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Full Length Research Paper

In vitro antibacterial activity of mixed *Garcinia buchananii* B. and *Curcuma longa* L. ethanolic extracts against *Streptococcus pneumoniae*

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***Streptococcus pneumoniae* is the main bacterial cause of community-acquired pneumonia. In children, 22% of the deaths are due to pneumonia as the single leading cause of death. The local people in Uganda use herbs like *Curcuma longa* Linnaeus and *Garcinia buchananii* Baker to manage upper respiratory tract infections (URTIs). The ethanolic extracts of the *C. longa* rhizome and *G. buchananii* stem bark have individually demonstrated antimicrobial activity against bacteria, protozoa, and viruses. Crude extracts of *C. longa* rhizome powder and *G. buchananii* fresh back were obtained through maceration using ethanol. *In vitro* disc diffusion method and serial dilution method were used to determine antibacterial susceptibility and minimum inhibitory concentration (MIC), respectively of the plant extracts against *S. pneumoniae*. Both ethanolic extracts of *C. longa* rhizome and *G. buchananii* stem bark individually showed activity against *S. pneumoniae* and this antibacterial effect was largely dose-dependent. However, ceftriaxone had superior antibacterial activity ($p < 0.0001$) than all the individual extracts and combinations. The MICs of *C. longa* and *G. buchananii* ethanolic extracts were 3.125 and 1.5625 mg/mL, respectively. The 50:50 *C. longa* - *G. buchananii* combination showed superior activity compared to other combinations, though it was not statistically significant ($p > 0.05$). The Fractional Inhibitory Concentration Index (FICI) was 11.68. This study concluded that the ethanolic extracts of both the rhizome of *C. longa* and the stem bark of *G. buchananii*, when used singly and in combination, demonstrated antibacterial activity against *S. pneumoniae.* However, the combination of the ethanolic extracts of these two plants demonstrates antagonistic activity.**

Key words: *Curcuma longa*, *Garcinia buchananii*, *Streptococcus pneumoniae*, combined antibacterial activity.

INTRODUCTION

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia. It is one of the most common pathogens that cause invasive diseases such as sepsis, meningitis, and pneumonia, bacteremia, and

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sinusitis (Soto-Noguerón et al., 2016; Wessels et al., 2012). Pneumonia due to *S. pneumoniae* accounts for numerous hospitalizations and deaths among all age groups (Baldo et al., 2015). In children, acute respiratory tract infections account for 22% of deaths; with pneumonia as the single leading cause of death. The World Health Organization (WHO) estimates that about 14.5 million episodes of serious pneumococcal disease occur, resulting in about 826,000 deaths in children under five years. The highest number of deaths due to *S. pneumoniae* occurs in developing countries than in industrialized country settings (WHO, 2012). Seventy percent of the deaths occur in African and Asian countries (Krumkamp et al., 2012).

The first-line management of URTIs caused by *S. pneumoniae* is penicillins like amoxicillin. However, due to the emergence of penicillin-resistant bacterial strains, many other drugs are often used to manage these infections including fluoroquinolones, cephalosporins like ceftriaxone, cefotaxime. Other drugs used include clindamycin, doxycycline, vancomycin, and linezolid (UCG, 2016). However, resistance to penicillins, macrolides, fluoroquinolones, tetracycline, clindamycin, and trimethoprim-sulfamethoxazole combination is on the rise and occurs through various mechanisms (Cherazard et al., 2017).

Medicinal plants in Africa constitute a large but still largely untapped pool of natural product remedies. World Health Organization (WHO) estimates that 80% of the population in developing countries still relies on plant-based medicines for some part of primary health care (Ekor, 2014). In parts of East Africa like the Lake Victoria basin, numerous medicinal plants including *Garcinia buchananii* B. and *Curcuma longa* L. are used individually or in combination to manage upper respiratory tract infections (URTIs). In Africa, *G. buchananii* (family Clusiaceae) bark extract is widely used traditionally for the management of gastrointestinal diseases like diarrhea, and dysentery (Balemba et al., 2010), and other conditions such as respiratory tract infections, eye diseases, hypertension, and diabetes (Okullo et al., 2014). *G. buchananii* contains an isoprenylated benzophenone derivative garcinol as one of its phytochemicals (Schobert and Biersack, 2019; Stark et al., 2015). Garcinol has been reported to have antibacterial activity against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis,* and *Enterobacter aerogenes* (Varalakshmi et al., 2010). *C. longa* L. (Tumeric) belongs to the Zingiberaceae family and is used largely as a coloring agent, as a spice, and also as a medicine (Gurning, 2020). As a medicine, it is used widely as an antimicrobial, anti-inflammatory, anticancer, antidiabetic, and antioxidant agent (El-Kenawy et al., 2019; Teow et al., 2016). Rhizome extracts of *C. longa* have been found to possess broad-spectrum antibacterial activity (Kumar et al., 2020), with this activity attributed to its major constituent curcumin (Teow et al., 2016). Studies on *C. longa* rhizome extracts have shown activity against *S. aureus*, *Klebsiella pneumonia, E. coli,* and *Staphylococcus epidermidis* (Feghali et al., 2018; Singh et al., 2017).

Mixtures of different plants extracts are widely used to manage various diseases. The rationale of the use of combinations is to benefit from the possible synergistic or potentiating effects (Ozioma and Okaka, 2019). There is however a great need to evaluate whether the different combinations of the crude plant actually achieve the desired benefits. In various studies, the antibacterial activity of individual extracts of *C. longa* rhizome and *G. buchananii* stem back extracts have been established for various micro-organisms but not *S. pneumoniae.* This study aimed at establishing the antibacterial activity of individual and combination of ethanolic extracts of *C. longa* rhizome and *G. buchananii* stem bark against *S. pneumoniae.*

MATERIALS AND METHODS

Reagents and chemicals

Ethanol (70%), distilled water, concentrated sulfuric acid, ferric chloride, sodium hydroxide, hydrochloric acid, iodine, potassium iodide, chloroform, dimethylsulfoxide (DMSO), and ceftriaxone standard.

Collection and identification of plant

Fresh forms of turmeric rhizome were obtained from a garden in Rakai-Uganda, while fresh forms of the stem bark of *G. buchananii* were obtained from a garden in Kisaasi, Uganda in February 2018. The plant materials were transported to the pharmacognosy laboratory at the Department of Pharmacy, Makerere University. Herbarium specimens were submitted to the Makerere University herbarium for authentication by a botanist.

