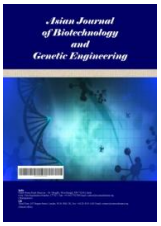

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Molecular and Antibiotic Susceptibility Profiling of Bacteria Isolated from Pre-sterilized Food Samples Used as Substrates for Outdoor Air Quality Assessment

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Authors' contributions

This work was carried out in collaboration among all authors. Author TS designed the study, wrote the protocol and the first draft of the manuscript. Authors GA and LOA managed the analyses of the study. Author AG also performed the statistical analysis and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A large proportion of human population spend significant part of their life in the outdoor environment due to activities relating to occupation and other lifestyle related events. This work was carried out fundamentally, to identify the bacterial community influencing the quality of outdoor air, vis-à-vis their antibiotic susceptibility pattern. The research was conducted at the River State University, Port Harcourt, Nigeria, using pre-sterilized food (yam, pawpaw, and meat) as air sampling substrates, by exposing the samples to air and studied during the wet and dry seasons. The bacterial species were identified using a culture-dependent molecular technique, and the result recorded *Escherichia coli* (CP040927), *Klebsiella pneumoniae* (MN177202), *Shigella flexneri* (EU009189), *Salmonella typhi.* (CP003278), *Bacillus subtilis* (EF194103) and *Staphylococcus aureus* (CP042650) as the predominant bacterial species. *E. coli* was however the most predominant species with a frequency of 34.3% and 26.7% for the dry and wet season, respectively. It was also observed from the study

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that the bacterial groups were higher during the wet season (35 isolates) than in the dry season (30 isolates). There was a statistical difference ($p < 0.05$) between the various substrates and seasons sampled. The antibiotic susceptibility pattern of the bacterial isolates showed that 100% of the isolates were resistant to Ceftazidime, Augmentin, Cefuroxime, Ceftriaxone and Cloxacillin, while Erythromycin, Ofloxacin, Ciprofloxacin and Meropenem were active against all the isolates (100%). Results from this study would be useful to public health professionals for deciphering the health risk associated with outdoor air quality.

Keywords: Antibiotic susceptibility; molecular; outdoor air quality; pre-sterilized food sample; profiling; sequencing.

1. INTRODUCTION

Air quality play a significant role in the wellbeing of individuals inhabiting a particular environment. The air quality of an environment is influenced basically by physical, chemical and biological factors. Microorganisms found in the air environment are versatile and known for various health issues relating to both upper and lower respiratory tract infections, amongst others associated with airborne microbial population. The effect of airborne bacteria on human health may include allergic asthma and seasonal allergies, and other forms of diseases [1,2]. These diseases can be transmitted as aerosolized droplets [3,4], causing various disease syndromes in human when inhaled [5], depending on the immunological and physiological status of the host. This implies that exposure to airborne microorganisms can affect health negatively [6] and generally depends on the particle size [7]. This has therefore, made the outdoor air environment a current and important area of research.

Bacterial species form part of the many types of biogenic aerosol particles, and they are known to be ubiquitous in the atmosphere [8]. They can exist in aerosolized forms as pathogenic and/or non-pathogenic dead or live microorganisms [9]. Due to their size, bacteria have a long atmospheric residence time (of the order of several days) and can be transported by wind over long distances. Bacteria enter the atmosphere as aerosol particles from practically all surfaces, including soil, water, and plant surfaces [10]. Once in the air, they are carried upwards by air currents and may remain in the atmosphere for many days before being removed by precipitation or direct deposition onto surfaces. Meteorological parameters like wind direction, wind speed, temperature, and relative humidity determine the suspension, transportation, and deposition of airborne microbes [11]. A sound knowledge of the concentration and distribution patterns of airborne bacteria on a global scale is needed in order to assess their importance in relation to the climate and health effects of atmospheric aerosol, including cloud formation and development, microbial biodiversity, and atmospheric chemistry.

The characteristics of atmosphere as a habitat include extreme temperature variations, light, low moisture content and organic matter. All these characteristics make the atmosphere unsuitable for growth of microorganisms.

