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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing *Escherichia coli* in the Calgary Health Region: emergence of CTX-M-15-producing isolates. *Antimicrob. Agents Chemother.* 51: 1281-1286.

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Physiological and sanitary potential of peanut seed treated with cinnamon powder

Adrielle Naiana Ribeiro Soares, Edilma Pereira Gonçalves*, Jeandson Silva Viana, Priscila Cordeiro Souto and Mácio Farias de Moura

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This study aimed to assess the physiological and sanitary potential of peanut seeds cv. BR1, treated with powdered cinnamon (*Cinnamomum zeylanicum*). The experiment was conducted in the laboratories of the Seed and Plant Pathology and Microbiology at the Federal Rural University of Pernambuco / Academic Unit of Garanhuns (UFRPE / UAG). The experimental design was completely randomized, with four replications of 50 seeds. The treatments consisted of treating the seeds with cinnamon powder at different doses (0: control; 2, 4, 6, 8 and 10 g) in the ratio of 2 g powder and 81.2 g. The seeds were soaked in 1% sodium hypochlorite for 3 min, and then immediately subjected to the following tests: germination, speed of germination index, seedling length, seedling dry matter and seed health testing. Fungi of the genus *Aspergillus*, *Penicillium* and *Rhizopus* were detected greatly in the treatments with the highest dose. Sodium hypochlorite was effective in combating fungal growth, but damaged the physiology of the seeds. Using cinnamon powder of 5 g was efficient in controlling fungi and did not affect the seed quality of peanuts.

Key words: *Arachis hypogaea*, natural fungal control, *Cinnamomum zeylanicum*.

INTRODUCTION

Peanut (*Arachis hypogaea* L.) belongs to the family Fabaceae; subfamily Papilionidae, and gender *Arachis*. The economic importance of peanuts is linked to their having pleasant taste and seeds having rich oil (40 to 50%) and protein (22 to 30%) (Silveira et al., 2011). In Brazil, production is more significant in São Paulo, which is the largest producer; it accounted for 96.67% of the country's total production in the 2012/2013 crop season, followed by Bahia State, with 3.33% (Conab, 2013). In

the semiarid region of Pernambuco State, it has been tested that doing intercropping with cactus leads to achieving high yields (Andrade et al., 2015).

Peanut crop is propagated by seeds, which constitutes a major component production. It is of great importance to maintain the quality of seeds used in the implementation of this culture, especially regarding its genetic, physical, physiological and phytosanitary attributes (Grigoletto et al., 2012). Seed quality is of

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utmost importance in the production process of any plant species, because it influences the development of culture.

The physiological quality of peanut seeds can be compromised, and even lost gradually. This can happen through deterioration influenced by fungus contamination (especially those of the genus *Aspergillus*, *Penicillium*, *Fusarium* and *Rhizopus*) during the stages of maturation and harvest (Pitt et al., 1991), post-harvest, drying (Fernandez et al., 1997) and processing (Almeida et al., 1998).

In conventional production, seeds are often treated with chemical fungicides. This reduces seed and seedling losses caused by seed-borne and soil-borne diseases. Most seed protectants are not an option for organic growers. Herbal and plant extracts treatment and the use of hot water can be used by organic farmers to improve seeds' performance. It is therefore necessary to search for control measures that are cheap, ecologically sound and environmentally safe to eliminate or reduce the incidence of these economic important pathogens, so as to increase seed germination.

The losses caused by pathogens has been reduced, mainly by the use of chemicals, either pre- or post-harvest, however, the use of agrochemicals has led the emergence of numerous high severity problems such as the emergence of microorganisms resistant to fungicides, environmental damage and harm to human health (Cruz et al., 2009). Alternatively, the use of pesticides has been researched as natural products. Shah et al. (1992) reported that *Argemone mexicana* seed extract was effective in eliminating most of the seed-borne fungi of cowpea but was not effective against *Alternaria alternata*, *Curvularia lunata*, *Mucor* sp. and *Macrophomina phaseolina*.

Some plants have in their chemical composition active substances that can act as fungitoxic in plant-pathogen interactions with other antimicrobial activities; they can even activate the defense mechanisms of the host plant with pathogen control programs, thus reducing the indiscriminate use of pesticides (Silva et al., 2011).