Preparation of plant extracts

The fresh rhizomes of *C. longa* were washed using distilled water. The rhizomes were then dried in the open air under shade for 10 days after which, they were ground using a motor and pestle. The obtained powder was macerated in ethanol (70%) in a closed glass container; in a ratio of 1:6 at room temperature, for 5 days with occasional agitation. The obtained mixture was then filtered using Whatman filter paper number 1. The filtrate was concentrated by evaporation in a rotary evaporator at 90°C. This process was also repeated for the fresh bark of *G. buchananii*. The percentage yield for the plant materials was then calculated using the formula:

Percentage yield of extract = (Mass of dried crude extract / Mass of powdered plant material macerated) ×100%

Phytochemical analysis

Phytochemicals screening for tannins, alkaloids, saponins, flavonoids, steroids, and phenols was done following standard methods (Evans, 2009) (Table 1).

Test for tannins (Ferric chloride test)

Ferric chloride (1 mL) was added to the extract. The formation of a blue-green precipitate confirmed the presence of tannins.

Test for alkaloids

Extracts (10 g) were dissolved in dilute hydrochloric acid and then filtered. To 2 mL of the filtrate was added Wagner's reagent (Iodine in Potassium iodide). The formation of a reddish-brown precipitate indicated the presence of alkaloids.

Test for saponins

The extract was added to distilled water (20 mL) in a measuring cylinder and agitation was done for 15 min. The formation of a 1 cm layer of foam confirmed the presence of saponins.

Test for flavonoids

Sodium hydroxide (2 mL) was added to the extract. The formation of a yellow color, that became colorless once diluted acid was added confirmed the presence of flavonoids.

Tests for steroids

To extract (1 mg) in a test tube was added chloroform (10 mL). Concentrated sulfuric acid (10 mL) was added slowly on the sides of the test tube. The formation of a brown ring at the intersection of the two layers and with the upper layer turning green confirmed the presence of steroids.

Test for phenols

The extract was dissolved in distilled water (2 mL) and then a few drops of ferric chloride were added. The formation of a deep blue or greenish colour confirmed the presence of phenols.

Antimicrobial susceptibility testing

The disc diffusion method was used to screen for antibacterial activity. *S. pneumoniae* ATCC 49619 (Provided by Microbiology department-Makerere University) was inoculated on blood agar plates by streaking. Sterile Whatman filter paper discs measuring 6 mm in diameter were sterilized by autoclaving at 121°C and a pressure of 15 psi for 15 min. These filter disks were then impregnated with crude plant extracts of different concentrations 3.125, 6.25, 12.5, 25, 50, 100 mg/mL, and 25:75, 50:50 and 75:25 fractional combinations of the extracts under aseptic conditions. The positive control used was ceftriaxone (100 mg/mL) and the negative control was DMSO (1.5%). This was done in triplicate for all the extracts and the controls. DMSO was the solvent used to make all the different concentrations of the extracts and positive control. Impregnated filter paper discs for both controls and those with plant extracts were then placed on the surfaces of the blood agar media, onto which *S. pneumoniae* had been inoculated, and incubated at 37°C for 24 h. After incubation, the plates were removed from the incubator and the diameters of the zones of inhibition were measured using a Vernier caliper.

Determination of the MIC and FICI

The minimum inhibitory concentration (MIC) of the ethanolic extracts was determined by the serial dilution method. Ethanolic extracts of both *C. longa* and *G. buchananii* were prepared using Brain Heart Infusion (BHI) agar to make different concentrations ranging from 0.78125 to 200 mg/mL in their respectively labeled test tubes. *S. pneumoniae* (strain ATCC 49619) was inoculated into the BHI with different concentrations of both the individual and with the most potent combination of *C. longa* and *G. buchananii* (50:50). Controls containing only the nutrient broth without the extracts were included. The test tubes were then incubated at 37°C for 24 h. The presence of turbidity after the incubation period denoted the presence of *S. pneumoniae,* while the absence of any turbidity indicated inhibition of microbial growth. The concentration of the extract in the test tube with the lowest dilution with no detectable growth by visual inspection was considered the MIC. Due to the colored nature of the plant extracts, which made the observation for turbidity difficult, the samples were subsequently sub-cultured at 37°C in an incubator for 24 h to observe for any growth. The Fractional Inhibitory Concentration Index (FICI) was calculated using the following standard formula. The effects of the combinations were then classified as: synergistic, additive, indifference and antagonistic, if the FICI is <1, =1, >1 ≤2, and >2, respectively.

FIC *(G. buchananii* extract) = MIC (*G. buchananii* extract in combination) / MIC (*G. buchananii* extract alone)

FIC (*C. longa* extract) = MIC (*C. longa* extraction in combination) / MIC (*C. longa* extract alone)

FICI = FIC (*G. buchananii* extract) + FIC (*C. longa* extract).

Data management and analysis

Graphpad Prism Ver.7.0 was used to compute descriptive statistics of the mean and standard deviation (SD) inhibition zone diameter. The data was then subjected to one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests to compare the antibacterial activities of the different extract concentrations and the controls. A value of $p<0.05$ was considered significant.

RESULTS

Extraction yield and phytochemicals

The percentage yield of ethanolic extracts of *C. longa* rhizome and *G. buchananii* stem bark was 14.3 and 9.4%, respectively. Both ethanolic plant extracts contained alkaloids, saponins, tannins, flavonoids, and phenols and lacked steroids (Table 1).

Antimicrobial susceptibility of *S. pneumoniae* to *C. longa* and *G. buchananii* ethanolic extracts

Ceftriaxone 100 mg/mL (positive control) had the largest zone of inhibition of 43.33 (± 3.05) on the *S. pneumoniae* strain ATCC 49619 used when compared with all other test substances. Among the 3.125 to 100 mg/mL ethanolic plant extracts of *G. buchananii* and *C. longa*, the highest dose concentrations of 100 mg/mL had the largest zones of inhibition of 26.67 (± 1.15) and 25.33 (±

Table 1. Phytochemical yields and composition of the *C. longa* rhizome and *G. buchananii* stem bark ethanolic extracts.