Soil is one of the major sources of microorganisms found in air, and are usually transmitted following environmental perturbations associated with wind and influenced as well by gravitational forces. When wind blows it dislodges the microorganisms from the soil and liberates them into the air and these microorganisms may remain suspended in the air for a long time. Another way of transferring microorganisms to the air is by manmade actions like plugging and digging. Organisms can also be released in the form of water droplets or aerosols which are produced by wind or tidal actions. Microorganisms from plant and animal surfaces are also transferred by air currents through human activities like coughing, sneezing, laughing and even talking.

Above the land surface in a natural environment, airborne dust consists of up to about 25% of biological particles [10]. In urban and agriculturally-dominated areas, the percentage is usually higher [8]. Airborne biological particles as a whole are also denoted as bio-aerosols. Airborne microorganisms have impacts not only on human health, but also on climate and microbial biogeography. For example, the plant pathogen *Pseudomonas syringae* and related phylloplane bacteria have strong ice nucleation ability at 33°C warmer (−5°C) than the homogeneous freezing temperature of cloud droplets composed of pure water [12].

Bacteria have evolved diverse and remarkable ways to avoid antimicrobials in several cases, resulting in resistance due to a minor structural alteration in the target so that it is no longer bound by the drug, yet still functions. For example, streptomycin normally binds to a part of prokaryotic 30S ribosomal subunit that is critical for protein synthesis. A slight alteration in the structure of ribosome result in a distortion, so that streptomycin is no longer able to bind but the ribosome can still functionally translate mRNA. Alteration in membrane permeability or its other function may also confer antibiotic resistance.

There is paucity of information regarding the abundance of microbial population resident in the air environment. Also, there is a limited understanding of the quantities and types of bacteria found in the atmosphere [13]. This scanty information may be due to methods used in the isolation and identification of microorganisms associated with the air environment. However, with recent advances in high-throughput sequencing, the dynamics of bacteria in the atmosphere can be better understood [14], and thus provide a more comprehensive data set for deciphering those bacteria found in the atmosphere and the control of their populations. Surveillance of antibiotic resistance has recently been given much attention by researchers as a major tool to assess the health risk of drug-resistant airborne microbes as well as bacteria from other environmental sources [15,16].

The potential of sterile food sources serving as a medium for bacterial growth in the outdoor air environment, with the ultimate aim of applying them as a novel technique in catching and probing bacterial community lurking the outdoor air environment has been evaluated and reported by Sampson, et al. [17]. This was done with the view of using these pre-sterilized food samples to determine the outdoor air quality at heights significantly above ground level. This paper however looks at the bacterial determinants of an outdoor air quality, using sequence-based molecular technique and as well, evaluate the antibiotic susceptibility pattern of the isolates to conventional antibiotics.

2. MATERIALS AND METHODS

2.1 Study Area and Sampling Techniques

The air sampling was conducted at the River State University, Harcourt, Nigeria as described by Sampson et al. [17]. All microbiological analyses were carried out at the Microbiology Laboratory of the River State University, Harcourt, Nigeria.

2.2 Study Period, Sampling Frequency and Duration

The study was carried out between the months of May 2018 to May 2019. The samples were studied at daily intervals for five consecutive days in two seasons (wet and dry).

2.3 Isolation of Pure Culture

To get a pure culture, an inoculum of the colonies was taken and sub-cultured on fresh agar plates using the streak plate method and incubated for 24 hours as described by [16,17].

2.4 Molecular Identification

2.4.1 DNA extraction and quantification

Extraction of DNA from the pure isolates was done using the boiling method. This was achieved by centrifuging five milliliters of an overnight broth culture of the bacterial isolates grown in Luria Bertani (LB) media at a speed of 14000 rpm for 3 min. This was followed by re-suspending the cells in a vial containing 500 µl volume of normal saline and was subjected to a heating process at a temperature of 95°C for a period of 20 min, after which it was allowed to cool on ice before later been spun for another period of 3 min at same speed of 14000 revolutions per minute (rpm). The DNA suspension was decanted to a micro-centrifuge tube of 1.5 ml volume and stored at -20°C for other downstream reactions.

The genomic DNA was quantified using a spectrophotometer (Nanodrop 1000). The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µl of sterile distilled water and blanked using normal saline. Two microliter of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button, and read via computer device attached to the system.