In this sense, medicinal plants have been extensively studied for the control of various pathogens, due to the active principles present with potential fungicidal and / or bactericidal. Among them, we can mention Cinnamon (*Cinnamomum zeylanicum* L.); in addition to its use as a spice or condiment for providing flavor and aroma in food, it has fungitoxic properties; the main antimicrobial constituent, cinnamaldehyde (Ranasinghe et al., 2002).

There is a growing need to evaluate the sanitary quality of peanut seeds, in order to select highly physiological quality and pathogen free ones. Thus, this study aims to evaluate the physiological and sanitary quality of peanut seeds cv. BR1 treated with cinnamon powder.

MATERIALS AND METHODS

The experiment was conducted at the Seed Analysis Laboratories

and Plant Pathology and Microbiology, Federal Rural University of Pernambuco / Academic Unit of Garanhuns (UFRPE/UAG), Pernambuco State. The peanut seeds cv. BR1 used were purchased from Embrapa Cotton - Campina Grande, Brazil.

At the Seed Analysis Laboratory, the peanut cultivar BR1 seeds were treated as follows: the control (0) and the experimental seeds were immersed in sodium hypochlorite for 3 min, according to Brasil (2009), using different doses of cinnamon powder (*Cinnamomum zeylanicum* L.): 2, 4, 6, 8 and 10 g. Seeds treated with cinnamon powder dose remained conditioned for 24 h in a plastic bag together with dust. Treatment with 1% sodium hypochlorite solution was done according to the recommendations given for Sanitary Analysis Manual seeds (Brasil, 2009). To obtain the hypochlorite 297 mL of distilled water was mixed with 3 mL of sodium hypochlorite. After the treatments, there was the germination and health test.

Physiological quality test

The germination test was performed according to guidelines of the Rules for Seed Analysis-RAS (Brasil, 2009), using germination chambers type Biochemical Oxygen Demand (B.O.D), equipped with fluorescent lamps. It was done under constant temperature of 30°C in substrate, Germitest brand; it was sterilized for 20 min at 105°C ± 3°C and moistened with distilled water. The amount was equivalent to 2.5 times the weight of the paper.

The percentage of germination was carried out at 10 days after sowing. Seedlings with well-developed essential structures were considered as normal (Brasil, 2009).

First count

First count was held on the fifth day after the beginning of the experiment, corresponding normal seedlings.

Speed of germination index

Speed of germination index was carried out simultaneously with the germination test; daily counts were done from the fifth day. At the end of the test, the index was calculated using the formula:

$IVG = \frac{G1}{N1} + \frac{G2}{N2} + \dots + \frac{Gn}{Nn}$, where IVG = germination rate index; G1, G2 and Gn = number of normal seedlings, computed in the first, second... and last count, respectively; N1, N2, Nn = number of days from sowing to first, second... and last count, respectively (Maguire, 1962).

Seedling length

Seedling length seedlings were measured on the 10th day after sowing, using a ruler graded in centimeters, measuring from the root to the epicotyl; the result was divided by the number of seedlings.

Dry mass of seedlings

Seedlings were wrapped in paper bags without the cotyledons, put in oven at 80°C for 24 h, and then weighed on an analytical balance; the result is expressed in grams (Nakagawa, 1999).