Extracts	*C. longa* rhizome	*G. buchananii* stem bark
Percentage yield	14.3 %	9.4%
Alkaloids	+	+
Saponins	+	+
Tannins	+	+
Flavonoids	+	+
Phenols	+	+
Steroids	-	-

(+)- Present, (-) – absent
Source: Authors

Table 2. Antibacterial activity of ethanolic extracts of *G. buchananii* and *C. longa* against *Streptococcus pneumoniae*.

Test substances and concentrations	Mean zone of inhibition (± SD) (mm) (N=3)
Ceftriaxone 100 mg/mL (positive control)	43.33 (±3.05)[‡]
DMSO 156.26 mg/mL (negative control)	0 (±0)
G. buchananii 100 mg/mL	26.67 (±1.15)
G. buchananii 50 mg/mL	24.6 (±1.15)
G. buchananii 25 mg/mL	20.67 (±3.05)
G. buchananii 12.5 mg/mL	18.67 (±1.15)
G. buchananii 6.25 mg/mL	17. 33 (±1.15)
G. buchananii 3.125 mg/mL	16 (±2.1)
C. longa 100 mg/mL	25.33 (±2.31)
C. longa 50 mg/mL	22 (±5.29)
C. longa 25 mg/mL	20 (±4.1)
C. longa 12.5 mg/mL	19.33 (±3.05)
C. longa 6.25 mg/mL	16.67 (±1.15)
C. longa 3.125 mg/mL	14 (±2.3)
75% *C. longa* + 25% *G. buchananii*	16 (±1.2)
50% *C. longa* + 50% *G. buchananii*	20 (±2.15)
25% *C. longa* + 75% *G. buchananii*	17 (±1.82)

[‡]The positive control (Ceftriaxone) had extremely superior activity ($p < 0.0001$) on *Streptococcus pneumoniae* in comparison to the individual extracts of *G. buchananii* and *C. longa* at concentrations of 3.125-100 mg/mL and when in combination.
Source: Authors

2.31), respectively. Of the *C. longa* and *G. buchananii* extracts combinations, the 50% *C. longa* + 50% *G. buchananii*, had the largest zone of inhibition (Table 2). There was no significant difference ($p > 0.05$) in activity between the different *G. buchananii* and *C. longa* extracts combinations, and between similar concentrations of *G. buchananii* and *C. longa* when used singly.

The MICs for ethanolic extracts *C. longa* rhizome and *G. buchananii* stem bark against *S. pneumoniae* were 3.125 and 1.5625 mg/mL, respectively. The most potent combination of extracts (50% *C. longa* and 50% *G. buchananii*) had a MIC of 12.162 mg/mL and a Fractional Inhibitory Concentration Index (FICI) of 11.68.

DISCUSSION

The percentage yield of ethanolic extract of *C. longa* from this study was lower than that observed in a previous study that reported 17.39% (Tanvir et al., 2017). Variations in yields and phytochemical composition of plant extracts are reported to be caused by variations in climatic and agronomic factors, and as well as differences in the extraction procedures used (Borges et al., 2018; Dhanani et al., 2017). Both *C. longa* and *G. buchananii* contained significant amounts of alkaloids, saponins, tannins, flavonoids, and phenols. These findings are similar to those in previous studies (Gurning,

2020; Tanvir et al., 2017) and the anti-bacterial inhibitory effects identified could be attributed to some or all of these phytochemicals in the plant extracts (Oghenejobo et al., 2017; Stark et al., 2015). For instance, Garcinol which is a flavonoid in *G. buchananii* has antibacterial activity against *S. aureus, E. coli, Bacillus subtilis,* and *Enterobacter aerogenes* (Varalakshmi et al., 2010). Curcumin, a phenolic compound in *C. longa* has been reported to be responsible for its broad antibacterial effects (Oghenejobo et al., 2017; Teow et al., 2016), acting through reducing bacterial membrane integrity resulting in membrane leakages in both Gram-positive and Gram-negative bacteria (Tyagi et al., 2015).

Both *C. longa* rhizome and *G. buchananii* stem bark extracts have been reported to have activity against various microbes (Afrose et al., 2015; Moghadamtousi et al., 2014; Wise et al., 1998). In this study, ethanolic extracts of *C. longa* at a concentration of 100 mg/mL showed sensitivity against *S. pneumoniae* while its concentrations of 50 and 25 mg/mL showed intermediate antibacterial activity. For *G. buchananii*, ethanolic extracts of concentration 50 and 100 mg/mL showed sensitivity against *S. pneumoniae,* while the 25 mg/mL had intermediate activity. *S. pneumoniae* was sensitive to ceftriaxone (positive control) with a mean diameter of the zone of inhibition of ≥ 23 mm (CLSI, 2013). There was no significant difference ($p > 0.05$) in the antibacterial activity of ethanolic extracts of *C. longa* rhizome and *G. buchananii* stem bark of the same concentrations on *S. pneumoniae.* Furthermore, the antibacterial effect of the two ethanolic plant extracts on *S. pneumoniae* was largely dose-dependent. The dose-dependent antibacterial property of *C. longa* rhizome extracts has been reported in other studies (Izui et al., 2016). There were however no significant differences in activity between *G. buchananii* concentrations of 50 and 100 mg/mL or *C. longa* concentrations of 12.5, 25, 50, and 100 mg/mL used in our study. Ceftriaxone 100 mg/mL was more active ($p < 0.0001$) on *S. pneumoniae* when compared with all the different ethanolic extracts of *C. longa* rhizome and *G. buchananii* stem bark at the different combinations used.

In this study, the MICs for ethanolic extracts *C. longa* rhizome and *G. buchananii* stem bark against *S. pneumoniae* were 3.125 and 1.5625 mg/mL, respectively. In previous studies, the MICs of *C. longa* ethanolic and methanolic extracts against other micro-organisms such as *S. aureus, S. epidermidis, E. coli*, and *K. pneumoniae,* have been reported to be in the range of 0.2 to 16 mg/mL (Niamsa and Sittiwet, 2009; Raji et al., 2018; Wise et al., 1998). Cephalosporins like ceftriaxone and cefotaxime, as well as β-lactams such as penicillin G, have MICs ≤ 2 mg/L on *S. pneumoniae* strains, and are effective treatments for pneumococcal bacteraemia and pneumonia caused by *S. pneumoniae* (Kaplan and Mason, 1998). Basing on the MIC ranges obtained in this study and in related studies, the plant extracts of *C. longa* rhizome and *G. buchananii* stem bark are potential treatments for infections caused by *S. pneumoniae* strains such as URTIs, pneumonia, otitis media, and sinusitis.

