for 10 minutes. Four hundred microliters were used as a specimen in the MagNA Pure Compact (MPC) System (Roche Applied Science, Indianapolis), using MPC Nucleic Acid isolation kit 1 according to the manufacturer’s instructions. Elution tubes containing 200 µL purified nucleic acids were stored at −80°C until further use. The LightCycler 2.0 instrument (Roche Applied Science, Germany) and Fast start LightCycler 480 HybProbes Master Kit (Roche Diagnostics, USA) were used for polymerase reaction. Specific primers and probes (Table 1) designed by TIB Molbiol (Germany) targeting the gene, species-specific gyrB, were amplified by singleplex real-time polymerase chain reaction (rPCR) following the protocol shown in Table 2.

2.6. Antimicrobial Susceptibility Testing. Antimicrobial susceptibility testing was performed by MicroScan autoScan−4 system using dehydrated broth microdilution method in the MIC Panel Type 44 (NM44) (Beckman Coulter, Inc. USA) following the manufacturer’s guidelines [20]. The following antibiotics were tested in the panels: amikacin, aztreonam, cefepime, cefazidime, ciprofloxacin, doripenem, gentamicin, imipenem, levofloxacin, meropenem, piperacillin/tazobactam, piperacillin, and tobramycin. MICs were analyzed and interpreted according to the recommended clinical breakpoints given in CLSI guidelines [21]. ATCC Quality control organisms used were P. aeruginosa ATCC 27853 and Escherichia coli ATCC 25922. Nonsusceptibility includes a combination of resistance and intermediate resistance. Multidrug (MDR) P. aeruginosa was defined as nonsusceptible to at least one agent in three or more antimicrobial categories according to Magiorakos et al. [22]. Multiple antibiotic resistance index (MARI) was calculated and interpreted for the isolates as described by Gufe et al. [23]. Briefly, it is described as the ratio of the number of antibiotics to which isolates were resistant (a), to the total number of antibiotics to which the isolates were exposed (b), that is, multiple antibiotic resistance index (MARI) = a/b. Bacteria having MARI >0.2 originate from high-risk sources of contamination where several antibiotics have been used, while MARI value ≤0.2 indicates strains from sources where antibiotics have seldom or never been used.

2.7. Molecular ESBL and MBL Detection by rPCR. Isolated P. aeruginosa colonies on Cetrimide agar and CHROMagar Pseudomonas were selected for genomic DNA extraction. Fifteen multidrug isolates were selected from the pool using a simple random sampling technique. Template DNA was extracted by MagNA Pure Compact (MPC) using MPC Nucleic Acid isolation kit according to the manufacturer’s instruction. Real-time PCR was carried out in the LightCycler 2.0 instrument (Roche Applied Science, Germany) using Fast start LightCycler 480 HybProbes Master Kit (Roche Diagnostics, USA). Specific primers and probes (Table 3) targeting the genes CTX-M, SHV, TEM, IMP, and VIM were amplified by singleplex rPCR using the same protocol described in Table 2. Primers were designed by TIB Molbiol (Berlin, Germany). rPCR assay was performed in 32 carousels using 20 µL capillaries with each capillary containing a total volume of 20 µL, including 2 µL of LightCycler FastStart DNA Master Hybridization Probes (Roche Diagnostics), 2 µL of primers (0.5 mM for each forward and reverse), 2 µL of the probe, 2.4 µL of MgCl2, 2 µL of extracted DNA, and water to make up the volume of 20 µL.

Absolute quantification was carried out using the LightCycler software 4.05. Data were obtained during the annealing period. Fluorescence was measured once every cycle immediately after the 60°C incubation (extension step). Fluorescence curves were analyzed with the LightCycler software, version 4.05. Results were expressed by determination of threshold cycle (Ct) value, which signified the cycle at which sample fluorescence became remarkably different from the baseline signal. Positive control strains used included Klebsiella pneumoniae ATCC 51503 (blaCTX-M), Klebsiella pneumoniae ATCC 700603 (blaESV), Escherichia coli NCTC 13351 (blaTEM), P. aeruginosa NCTC 13437 (blaVIM), and Escherichia coli NCTC 13476 (blaIMP). These were obtained from the National Institute of Communicable Diseases (NICD), Johannesburg, South Africa.