2.4.2 16S rRNA amplification

The amplification of the gene was done using Polymerase Chain Reaction (PCR) method. The

16s rRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microliters for 35 cycles. The PCR mix included: The X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5 µM and the extracted DNA as template. The gene amplification was done using a set of conditions that involved a first step of denaturing the DNA at a temperature of 95°C for a duration of 5 minutes, followed by another cycle of DNA denaturing at same temperature, albeit for 30 seconds. The next was a 35 cycle series of annealing at 52°C and extension at 72°C, for 30 seconds each, except for the final extension that was maintained for a period of 5 minutes. The final PCR product was electrophoresed at 130V for 30 minutes using 1% agarose gel concentration, and the DNA bands were visualized with the aid of a blue light trans-illuminator.

2.4.3 Sequencing

Sequencing analysis was performed at Inqaba Biotechnical Pty Ltd, South Africa. This was done using a BigDye Terminator kit on a 3510 ABI sequencer maintained at a final volume of 10 µl. The components included 0.25 µl Big Dye® terminator v1.1/v3.1, 2.25 ul of 5 x Big Dye sequencing buffer, 10 µM, Primer PCR primer, and 2-10 ng PCR template per 100 bp. The sequencing conditions involved 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4 min.

2.5 Antibiotics Susceptibility Test

Antibiotics Sensitivity Test was performed according to NCCLS [18]. A set of antibiotics discs (multi-disc) was dispensed onto the surface of the agar plate inoculated with the isolates, using the Kirby Bauer disc diffusion method. With the aid of a sterile forceps, the respective antibiotic discs were placed onto the agar and slightly pressed down to ensure its contact with the agar. Within 30 minutes of applying the discs, the plates were inverted and incubated at 37°C for 18 hrs. After overnight incubation, the test plate was examined. Using a ruler on the underside of the plate, the diameter of each zone of inhibition was measured in mm. The measurement included the diameter of the disc and susceptibility or resistance of the isolates was reported by referring to Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints of the NCCLS [18] and the organisms were reported as either susceptible, intermediate, or resistant to the agents that were tested.

3. RESULTS AND DISCUSSION

3.1 Molecular Characterization of Bacterial Isolates

The identification and characterization bacterial isolates is fundamental in understanding the ecology and environmental health concerns of a habitat, as the resident micro flora of a place influences to a large extent the quality of such environment. Different microbiological approaches have been used by researchers to probe the microbiological parameters of an environment (aquatic, terrestrial, air and other environments). The use of a high-throughput approach undeniably ensures the reliability of a method. This research therefore explored the use of a culture-dependent molecular technique in accessing the bacterial diversity of an outdoor air environment.

As stated in the methodology, pre-sterilized food samples (yam, meat and paw paw) were exposed to an outdoor air environment and left to be contaminated by microflora, after which the bacterial isolates were subjected to a sequence based molecular characterization. It follows that the obtained 16s rRNA sequence from the isolates produced an exact match during the mega blast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolate *Klebsiella spp* showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolate MN177202 within the *Klebsiella* spp and revealed a close relatedness to *E. coli, Shigella* spp*., Salmonella* spp*., Bacillus* spp*.* and *Staphylococcus aureus* as shown in Fig. 1.

Culture-based probing of atmospheric samples identify far fewer taxonomic groups of bacteria, and presents the Gram-positive spore-forming bacteria as the most commonly cultured airborne bacterial taxa [19]. This research is however opposed to this as most of the isolates were found to be relatively rare members of the airborne bacterial communities and highly dominated by Gram negative bacteria. This

observed difference is attributable to the method adopted in this study which involved the use of a novel method of air quality assessment alongside molecular characterization of the isolates. We have stated in our previous publication [17] that the bacteriological quality of air is determined by the type of substrate used to capture the bacterial population lurking the air environment. The identification of these isolates in an outdoor air environment may be due to human activities and other environmental sources. The presence of these organisms therefore makes the air unsafe as they are associated with different disease forms like pneumonia, diarrhea, tuberculosis, typhoid fever, pertussis, etc.