Synergistic antibacterial activity has been reported for extracts of *C. longa* when used in combination with various conventional antibiotics such as ampicillin, ciprofloxacin, gentamycin, amikacin, and cefepime among others (Moghadamtousi et al., 2014; Teow et al., 2016). Furthermore, mixtures of various plant extracts are widely used to benefit from potential synergistic or additive effects; however, combining plant extracts can also result in antagonism. Antagonistic effects from combination of plant extracts are reported to be a result of the masking of the active principles by other components in the complex mixture (Caesar and Cech, 2019).

Conclusion

In this study, the Fractional Inhibitory Concentration Index (FICI) of the most potent combination of extracts (50% *C. longa* and 50% *G. buchananii*) was 11.68. A calculated FICI greater than 2 indicates antagonism (Ofokansi et al., 2012). This indicates that combining *C. longa* and *G. buchananii* ethanolic extracts using earlier combination ratio provides no advantage to the antibacterial activity of each of these plant extracts on *S. pneumoniae*. The use of a broader range of *C. longa* and *G. buchananii* ethanolic extracts concentrations ratios is required to confirm this reported antagonism. Variations in concentration ratios of different plant extracts can result in either synergism or antagonism (van Vuuren and Viljoen, 2011).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Emergence of Diarrhoeagenic *Klebsiella pneumoniae* Carrying *ast*A and *sen*B genes in Nigeria

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***Klebsiella pneumoniae* is increasingly being isolated from the stool of Nigerian patients. *K. pneumoniae* was isolated from stool samples submitted to two clinical laboratories in Nigeria from patients presenting with various symptoms of gastrointestinal tract infections, including diarrhoea. The authors characterized the virulence and antimicrobial resistance genes of these *K. pneumoniae* strains. Sixteen pure cultures of heavy growth of *K. pneumoniae* isolated from two facilities in Nigeria were subjected to susceptibility testing using a panel of antibiotic, with agar dilution method. Paired-end Illumina whole genome sequencing was completed using a NextSeq instrument. Virulence genes including *ast*A, *sen*B, and *gad* were found in 5 isolates. Multiple plasmid replicons were present; IncF and Col were common plasmids while others were IncR and IncY. Four different STs were found; ST914, ST1962, ST494, and novel ST. These isolates carried various important resistance genes to cephalosporins, fluoroquinolones, aminoglycosides, and so on, including blaCTX-M-15 in one of the isolates. Diarrhoeagenic *K. pneumoniae* is present, which is caused by plasmid-mediated virulence genes such as *ast*A, *sen*B, and *gad*. Fluoroquinolone and third generation cephalosporin resistance were discovered.**

Key words: *Klebsiella*, diarrhoea, virulence genes, antibiotics resistance, genomics, Nigeria.

INTRODUCTION

The common diarrhoea-inducing pathogens include, rotavirus, norovirus, diarrhoeagenic *Escherichia coli*, *Salmonella spp*., *Shigella spp*., and *Yersinia* species (Operario and Houpt 2011; Sjoling et al., 2015). *Klebsiella pneumoniae* can also cause diarrhoea, but most studies focus on extra intestinal infections. Although, *K. pneumoniae* occurs as a commensal in the intestine, it may induce diarrhoea through the production of toxins

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(Guarino et al., 1989; Panigrahi et al., 1991). Some of these diarrhoeagenic strains encode thermostable toxins similar to enterotoxigenic or enteroaggregative toxins of *E. coli*. Enteroaggregative heat stable toxin 1 (EAST-1) is encoded by *ast*A gene on a 60-MDa pAA plasmid (Telli et al., 2010). *ast*A produces a toxin that stimulates the production of high levels of cyclic guanosine monophosphate (cGMP) in cells such that sodium/ chloride co-transport is inhibited and absorption of water and electrolytes from the intestine at villus tips is reduced, resulting in diarrhoea (Telli et al., 2010). Similarly, *sen*B a plasmid-mediated gene has been described to produce the TieB enterotoxin in enteroinvasive and uropathogenic *E. coli* strains (Nataro et al., 1995; Touchon et al., 2009). *K. pneumoniae* isolation from stool is increasing in Nigerian hospitals, and they are usually ignored as native intestinal flora even when isolated as a pure culture from cases of diarrhoea in children and adults. The authors therefore investigated the presence of *K. pneumoniae* and characterized their virulence and antimicrobial resistance genes in Nigeria.

METHODS

Bacterial isolates

Sixteen pure cultures of heavy growth of *K. pneumoniae* isolated from stool of different patients from two clinical laboratory facilities in Nigeria (diagnosed with various diseases) were identified using cultural morphology, Gram reaction (Bartholomew and Mittwer, 1952), standard biochemical tests; indole, citrate, motility, methyl red, voges proskauer and sugars (Ewing, 1986; Barrows and Feltham, 1993), and API 20E strips (BioMérieux, Basingstoke, UK). Ethical approval was obtained for the study from Federal Capital Territory, Health Research Ethics Committee with approval number FHREC/2018/01/95/14-08-18, including informed written consent from the participants.

Antibiotic susceptibility testing

Susceptibility of all isolates to a panel of antibiotics classes in common clinical use in these hospitals were determined by the agar dilution method on Mueller–Hinton agar according to the recommendations of CLSI breakpoints (Wayne, 2018). All runs included the control organism *E. coli* (ATCC 25922).

Whole genome sequencing and bioinformatics

DNA was extracted using a QIAamp1 DNA Mini Kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions. Paired-end Illumina whole genome sequencing was completed using a NextSeq instrument at the Quadram Institute Bioscience. Bioinformatics used an in-house pipeline hosted on an IRIDA instance; sequences were assembled with shovill and annotated with prokka and core snps identified with snippy. Furthermore, assemblies were used to search for plasmid content using the 'PlasmidFinder' tool hosted at the Centre for Genomic Epidemiology(https://cge.cbs.dtu.dk/services/PlasmidFinder), and for isolates likely to carry significant resistance genes in plasmids, 'plasmidSPAdes' was used to assemble likely plasmid contigs from the trimmed reads.