Health test

The seeds were distributed on two sheets of paper germitest,

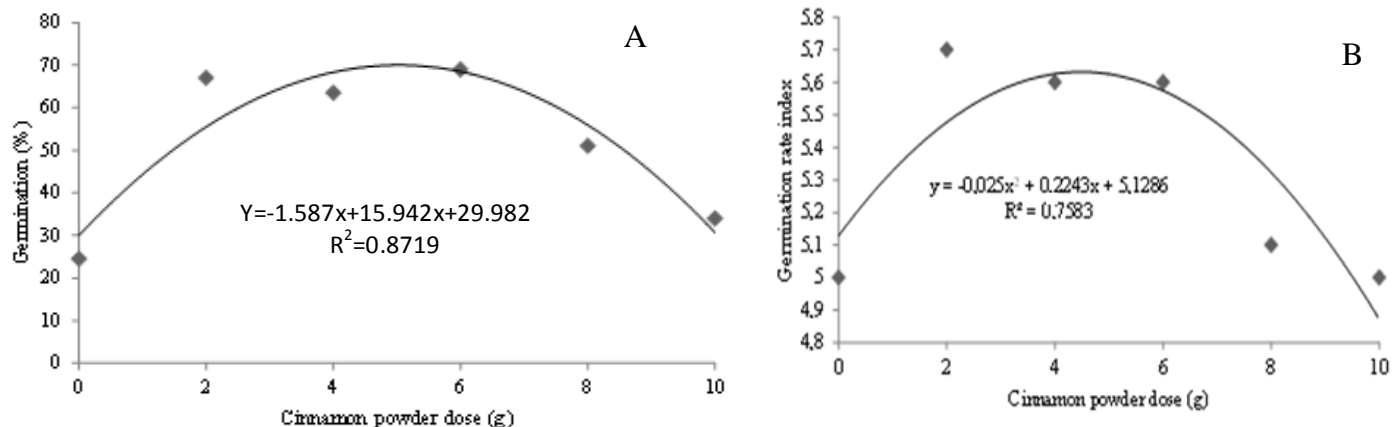


Figure 1. Germination (A) and germination speed index (B) of *Arachis hypogaea* seeds treated with cinnamon powder (*Cinnamomum zeylanicum* L.).

sterilized for 20 min in an oven at $105 \pm 3^\circ\text{C}$ and moistened with distilled water (control) and the concentrations of the extract. The amount is equivalent to 2.5 times the weight of the paper. The rolls were maintained in a growth chamber (B.O.D.), equipped with fluorescent lights at 20°C temperature. The test duration was 8 days; the evaluations were done on the eighth day after the beginning of the experiment, according to the rules of Sanitary Analysis Manual Seeds (Brasil, 2009).

To identify and quantify the percentage of fungi, each seed was visualized with the aid of a magnifying glass (60 times magnification) and an optical microscope, allowing the detection of fungal structures. The aggregation of fungi was performed by percentage of seeds contaminated, resulting in the average per treatment.

For physiological and health quality tests, we used the completely randomized design with four replications of 50 seeds, totalling 200 seeds per treatment. The data of the variables measured in the experiment were subjected to analysis of variance using the F test and the means compared by Dunnett "t" test at 5% probability. Quantitative data was submitted to polynomial regression analysis, testing the linear and quadratic model, choosing the highest R^2 . The analyses were done using the software SAEG (Ribeiro, 2001).

RESULTS AND DISCUSSION

Figure 1 shows the results of germination (A) and germination rate index (B) on *Arachis hypogaea* seed cv. BR1 treated with doses of cinnamon powder. When the seeds were treated with a dose of 5.0 g of powder, there was an increase in the percentage of germination (71%); the highest rate was 5.6 at a dose of 4.5 g. It can be seen that the treatment of seeds with cinnamon powder did not impair germination and germination speed index. Ferreira and Áquila (2000) found that germination is less sensitive to allelochemicals compared to the growth of seedlings. The better performance of treated seeds compared to the control is because cinnamon powder is reliable to control fungi.

The answer to the interference of allelochemicals on

seed germination depends on the concentration used (Ciarka et al., 2002). Furthermore, the influence of the type of extract also depends on the sensitivity of the tested plant to allelopathic compounds present; with certain substances it can inhibit germination or growth, and with others it can be the same or stimulating innocuous (Almeida et al., 1998).

This study confirms that natural mycoflora present in seeds of *A. hypogaea* was capable of causing poor seed germination and had negative influence on seedling growth. *C. zeylanicum* L. increase germination and germination speed index in some doses. This could be attributed to the suppression of the incidence of the seed borne fungi that could have killed the embryo of the seeds. This result is consistent with that of Parimelazhagan and Francis (1999) who established that leaf extracts of *Clerodendrum viscosum* increased seed germination and improved seedling development of rice seeds.

Cinnamon has a chemical composition in the presence of substances such as cinnamic aldehyde (75-90%), aldehyde benzoic acid, cinnamic acid, coumarin, methyl-ortocumaraldehyde, salicylic acid methyl ester to cinnamic acid (Gerhardt, 1973). The cinnamaldehyde is the main component in fungal activity present in cinnamon extracts. Other chemicals have additive or synergistic effect of totally fungitoxicity (Jham et al., 2005).