2.8. Statistical Analysis. All the data was entered into an Excel sheet and uploaded onto the SPSS software (version 23.0 IBM, Armonk, NY). The prevalence of multidrug-resistant (MDR) P. aeruginosa and their distribution from different sources (water and abattoir wastewater) were determined and expressed as percentages.

3. Results

3.1. Isolation and Antimicrobial Susceptibility Testing. During the period of study, fifty-one isolates of Pseudomonas species were recovered, out of which thirty-six isolates were P. aeruginosa (70.6%) and fifteen were P. fluorescens/putida (29.4%). P. aeruginosa was the predominant species, of which nineteen (52.8%) and seventeen (47.2%) originated from surface water and abattoir wastewater, respectively. Of these, the 36 strains of P. aeruginosa were selected for further confirmation. They were confirmed by the real-time amplification of the gyrB gene including the reference strain, ATCC 27853 (Figure 1). The results of antibiotic susceptibility testing of P. aeruginosa strains showed varying levels of resistance. Of the clinically relevant antibiotics in the panel, there was resistance to aztreonam (86.1%), cefazidime (63.9%), piperacillin (58.3%), cefepime (55.6%), imipenem
Table 1: Primer sequences for detection of gyrB genes.

| Primers       | Primers sequences (5′-3′) | Tm in °C | References |
|---------------|---------------------------|---------|------------|
| gyra forward primer | CCT GAC CAT CCG TCG CCA CAA | 55.3    | [19]       |
| gyra reverse primer   | CGC AGC AGG ATG CCG ACG CC | 53.1    |            |
| gyra probe 1          | FAM-CCG TGG TGG TAG ACC TGT TCC CAG ACC-BHQ |       | This study |
| gyra probe 2          | FAM-CCG TGG TGG TAG ACC TGT TCC CAG ACC-BBQ |       |            |

Table 2: rPCR cycle protocol.

| Protocol       | Temperature (°C) | Acquisition mode | Time | Ramp rate | Cycle |
|----------------|------------------|------------------|------|-----------|-------|
| Denaturation   | 95               | None             | 5 minutes | 4.4      | 1     |
| Quantification: Annealing | 95               | None             | 30 seconds | 4.4      | 45    |
| Extension      | 60               | Single           | 1 minute | 4.4      |       |
| Cooling        | 40               | None             | 30 seconds | 4.4      | 1     |

Table 3: Primer sequences for detection of blaCTX-M, blaSHV, blaTEM, blaIMP, and blaVIM genes.

| Primers       | Primers sequences (5′-3′) | Tm in °C | References |
|---------------|---------------------------|---------|------------|
| CTX-M forward primer | ATGAGYACCAGTAGAARGTKATGGC | 58.7    | [24]       |
| CTX-M reverse primer   | ATACCKCGGRTGCCGGGGRAT | 59.3    |            |
| CTX-M probe           | FAM-CCCAGACCTGGGAGACGAGAC-GG-BBQ | 70.2    |            |
| SHV forward primer    | TCCCATGATGACACTTTAAA | 56.8    | [25]       |
| SHV reverse primer    | TCCGCTGGCAGATGTTGGA | 58.6    |            |
| SHV probe             | FAM-TGCCCGGTGACAGACGAGCTGGAG-BBQ | 68.3    |            |
| TEM forward primer    | GCATCTTATGGAGATCAGTA | 56.6    | [25]       |
| TEM reverse primer    | GTTCGGATCTGTTTGTCAAGA | 57.7    |            |
| TEM probe             | FAM-CGATCTGTACCATGCAGATGTA-BHQ | 62.2    |            |
| IMP forward primer    | GGCGGGAATAGTGGCCTTA | 57.6    | [26]       |
| IMP reverse primer    | GCCGCTAGACCTTACCTTCCTTTTT | 59.3    |            |
| IMP probe             | FAM-CGATCTATCCCACTCAGATGTAATTAAAC-BHQ | 67.4    |            |
| VIM forward primer    | TGCGCCTCGGTCCAGTAGA | 59.0    | [26]       |
| VIM reverse primer    | TGAGGGGACGTGATACACAGAT | 58.5    |            |
| VIM probe             | FAM-CTCTTATCCCTTCTGGCTGCGCATTCC-BHQ | 67.6    |            |

4. Discussion

In this study, thirty-six isolates of P. aeruginosa were recovered from abattoir wastewater and surface water. In agreement with this study, Ighinosa et al. [27–29] have all reported the occurrence of P. aeruginosa from hospital drains, environmental, and wastewater networks from various parts of the world. The occurrence of this microorganism is a cause of concern given that it is an opportunistic human pathogen and can infect people whose immunity is compromised [30]. A similar study in Nigeria found that the discharge of effluents from abattoir directly into water bodies without prior treatment has triggered serious health risks subsequent to its contamination by bacteria [14].