Comparative genomics

Strains belonging to sequence types implicated in globally disseminated disease were compared against completed genomes were available to determine relationships between sources of strains, Nigerian strains and others in global circulation. Reads were also mapped against reference strain HS11286, SNPs identified and phylogenetic relationships determined to determine whether clones in Nigeria are divergent or highly similar from those seen globally.

RESULTS AND DISCUSSION

In the 16 isolates of *K. pneumoniae,* 5 encoded virulence genes namely, *ast*A, *sen*B, and *gad*. All of these 5 isolates had the gad gene while only 2 had all the 3 genes (Figure 1). *ast*A encodes enteroaggregative *E. coli* heat-stable enterotoxin (EAST 1), *sen*B encodes TieB enterotoxin, while gad is a glutamate decarboxylase protein. The contribution to pathogenicity of gad has been debatable; this enzyme has been reported to be essential in the survival of enteric pathogens in the acidic conditions of the mammalian stomach (Lin et al., 1996). Two isoforms of glutamate decarboxylase (GAD) encoded by *gad*A and *gad*B are the most effective *E. coli* acid resistance system (De Biase et al., 1999). The major role of this system might be to facilitate the colonization of the intestines by commensal strains of *E. coli*. This is a housekeeping gene in *E. coli*, but not part of the *K. pneumoniae* core genome and here it was 31.3%.

EAST 1 has been identified as a plasmid-mediated enterotoxin of low molecular weight and associated with enteroaggregative *E. coli*. It shares about 50% protein identity with heat-stable enterotoxin (STa), and the gene has also been found in many enterotoxigenic *E. coli* strains (Contreras et al., 2011) and other members of Enterobacteriaceae such as *Salmonella* (Paiva de Sousa et al., 2001). The authors demonstrated the presence of the gene in different strains of *K. pneumoniae* (ST914 and ST1962) from two different geographic regions of Nigeria, and these patients presented with diarrhoea episodes. There are reports debating whether astA is sufficient to cause diarrhoea without other virulence factors in *E. coli,* but Soto et al. (2009) and Mirzarazi et al. (2015) reported that *E. coli* acquired this toxin to become a diarrhoea-causing agent, which may be the situation in these *K. pneumoniae* strains.

The two *K. pneumoniae* that encoded *ast*A also encoded *sen*B, a plasmid-mediated gene associated with enterotoxicity of enteroinvasive *E. coli* (EIEC) that codes the TieB protein. It has also been described to have some role in uropathogenic *E. coli*. Multiple plasmid replicons were present in all strains; however IncF and Col plasmids play a major role in the dissemination of these genes (Figure 1).

Susceptibility testing identified all 5 isolates with

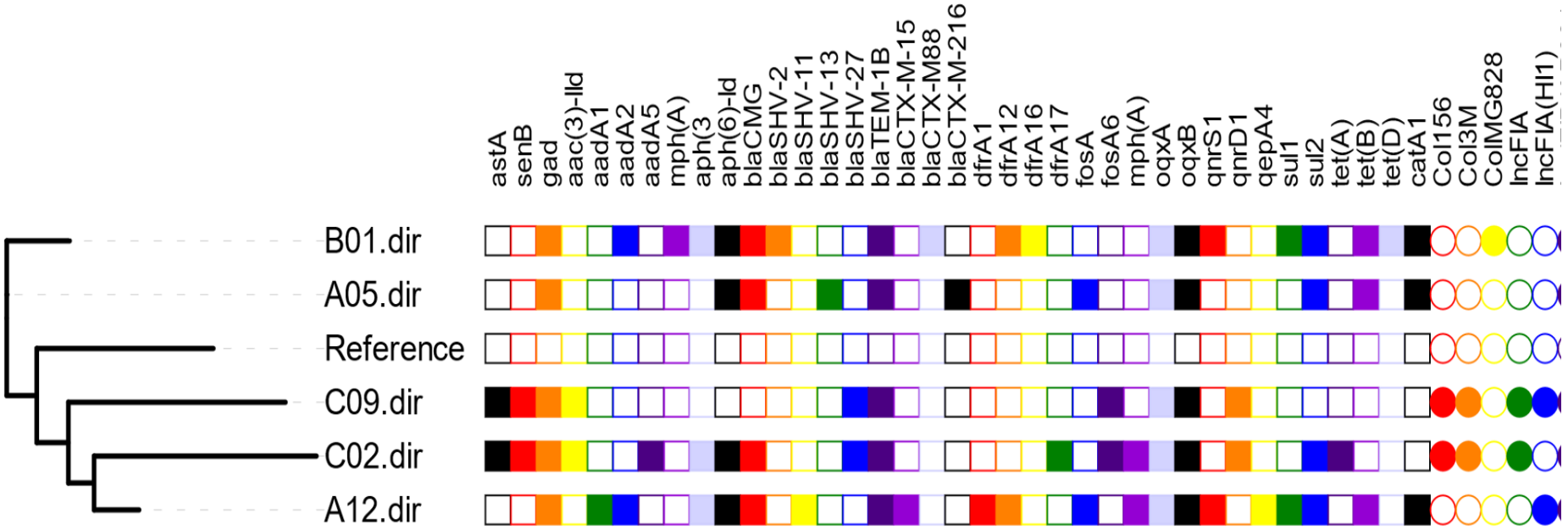

Figure 1. Relationship of the 5 *K. pneumoniae* strains carrying virulence genes (the first three filled boxes indicate presence of virulence genes). Filled boxes indicate antimicrobial resistance genes. Filled circles indicate plasmid replicons. Filled stars indicate antimicrobial resistance, MPM: Meropenem; CTX: Cefotaxime; CIP: Ciprofloxacin; GEN: Gentamycin; AK: Amikacin; COL: Colistin; AZM: Aztreonam.
Source: Authors

these virulence genes were sensitive to meropenem (MIC values of ≤ 0.03 µg/ml) while isolates with both *ast*A and *sen*B had low level resistance to ciprofloxacin and cefotaxime (MIC value of 8 µg/ml each), whereas other strains had high level resistance to ciprofloxacin (MIC value of >64 µg/ml). Resistance to colistin varied with MICs between 1 and >64 µg/ml. The isolates carried a range of important resistance genes to cephalosporins, fluoroquinolones, aminoglycosides, with the presence of various PMQR genes, aph(3'')-Ib, aac(3)-IId, variants of blaCTX-M including blaCTX-M-15 found in one of the isolates.