3.2 Seasonal Prevalence of Bacteria in the Air Environment Studied

The study on the effect of seasonal changes on the occurrence of bacterial population in the outdoor environment revealed that the bacterial genera were isolated mostly during the wet season than dry season. The result as presented in Table 1 shows that the wet season sampling had a total of 35 isolates while 30 was recorded during the dry season sampling. Also seasonal variation influenced the occurrence of the individual isolates in the various food samples used. It follows that, *E. coli* constituted 34.3% and 26.6% of the total bacteria isolated during the wet and dry season, respectively. This shows that the frequency of occurrence of *E. coli* was higher during the wet season than in the dry season. Similar pattern was observed for both *Bacillus and Klebsiella species,* while *Staphylococcus*, *Salmonella* and *Shigella species* had higher frequencies during the dry season (Table 1, Fig. 2). This difference in the frequency of occurrence of the individual isolates with respect to season (dry and wet) is attributable to their metabolic and physiological variations. It follows that while some organisms can adapt to some environmental conditions, others may be negatively affected by such environmental conditions of temperature, desiccation, and other atmospheric and meteorological factors.

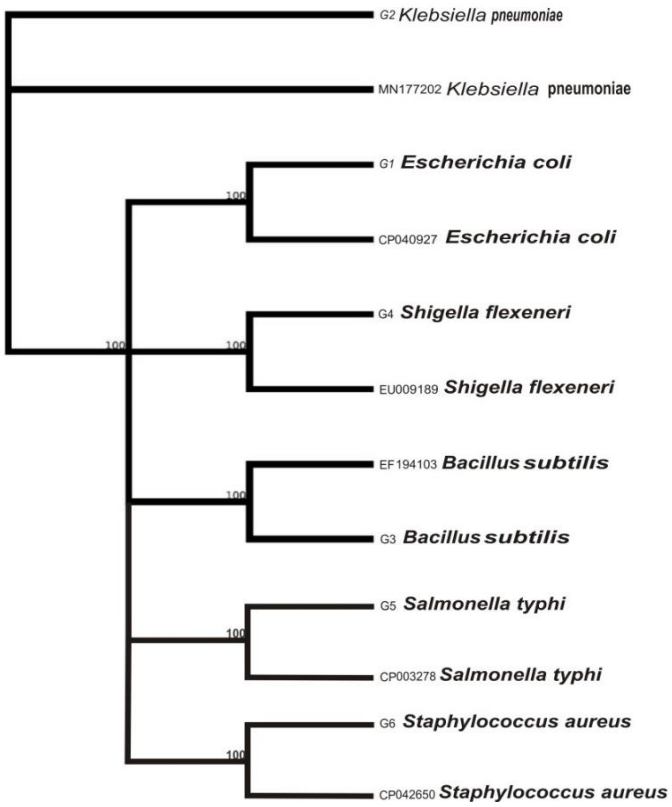

Fig. 1. Phylogeny of the bacterial isolates

Table 1. Seasonal pattern of bacterial colonization of pre-sterilized food samples in air

Isolate	Frequency (Number) of isolates							
	Wet season				Dry season			
	Yam	Paw	Meat	Total (%)	Yam	Paw	Meat	Total (%)
E. coli	4	3	5	12 (34.3)	2	2	4	8 (26.6)
Salmonella sp.	-	-	2	2 (5.7)	-	-	4	4 (13.3)
Staphylococcus aureus	1	3	1	5 (14.3)	2	2	1	5 (16.7)
Bacillus sp.	3	1	-	4 (11.4)	2	1	-	3 (10)
Klebsiella pneumoniae	2	2	4	8 (22.9)	1	2	2	5 (16.7)
Shigella sp.	-	2	2	4 (11.4)	-	1	4	5 (16.7)
Total	10	11	14	35 (100)	7	8	15	30 (100)