The values for the length (A) and seedling dry weight (B) derived from peanut seeds treated with cinnamon powder are shown in Figure 2. It was observed that when the seeds were treated with 5.2 g of powder, there was the greatest seedling length of 7.7 cm and the greatest value for the dry mass was 0.025 g at a dose of 4.8 g. That dose reduced early growth and dry mass. Probably, this interference is related to the high concentration of allelochemicals present in cinnamon powder, as cinnamic acid. The compound (E) - cinnamaldehyde (97.7%) was

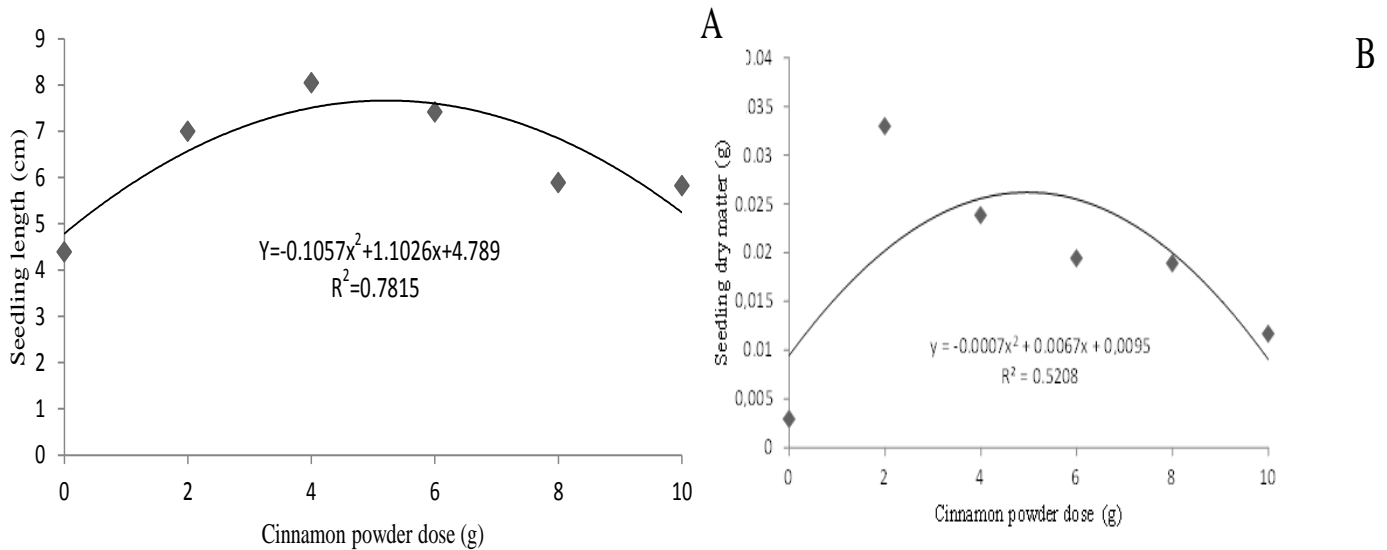


Figure 2. Length (A) and seedling dry weight (B) of *Arachis hypogaea* treated with powdered cinnamon (*Cinnamomum zeylanicum* L.).

found in large amounts (97.7%) in the essential oil of *C. zeylanicum*, being considered as the main component (Singh et al., 2007). The initial growth phase appears to be more sensitive to allelochemicals than germination, which may lead to abnormal seedlings, root necrosis, or alterations in the growth (Ferreira and Borghetti, 2004). The influence of natural extracts on the growth of seedlings shows the ability of compounds acting at this stage of development (Lousada et al., 2012). The potential of natural products to inhibit the growth of seedlings may be related to the chemical constituents present in the plant or the interaction of its components (An et al., 1993).