Conclusion

There is presence of diarrhoeagenic *K. pneumoniae* linked to carriage of plasmid mediated virulence genes such as *ast*A, *sen*B, and *gad* in diversity strains in Nigeria. This may represent a greater burden of diarrhoea caused by *K. pneumoniae* in future.

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CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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Full Length Research Paper

Biosorption of heavy metals using bacterial isolates from e-waste soil

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The discharge of untreated wastes containing heavy metals into the environment is a challenge to living cells, and in the search for treatment method, biosorption has been an economical and easy technique for heavy metals removal. This study was carried out to determine the biosorption capacity of bacterial isolates from electronic wastes soil. The isolates from e-waste soil were screened and selected for heavy metals such as Chromium (Cr), Lead (Pb), Copper (Cu), and Cobalt (Co), using agar plate method incorporated with 10 ppm of analogous solution of the test heavy metals with each isolate spotted on the agar surface and incubated for 4 days, revealed that *Bacillus cereus* S13 had the highest biosorption efficiency (highest zone of clearance) of 98% for Pb and Cu, while the least adsorbed metals were Pb (93.5%), Co (93.7%), and Cr (93.9%) by *B. cereus* S25. The biosorption potential of the selected bacteria was measured with atomic absorption spectrophotometer (AAS). The spectrophotometric analysis of heavy metals biosorption by isolates showed that *B. cereus* S13 efficiently removed 97.4% Cr and 95.9% Pb, while *B. cereus* S36 adsorbed 95.5% Pb and Cr at 20 ppm. *B. cereus* S27 biosorption capacity increased with increase in concentration of heavy metals used except for Pb (96.9%) where larger percentages were removed from the solution at lower concentration. Conclusively this study affirmed that *B. cereus* strains from electronic waste remediated heavy metals in aqueous solution and therefore, could be promising adsorbent of heavy metals particularly chromium, lead, and cobalt.

Key words: Biosorption, heavy metals, bacteria, potential.

INTRODUCTION

Heavy metals are natural elements with atomic numbers greater than 20, characterized by a relatively high density (at least 5 gcm^{-3}), with a health-impactful toxicity even at low concentrations on living organisms such as plants, animals and microbes (Murthy et al., 2012). They are characteristically existing components found in changing variation in the environments and are part of human daily activities, they are also found in important structures and in a range of other artificial mixes. The activities of human such as wood burning, mining, fertilizer application to the soil, combustion of coal, smelting, incineration and landfill disposal of wastes containing heavy metals have greatly

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impacted their biochemical cycles (Srivastava et al., 2017; Ali et al., 2019). Scientist have searched and are still on the lookout for the cheapest source of removal of these metals from wastes. However, presently biosorption is a very cheap and effective means of removing heavy metals in liquid wastes, and being a non-living method of treating wastes contaminated with heavy metals using dead microbial cells, has proven to be effective over bioaccumulation (active uptake) via the feasibility study conducted on the large scale application of microorganisms in active uptake of heavy metals from liquid wastes, where the latter required continuous addition of nutrients in to the biomass medium thereby raising the biological oxygen demand (BOD) or chemical oxygen demand (COD) of the liquid wastes. Also not limited to this factor, is the difficulty in maintaining a healthy microbial population due to metal toxicity as well as the potential recovery of intracellularly absorbed metals are also narrow since these metals can form complexes with other metabolites in solution (Briffa et al., 2020). Diverse species of bacteria are present in the soil some of which have been used to rid-off heavy metals from liquid effluent. Bacterial species which have been isolated from the soil and reported to have been used for heavy metals biosorption are *Pseudomonas, Micrococcus, Escherichia, Streptococcus, Enterobacter, Staphylo*c*occus* etc. (Murthy et al., 2012). Thus, this study aimed at isolating bacteria from e-waste polluted soil and using the isolates to adsorb heavy metals in aqueous solution.

MATERIALS AND METHODS

Collection of electronic waste soil

The Electronic waste soil was collected from an electronic dumping site at Apete Akufo area, Ibadan, Oyo State Nigeria. The soil was collected at four different locations on the site. Into a sterile polythene bags and were immediately transported to the microbiology laboratory of Oyo State College of Agriculture and Technology Igboora, for further microbiological analysis.

Isolation of bacterial from E-waste soil

Ten grams (10 g) of the soil sample was weighed in to 9 ml of sterile water in a conical flask and mixed together on an electric shaker. Serial dilution was performed on the soil solution by pipetting 1 ml of soil solution into 9 ml of the diluent (sterile water) in a test tube with gentle mixing. Desired serial dilutions were carried out on the soil solution and were plated on the nutrient agar plate in triplicated. The inoculated plates were incubated at 25-28°C for 48 h. Pure isolates of the bacteria from soil sample was obtained and isolates were stored on agar slant for further study (Kumar et al., 2010).

Screening of bacteria isolates for biosorption of heavy metals

The method of Kumar et al. (2010) was used with modification to screen heavy metals biosorption capacity of the bacterial isolates. The bacterial isolate was standardized using 0.5 McFarland standard. Sterile molten nutrient agar containing 1 ml of 10 ppm of each heavy metals (Co, Cr, Cu, and Pb) prepared in 100 ml standard flask was poured on plates and each standard isolate was inoculated on agar surface by swabbing and in replicates. Plates were incubated at 28 ± 2°C for 3 days. Colonies surrounded by a cleared zone were selected for further study. Bacterial isolates were selected for biosorption of the heavy metals.

Phenotypic identification of bacterial isolates with biosorption potential from e-waste soil

The preliminary phenotypic identity of the bacterial isolates with biosorption potential was done through the subjection of the isolates to various biochemical test such as catalase, oxidase, grams staining, spores staining, sugar utilization etc. as shown in table 2. The isolates were identified using Bergey's manual of bacteriological identification.