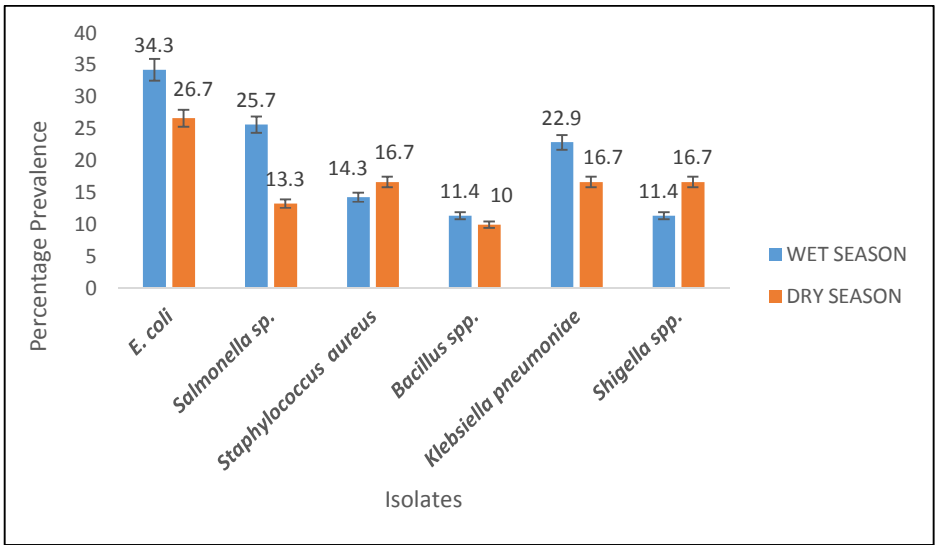

Fig. 2. Occurrence of the bacterial isolates in the wet and dry season

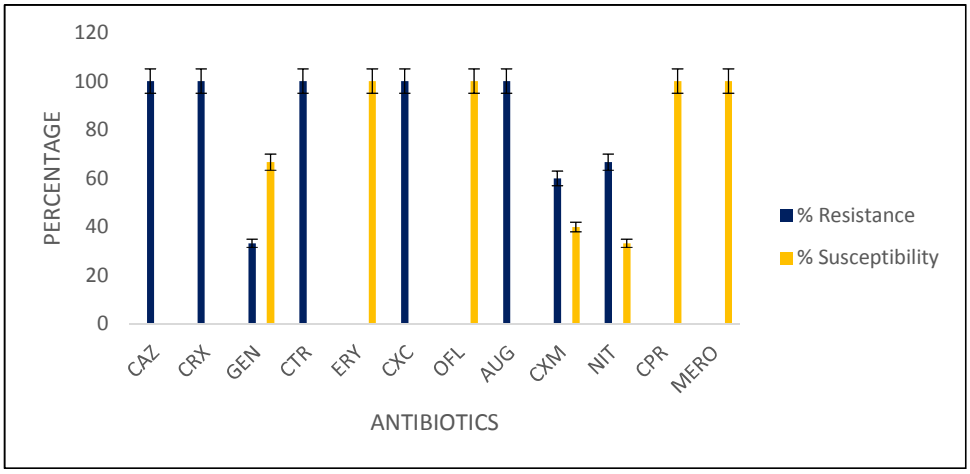

Fig. 3. Antibacterial activity of the various antibiotics tested against the isolates

Keys: CAZ – Ceftazidime; CRX – Cefuroxime; GEN – Gentamicin; CTR – Ceftriaxone; ERY – Erythromycin; CXC – Cloxacin; OFL – Ofloxacin; AUG – Augumentin; CXM – Cefixime; NIT – Nitrfurantion; CPR – Ciprofloxacin

Other factors observed that influenced the seasonal variation of these bacterial populations in the outdoor environment was the nature of the substrate. From Table 1 it is deducible that all the food samples used as substrate, except for meat, had higher number of isolates during the wet

season than the dry season. This shows that weather-related conditions affect the nature of the substrates which in turn influences the proliferation of bacteria in the outdoor air environment.

The features of the atmosphere such as extreme temperature variations, light, low moisture content and organic matter influence its habitat potentials and to this extent determine the type of flora and fauna that inhabits this environment; since only well adapted species can survive in this milieu [20].

The findings from this study show that transmission or spread of diseases is influenced by weather as well as other environmental factors. Climate conditions like temperature, winds and relative humidity in any territory, either all year round or at isolated moments (days or weeks) are the main factors affecting the spread, duration and infectiousness of droplets containing pathogens. For instance, influenza virus, is spread easily in northern countries (north hemisphere), because of climate conditions which favors the pathogenicity/virulence of the virus but on the other hand, lots of bacterial infections cannot spread outdoor most of the year, keeping it in a latent stage [21].