The prevalence rate of P. aeruginosa (70.6%) seen in the current study is comparable to previous reports from Nigeria on water samples from fish pond sites and cattle waste, where P. aeruginosa was found to be the most prevalent with the highest occurrence rate of 62.8% and 71.5%, respectively, among other species [31, 32]. This possibly could be due to the physiological versatility and limited nutritional requirements that enable it to adapt in adverse conditions [33]. Likewise, in agreement with this...
study, a study carried out in Mafikeng in the North West Province of South Africa isolated *P. aeruginosa* from both drinking and surface waters [34]. However, contrary to our findings, a study carried out in Alice, Eastern Cape, South Africa, on wastewater samples found a lower occurrence rate of 11.1% [35]. This disparity is most likely due to different treatment processes used in water purification, or it can be assumed that wastewater treatment plant (WWTP) does not totally eliminate bacteria especially MDR strains since these organisms are resilient to the treatment processes and eventually play a role in the transmission and spread of antimicrobial resistance.

The resistance profiles of the isolates revealed 63.9% and 55.6% resistance to the second- and third-generation cephalosporin, respectively (ceftazidime and cefepime) and

**Table 4: Antibiotic resistance pattern of *P. aeruginosa* isolates.**

| Antibiotic     | No (%) resistant | No (%) susceptible |
|----------------|------------------|--------------------|
| Amikacin       | 6 (16.7)         | 30 (83.3)          |
| Aztreonam      | 31 (86.1)        | 5 (13.9)           |
| Ceftazidime    | 20 (55.6)        | 16 (44.4)          |
| Cefepime       | 23 (63.9)        | 13 (36.1)          |
| Ciprofloxacine | 8 (22.2)         | 28 (77.8)          |
| Doripenem      | 5 (13.9)         | 31 (86.1)          |
| Gentamicin     | 6 (16.7)         | 30 (83.3)          |
| Imipenem       | 18 (50)          | 18 (50)            |
| Levofloxacine  | 11 (30.6)        | 25 (69.4)          |
| Meropenem      | 15 (41.7)        | 21 (58.3)          |
| Piperacillin   | 21 (58.3)        | 15 (41.7)          |
| Piperacillin/tazobactam | 17 (47.2) | 19 (52.8) |
| Tobramycin     | 3 (8.3)          | 33 (91.7)          |

**Figure 1:** Amplification of gyr B (real time with LightCycler 2.0) in *P. aeruginosa* strains including reference strain ATCC 27853.

**Figure 2:** Mean MARI values in abattoir wastewater and aquatic *P. aeruginosa* isolates.
低耐药性对氨基糖苷类抗生素。观察到头孢菌素在目前的研究中 Antibiotics 可能不会是可选的,因此将决定在有限的治疗选择。本研究发现 16.7% 的菌株对氨基糖苷类药物、阿米卡星和 gentamicin。相似的低对氨基糖苷类药物、阿米卡星 (19%) 但更高的对 gentamicin (28.5%) 被报告由一个研究发布的埃及 [36]。然而,一个调高的抗药性对 gentamicin 79% 在 gentamicin 以 PCR 被检测 ESBL。这被检测在 14 菌株 (93.3%)。blaTEM 被检测在 40% 的菌株而的最少 CTX-M (20%)。这研究是在在埃及 [36]。这项变异能被以差异在在抗药性模式的氨基糖苷类抗生素 [38]。

对内酰胺酶包括 imipenem (50%) 和 meropenem (41.7%) 也在在目前的研究。这不被出料,因为的事实 carbapenem 代表一个最多的有效的和作为的最好的选项为了治疗 Gram-negative 感染特别是 MDR 感染。 carbapenem-resistant P. aeruginosa 菌株频繁的相关与一个更高的死亡率由于对酶 carbapenemase 传递的抗药性的一个和一个更高的可能的在广泛的蔓延的抗药性通过移动基因元素 [39]。对别的抗微生物在人类和兽医医学通常导致了对一个扩增的抗药性抗药性细菌 (ARB) 和抗药性-抗药性基因 (ARGs) 可以被移动到人类病原性细菌。这个转移最终 nullifies 抗药性的当前和传递的抗微生物,因此导致了治疗失败对一些生命威胁性的疾病 [40]。