Molecular characterization of bacterial isolates

Extraction of the DNA

The method of Patra et al. (2010) was used for extraction with slight modification. The DNA was extracted from each bacterium by growing the culture in a 10 ml volume of broth medium. The cultures were grown for 5 days in a shaking incubator (80 rpm) at 25°C and 2 ml of each culture was centrifuged in a sterile micro centrifuge at 13,000 rpm. The supernatants were discarded and the pellet was transferred to Mo Bio Ultra Clean Soil DNA Kit (Laboratories Inc, CA, USA) and DNA extracted according to the manufacturer's instructions. The extracted DNA was electrophoresed on 1% agarose gel in TAE buffer and visualized under UV (Gel Doc, Bio-Rad Laboratories, USA) to check for integrity. The DNA was stored at 20°C until further analysis.

Amplification of the DNA

A Mini Cycler (MJ Research, Inc., Watertown, MA) heated lid thermos cycler was used to amplify DNA; 25-ll reactions were prepared by adding 8 pmol of each primer (0.8 ll of primer mixture), 0.5 ll of DNA sample, 12.5 ll Master Mix from Promega, and pure sterile water to 25 ll. All amplification reactions were hot started at 95°C for 3 min. The polymerase chain reaction (PCR) protocol used with short universal primers was: 94°C 90 s and 33 cycles, a final extension step at 72°C for 3 min, 4°C. When Golden Mixtures (G1–G13) were used, PCR parameters were the same as above except for annealing temperature which was set at 5°C for 1 min (Patra et al., 2010).

Purification of the DNA

Agarose Gels Containing Ethidium Bromide (0.1 Lg Per Ml) were used throughout the study at concentrations of 1.2. 1.6% (w/v). LKB power supply (Biochrom, Cambridge, England) and UV Trans-illuminator (Dinco and Rhenium Industrial Ltd.,), 100-bp ladder was used as molecular weight markers. Gels were photographed using a digital camera (Casio Exilim, Tokyo, Japan) at 3-8 mega pixels with sepia or black and white filter (Sameer et al., 2010).

Sequencing of the amplified DNA

The 16SrRNA gene sequence of the bacterial strain was determined through lysis of the cells. The 16SrRNA fragments

Table 1. Heavy metals biosorption capacity of Bacterial isolates screened at concentration of 10 ppm.

Isolate code	Pb (%)	Cr (%)	Co (%)	Cu (%)
S25	93.5^{a}	93.9^{a}	93.7^{b}	91.0e
S13	98.0^{bcd}	96.0^{c}	97.0^{b}	98.0^{bcd}
S36	97.2^{b}	94.7^{a}	96.7^{c}	96.2^{cd}
S27	97.7^{b}	96.7^{b}	97.0^{b}	98.0^{c}

Values within the column with different letters are significantly different P≤0.05.
Source: Authors

obtained was amplified by PCR using the universal primer forward 5'-AGATTT-GATCATG GCTCGA-3' and the reverse 5'-GGCTACC-TTGTTACGACTT-3' (position 1510-1492). The sequences of the amplified 16 rDNA fragments amplified was analysed using gene bank and compared with national centre for biotechnology information (NCBI) (Igiri et al., 2018).

Biosorption of the selected heavy metals

Five millimetres of sterile nutrient agar broth containing 1 ml of each standard 10 ppm and 15 ppm of each heavy metal were prepared separately in MacCartney bottles. 1 ml of each standard isolate was inoculated into each broth medium and inoculated bottles were incubated for 5 days at room temperature with constant shaking. After, centrifugation was carried out at 1792G for 25 min. The supernatant was digested using nitric acid of heavy metal solution sample. The concentration of metal was determined by absorption spectrophotometry (UV–Vis 752, UK) (Ahemad and Kibret, 2013). The percentage of biosorption was be determined with the formula; (%) biosorption= initial metal concentration − final metal concentration*100/initial metal concentration.

RESULTS AND DISCUSSION

The increase in industrialization has brought about a daunting increase in the discharge of heavy metals and other pollutants to the surrounding environment particularly soil and water resources. Diverse microorganisms are found in metal polluted environment, and some are said to have adapted and able to tolerate the toxic condition of heavy metals due to stress induced in solution. These microbes could be used to decontaminate the environment from heavy metals through various processes such as adsorption, oxidation and reduction, bio accumulation, methylation and demethylation (Briffa et al., 2020). The bacterial isolates were subjected to biosorption of the same concentration of heavy metals (10 ppm) under the same environmental conditions in order to know their sorption capacity. All the strains of bacteria as shown in Table 1, were able to adsorb heavy metals at different capacity (except for lead (Pb) where only S25 had the least adsorption percentage of 93.5%) which may be due to strains inherent properties such as nature of the metal binding sites and cell wall. The bacterial isolates S13 and S27 which were later identified as *B. cereus* strains, removed 96.7% of chromium from aqueous solution while S13 adsorbed 98% cupper more than the *Pseudomonas aeruginosa* used by Oyewole et al. (2019) to remove the same concentration of the metals in aqueous solution; an implication that the sorption efficiency of *B. cereus* than *P. aeruginosa*, may be due to its cell wall components and being gram positive bacterium have more peptidoglycan layers than *P. aeruginosa* (gram negative bacterium). Peptidoglycan layers are negatively charged by the presence of hydroxyl, amino and phosphate ions on their surfaces which are capable of binding positive metals ions in aqueous solution for removal (Kumar et al., 2010).

The biochemical identification of bacterial isolates with biosorption potential for heavy metals is shown in Table 2. Isolates S36, S13, S25 and S27 were gram positive bacteria, motile, spore formers, catalase and citrate positive. Sugar utilization revealed that all the isolates were positive for glucose and galactose. S13, S25 and S27 were negative for gelatin hydrolysis while S25 demonstrated variable reactions for growth in potassium cyanide and oxidase reaction. All the isolates were indole and methyl red negative. However, S36, S13 and S25 were lactose negative. The probable bacteria identified in this study were various types of *B. cereus*.