3.3 Susceptibility Pattern of Isolates to Various Antibiotics

The result of the antibiotic susceptibility assay (Fig. 3) showed that all the isolates (100%) were susceptible to Ceftazidime, Augumentin Cefuroxime, Ceftriaxon and Cloxacillin. It also followed that Erythromycin, Ofloxacin, Ciprofloxacin and Meropenem were inhibitory to all (100% of) the isolates tested.

The goal of susceptibility testing is to predict the likely outcome of treating a patient's infection with a particular antimicrobial agent [22]. This research has shown the susceptibility pattern of bacteria isolated from the outdoor air environment. From the report, it was observed that while some antibacterial agents were potent against all the isolates, some showed no inhibitory activity on all the isolates. This implies that in the event of any infection involving these bacterial organisms isolated in this study, Ceftazidime, Augumentin Cefuroxime, Ceftriaxon and Cloxacillin may not have any therapeutic value. This is attributable to the fact that these isolates may have developed some form of resistance to these drugs. Also, the susceptibility of all the isolates to Erythromycin, Ofloxacin, Ciprofloxacin and Meropenem is indicative of lack of prior exposure to these agents. From the foregoing analysis, it can be inferred that while some of the isolates may be from human sources, others may be of an environmental origin. This is in agreement with the fact that the susceptibility pattern of an isolate to various antibiotics is dependent on the source of the bacterial contaminant, as organisms without prior exposure may show low level resistance compared to pathogens of human origin with prior exposure [23].

A study by Hakam et al. [24] on antimicrobial efficacy of some herbs on resistant strains of *Pseudomonas species* isolated from West African Mud Creeper (*Tympanotonus fuscatus*), showed that some of the *Pseudomonas species* were found to be resistant to some conventional antibiotics previously used against the organisms but were susceptible to methanol extracts of ginger, garlic, bitter cola seed, bitter cola bark, turmeric. This finding by Hakam et al. [24] further buttresses the fact that resistance to an antimicrobial agent may be linked to prior exposure to the antimicrobial agent.

4. CONCLUSION

The molecular profiling of the bacterial population of an outdoor air environment has shown that the region of the air environment sampled was composed of some bacterial species such as *Escherichia coli* (CP040927), *Klebsiella pneumoniae* (MN177202), *Shigella flexneri* (EU009189), *Salmonella typhi.* (CP003278), *Bacillus subtilis* (EF194103) and *Staphylococcus aureus* (CP042650) known to cause various degrees of ailment in man, ranging from respiratory related illness to gastroenteritis. Their growth and survival in the atmosphere is however influenced by both substrate type and availability as well as other environmental dynamics associated with seasonal variation.

From the study it was observed that while some organisms were predominant during the wet season, others were predominant during the dry season. This variation is therefore attributable to the physiological and metabolic dynamism as well as functional variation that exist among the organisms. Also, the contribution of the nature of the substrate in the growth and survival of bacteria in the air environment was discovered in this study. It was observed that number of isolates from the different food samples used as

substrate for the air quality determination varied with respect to season. This may be as result of the impact of climate on the water activity of the substrate, which had a concomitant effect on the proliferation of the organisms. This observation therefore implies that the transmission of disease causing agents is influenced by various factors including particulate matter in the air, climatic factors and the physiological aspect of the organism.

From the study on the susceptibility of the isolates to conventional antibiotics, it was observed that some of the antibacterial drugs were inhibitory to all the bacterial isolates, while all the isolates were resistant to some of the drugs tested. This implies that a community of bacteria in the environment presents similar pattern of susceptibility to drugs depending on exposure/contact status. The presence of drug resistant strains or species in the air environment is of a great public health concern. These drug resistant populations are attributable to anthropogenic sources with prior exposure to these antibacterial drugs, and thus human activities in the environment relating to biogenic waste generation and disposal should be regulated. Also, Metagenomic evaluation of air quality using sterile food substrates is recommended.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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