在目前的研究,的流行率 55.6% MDR P. aeruginosa (MDRPA) 在非临床设定是高和 alarming。因此,这个研究证明了存在 MDR P. aeruginosa 在临床环境在的

| Table 5: Extended-spectrum β-lactamase (ESBL) and metallo-β-lactamase (MBL) gene types detected in nonclinical isolates of P. aeruginosa. |
|--------------------------------------------------|--|--------|
| Positive by PCR for ESBL | Number amplified | Total |
| (N = 15) | (%) |
| A. Single ESBL gene | | |
| blaSHV | 14 | 93.3 |
| blaTEM | 6 | 40.0 |
| blaCTX-M | 3 | 20.0 |
| blaVIM | 1 | 6.7 |
| blaIMP | 0 | 0 |
| B. Two or more ESBL genes | | |
| blaTEM + blaSHV | 6 | 40 |
| blaTEM + blaCTX-M | 3 | 20 |
| blaSHV + blaCTX-M | 3 | 20 |
| blaSHV + blaVIM | 1 | 6.7 |

Eastern Cape Province of South Africa。这个发现是一致于的报告的 Algammal 等人 [41] 与 55.5% MDR Strains of P. aeruginosa 从新鲜的鱼类样本。然而,Olga 等人 [42] 报告了一个低率的 MDRPA 为 32% 从水体环境。这种不适当的使用抗微生物药能传递的抗药性,因此引发抗药性治疗无效 [43]。

MAR indexing 是一个简化快速的方法区分抗微生物抗原从不同的来源,无论是从高风险的来源的污染,如抗生素在低风险的来源,如抗生素在高风险的来源的污染可能对人类产生潜在的不适 [45]。一个 MAR index >0.2 指出了抗药性 originate 从高风险的来源的污染 [46]。在目前的研究,的分析的 MAR index of the P. aeruginosa Strains showed 了全部的他们有 MAR index 上 0.2 (Figure 2)。Odjajare 等人 [47] 和 Gufe 等人 [23] 报道相似的结果。这些发现说明抗生素对动物的生产能高亮了这些抗微生物,这些常常 translocate 到水体并能对人类健康造成威胁。

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4.1. Limitations。这个限制的这个研究是在一个有限的采样大小。

5. Conclusions

这个发现是这个研究表现了在抗药性抗生素如cefazidime, ceftime, imipenem, 和 meropenem 包括 piperacillin 和 piperacillin/tazobactam, 哪些是抗生素的不适当的使用的一个抗药性 MDR 的 P. aeruginosa。这个造成了抗药性对成功的治疗的抗药性 MDR 的 P. aeruginosa。包括这个 Public health relevance, the results of this study reveal the importance and necessity of concerted surveillance of antimicrobial resistance and resistance genes in the nonclinical environment at both local and regional levels and the implementation of the One Health approach. In addition, the occurrence of ESBL-producing P. aeruginosa presents a potential public health threat since the genetic elements responsible for this resistance are present on mobile genetic elements (MGEs) that can be transferred to other Gram-negative bacteria through horizontal gene transfer.
Data Availability

All data generated or analyzed during this study are included in this published article.

Ethical Approval

This study was approved by the Human Research Ethics Committee of the Faculty of Health Sciences of Walter Sisulu University approved this study (Reference number: 024/2016).

Disclosure

The funders had no role in study design, data collection, analysis and interpretation of data, writing of the manuscript, and decision to publish.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors’ Contributions

Apalata T., Vasaikar S., and Hosu M. C. conceived and designed the experiment. Hosu M. C. and Okuthe G. E. were involved in the field and experimental studies. Hosu M. C., Okuthe G. E., and Apalata T. collected the data, analyzed the data, and drafted the manuscript. Apalata T., Vasaikar S., Hosu M. C., and Okuthe G. E. revised the manuscript critically for intellectual content. All authors read and approved the final manuscript.

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