The phylogenetic tree of ancestral relationship of the bacterial isolates with potential for heavy metals biosorption using 16SrRNA relationship between nucleotide sequences is showing Figure 1. The phylogenetic tree was compiled based on the alignment of partial 16SrRNA sequence. The bacterial isolates S13, S36 and S27 were observed as *B. cereus* but of different strain. Table 3 showed the Atomic Absorption Spectrophotometric (AAS) analysis of heavy metals adsorbed by bacterial isolates from electronic waste soil. The three strains of *B. cereus* (S13, S36, and S27) selected for used in this study demonstrated comparable removal of heavy metals in aqueous solution. At the concentration of 20 ppm of the heavy metals in aqueous solution, S27 largely removed cobalt and lead (98.8 and 96.9%) followed by S13 and S36 which adsorbed 95.9 and 95.5% lead (Pb) respectively, this may be due to the difference in resistant mechanism of each strain to heavy metals. However, *B. cereus* S13 was able to effectively remove almost all the heavy metals in aqueous solution in contrast with the remaining *B. cereus* S36 and S27 because the percentage of heavy metals removal

Table 2. biochemical identification of bacterial isolated from electronic waste soil.

Isolate code	Catalase	Citrate	Gelatin hydrolysis	Gram' reaction Growth in KCN	Haemolysis	Indole	Mortality	Methyl Red	Nitrate Reduction	Oxidase	Shape	Voges-proskauer	Fructose	Glucose	Galatose	Glycogen	Lactose	Maltose	Sucrose	Probable bacteria
S25	+	+	±	+	+	+	-	+	-	-	-	rod	+	+	+	+	+	-	+	-	*Bacillus cereus*
S13	+	+	-	+	+	+	-	+	-	-	-	Rod	+	±	+	+	+	-	+	+	*Bacillus cereus*
S36	+	+	-	+	±	-	-	+	-	±	±	Rod	-	+	+	+	+	-	-	+	*Bacillus cereus*
S27	+	+	-	+	+	-	-	+	-	+	-	Rod	-	+	+	+	-	+	-	+	*Bacillus cereus*

+ Means positive reaction, - means negative reaction .
Source: Authors

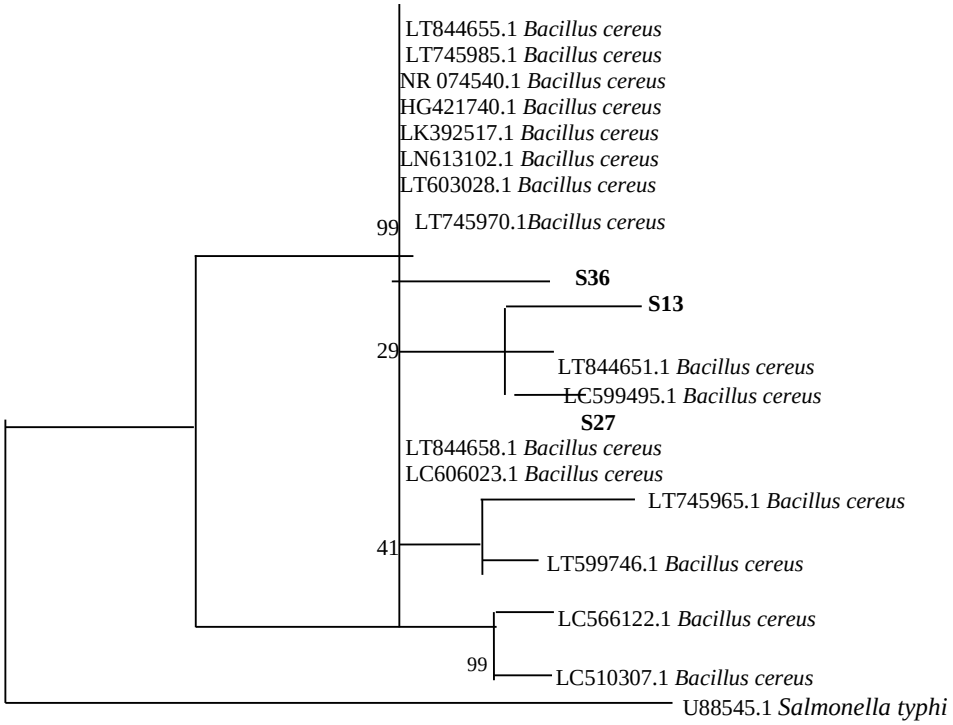

Figure 1. Phylogenetic tree of relationship of the isolates with their ancestors.
Source: Authors

Table 3. Biosorption of heavy metals by selected bacterial isolates at concentration of 15 and 20 ppm.

Isolate codes	Concentration (%) at 15 ppm				Concentration (%) at 20 ppm			
	Pb	Cr	Co	Cu	Pb	Cr	Co	Cu
S13	90.5^{a}	93.0^{b}	93.0^{b}	88.6^{ad}	95.9^{e}	97.4^{c}g	94.9^{ef}	94.5^{ef}
S36	93.7^{b}	92.5^{b}	83.2^{b}	91.4^{ab}	95.5^{e}	95.5^{e}	95.2^{e}	94.8^{ef}
S27	96.9^{c}	91.7^{ab}	88.2^{d}	90.3^{a}	94.5^{ef}	94.5^{ef}	98.8h	94.6^{ef}

Mean values with different letters are significantly different at P≤ 0.05.
Source:Authors.

increased with increase in concentration. This may be the result of increase in the affinity of the cell-metal binding sites for the metal ions since microbes under an extreme environmental condition have high surface area to volume ratio which provides them large interaction with matters in the environment. This result disagrees with the findings of Murthy et al. (2012) where a decrease in percentage removal of Pb resulted from increased concentration of the metals. The phylogenetic relationship of the isolates with their ancestors is shown in Figure 1. S36, S13 and S27 were seen to be 99% closely related to their parent which was *B. cereus.*

Conclusion

Biosorption of heavy metals (Chromium, Lead, Copper and Cobalt) were conducted using bacterial isolates from electronic waste soil. The results obtained in this experimental work showed that the isolates were able to adsorb various concentrations of heavy metals. An implication that strains of bacteria used are effective adsorbent for removal of these heavy metals from electronic waste soil. The results of this work revealed that the bacterial isolates *B. cereus* used was able to remove the heavy metals significantly from the aqueous solution. Therefore, it is recommended that further research should be focused on the use of these isolates to remediate these metals from other wastes and also from electronic wastes at higher concentration with regards to variable factors such as pH, time of exposure of adsorbent to the heavy metals solution in order to further ascertain their potency.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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