The results obtained by Nwachukwu and Umechuruba (2001) showed that crude extracts from all the plant leaves tested increased seed germination, led to the emergence of African yam bean seeds and gave significant ($P \leq 0.05$) reduction of mycelial growth of all the fungi tested compared to their aqueous extracts. Leaf extracts of neem, basil, bitter leaf and paw-paw, which are cheap and environmentally safe, are promising for protecting African yam bean seeds against major seed-borne fungi and can also improve the crop. Perelló et al. (2013) suggest that seeds treated with garlic juice had a relatively better germination percentage, plumule and radicle length, and seedlings than those seeds treated with high inoculum density of the target pathogen, *Bipolaris sorokiniana*. The inoculum level of naturally infected wheat seeds could be reduced through the use of garlic juice as seed dressing biofungicide, before sowing. Kiran et al. (2010) and Yassin et al. (2012) recorded the efficacy of the antifungal properties of some herbaceous and medicinal plants against cereal seed-borne mycoflora. The variation in results related to seedling growth can be explained by the fact that there is

a pattern of response to the concentration. Certain extracts even at low concentrations can interfere with growth of some species and not others. Hence, there is need to always perform specific tests involving species of plants and extracts concentrations (Premasthira and Zungsontiporn, 1996).

Figure 3 shows the results for the control of phytopathogens using peanut seeds treated with cinnamon powder. The use of cinnamon powder proved to be efficient in controlling these types of fungi (*Aspergillus niger*, *Penicillium* spp. and *Rhizopus* spp.); it led to a marked decrease in the percentage of these fungi with increasing doses. This result agrees with the findings of Zaman et al. (1997), that the efficacy of garlic, neem, ginger and onion extracts on seed borne fungi of mustard declined with increased dilution.

Similar results were found by Mamprim et al. (2013) on plant extracts of cinnamon, rue (*Ruta graveolens*), cinnamon (*Melia azedarach*), rosemary (*Rosmarinus officinalis*), lemon grass (*Cymbopogon citratus*) and citronella (*Cymbopogon winterianus*), which caused a reduction in diameter growth of *Metarhizium anisopliae* (Metsch.) Sorok from 1.8 to 7.6%. The essential oils of cinnamon and oregano (*Origanum vulgare*) at a concentration of 100 ppm have antifungal effect against *Aspergillus flavus* (Camarilho et al., 2006).

The results observed in the physiological quality test (Figures 1, 2 and 3) indicate that the control treatment showed lower seed behavior for germination and vigor compared to those treated with cinnamon powder. This can be justified by the presence of fungi in seeds untreated. The incidence of seed-borne pathogens can affect the physiological quality and, in some cases, inhibit seed germination. It is therefore necessary to search for control measures that are cheap, ecologically sound and

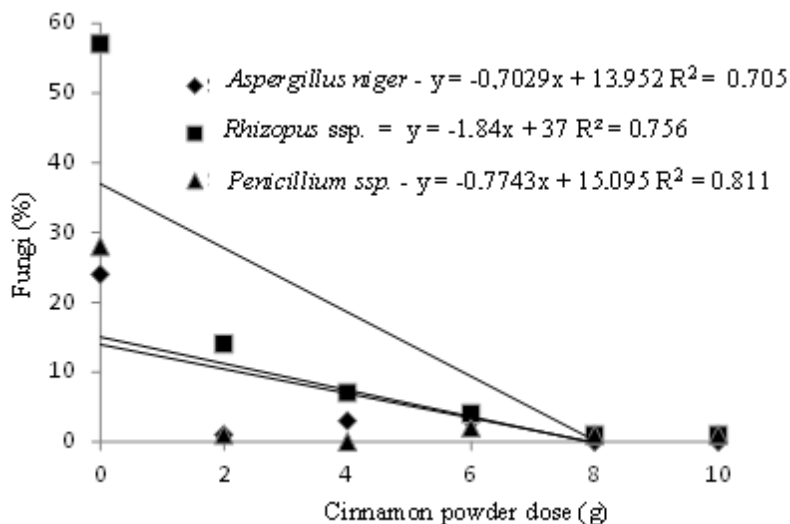


Figure 3. Percentage of fungi in *Arachis hypogaea* seeds treated with doses of powdered cinnamon (*Cinnamomum zeylanicum* L.).

environmentally safe to eliminate or reduce the incidence of these economic important pathogens, so as to increase seed germination.

Chavan and Kakde (2008) reported that fungi such as *A. niger*, *Aspergillus flavus*, *Alternaria dianthicola*, *Curvularia lunata*, *Curvularia pellescens*, *Fusarium oxysporum*, *Fusarium equiseti*, *Macrophomina phaseolina*, *Rhizopus stolonifer*, *Penicillium digitatum* and *Penicillium chrysogenum* cause discoloration, rotting, shrinking, seed necrosis, loss in germination capacity and toxification to oil seeds. *Aspergillus* is a common mould in tropical and sub-tropical countries and causes aflatoxin contamination as a result of moulding of badly stored commodities, such as groundnut, cereal and cotton seeds.

Saha et al. (2014) concluded that seeds like garlic tablet, allamanda tablet or neem leaf extract can be recommended for okra seed treatment to get higher germination and healthy seedling that will eventually produce more fruit.

Aspergillus spp. and *Penicillium* spp. are storage fungi. The interaction of seeds with these fungi can significantly increase the speed of reducing the quality of seeds (Neergaard, 1979). Also, the genus *Rhizopus* spp. is also considered a storage fungus that hinders the identification of other pathogens present in peanut seeds, due to its rapid growth. It covers the seed surface. The growth of storage fungi in seeds can cause death of the embryo, reduce twinning, overturning and cause rot (Mazzani and Layrisse, 1992; Bhattacharya and Raha, 2002). Thus, assessment of health quality is extremely important to check the quality of seeds.

Several studies have been developed in the search for natural products with fungitoxicity and their application in the control of fungi that cause damage to crops of

economic interest (Hillen et al., 2012). Among these products is cinnamon. Cinnamic aldehyde is present in high concentration (77.72%) of cinnamon; it is the main chemical component, and is primarily responsible for inhibiting the growth of pathogenic micro-organisms (Andrade et al., 2012).

Table 1 shows the results of the variables analyzed for germination, as well as the vigor and health of peanut seeds immersed in sodium hypochlorite and those treated with cinnamon powder doses. The first count, the germination speed index and the germination of treated peanut seeds showed significant effects compared to those seeds immersed in sodium hypochlorite solution 1%, for 3 min; except for those treated with 10 g, they were no significant differences. In addition, we observed higher number of abnormal seedlings when seeds were immersed in sodium hypochlorite and higher number of normal seedlings from seeds subjected to doses of cinnamon powder.

These results indicate that cinnamon powder doses were superior to the control and sodium hypochlorite treatment; and even though the use of sodium hypochlorite is a method recommended by RAS, it impaired the germination of peanut seed; this is presumably because soaking causes damage to seed membranes. Evangelista et al. (2007) state that the membranes can suffer from injuries when they are immersed in water when the seeds have water content less than 12%. The first soaking is considered critical because there is a rapid release of electrolytes to achieve a balance in the membranes. In that work, the peanut seeds were under 10% humidity (Kraft, 1997). The seeds of this species have a high degree of susceptibility to injury caused by rapid soaking through immersion in distilled water.

Table 1. *Arachis hipogaea* seeds treated with cinnamon powder dose (*Cinnamomum zeylanicum*) and immersed in sodium hypochlorite (1%).

Treatment	Control (sodium hypochlorite)									
	FC (%)	G%	GSI	LS	DS	ABS	SN	AS	PEN	RIZ
2 (Control)	22.0*	-9.00 ^{ns}	1.39*	1.29 ^{ns}	-0.001	18.0*	-9.00*	43.5*	26.5*	14.5*
3 (Dose 2 g - NaHCl)	27.5*	33.5*	2.03*	1.30 ^{ns}	0.028*	-18.0*	-15.0*	4.00 ^{ns}	-1.50 ^{ns}	-1.5 ^{ns}
4 (Dose 2 g - NaHCl)	25.5*	30.0*	1.81*	2.35*	0.019*	-16.5*	-13.5*	4.00 ^{ns}	-2.00 ^{ns}	-0.5 ^{ns}
5 (Dose 4 g - NaHCl)	25.5*	35.5*	1.88*	1.72*	0.015*	-21.0*	-14.5*	-2.00 ^{ns}	-0.00 ^{ns}	-0.0 ^{ns}
6 (Dose 6 g - NaHCl)	16.5*	17.5*	1.15*	0.19 ^{ns}	0.014*	-10.5 ^{ns}	-7.50*	0.00 ^{ns}	-1.50 ^{ns}	-1.5 ^{ns}
7 (Dose 8 g - NaHCl)	21.5*	0.50 ^{ns}	1.06*	0.13 ^{ns}	0.007 ^{ns}	4.50 ^{ns}	-5.00 ^{ns}	-1.00 ^{ns}	-1.00 ^{ns}	-2.0 ^{ns}

FC, First germination count; G, germination; GSI, germination speed index; SL, seedling length; DS, dry seedling mass; SN, normal seedlings; ABS, abnormal seedlings; AS, percentage of *Aspergillus niger*; PEN, percentage of *Penicillium* spp.; RIZ, percentage of *Rhizopus* spp. ns and * = not significant, * significant at the 5% probability by Dunnett's test.

For coffee beans (*Coffea arabica* L.), Sofiatti et al. (2009) found that seed soaked in sodium hypochlorite at concentrations of 4, 5, and 6% caused a reduction in seed germination and vigor.

For the length of peanut seedlings, only 8 and 10 g cinnamon powder was superior to sodium hypochlorite treatment. The dry weight of seedlings from seeds treated with cinnamon powder dose showed significant results, except for the dose of 10 g; and when compared to seedling dry matter from the control with those of hypochlorite sodium, it was observed that, despite having a significant effect, bleach gave the best result. This is due to pathogen control.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Congruence between the drug resistance pattern of *Escherichia coli* and *Proteus* spp. isolated from humans and those from wild animals

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As human populations grow and transform landscapes, contact with wildlife concomitantly increases. Disease emergence has been an important consequence of these contacts, with many of emerging infectious diseases in humans arising from wildlife reservoirs. Drug resistance is a very important dimension of disease emergence and tracing the source is a veritable containment strategy. *Escherichia coli* and *Proteus* spp. have important roles in the increasing cases and dissemination of antibiotic resistance, potentially acquiring resistance determinants and acting as reservoirs for resistance genes. This study was conducted to determine the similarity or otherwise of the drug resistance pattern of *Escherichia coli* and *Proteus* spp. isolated from humans and those from wild animals (a source with only minimal, hypothetical or no antibiotic exposure). The human samples were obtained from two human groups: persons not on antibiotics and humans on antibiotics. Animal samples were taken from rats (*Rattus* spp.), grasscutters (*Thryonomys swinderianus*), squirrels (*Xerus erythropus*) antelopes (*Tragelephus scriptus*), rabbits (*Oryctolagus cuniculus*), and farm lizards (*Agama* spp.). Organisms were isolated and identified based on basic microbiological methods and subjected to antibiotic disc diffusion tests and electrophoretic plasmid analysis. Results show that *E. coli* strains isolated from persons on antibiotics were resistant to ampicillin (46.62%), augmentin (39.72%), clarithromycin (5.16%); while resistance to antibiotics by *E. coli* isolated from those that are not on antibiotics were ampicillin (27.27%), augmentin (20.00%), ceftriaxone (18.18%) and nitrofurantoin (14.55%). Also, *E. coli* strains isolated from wild life were resistant to ampicillin (85.71%) and clarithromycin (14.29%). *Proteus* spp. isolated from human sources were susceptible to all test antibiotics except ampicillin; while those from wildlife were resistant to ampicillin (54.17%), chloramphenicol (16.67%), nitrofurantoin (8.33%), clarithromycin (8.33%), ofloxacin (4.17%), augmentin (4.17%) and pefloxacin (4.17%). The correlation matrices determined at $p \leq 0.05$, revealed high resistance correlation among the different genera of bacteria isolated from one source to those from the other sources. Resistance plasmid analysis revealed the presence of 23 kb plasmid DNA in organisms obtained from the different sources. This suggests the possibility of bidirectional resistant gene transmission at the human-wildlife interface, indicating congruence between the drug resistance pattern of *E. coli* and *Proteus* spp. isolated from humans and wild animals. This calls for a holistic and forward-looking approach that will take the complex interconnections among species into full account, recognizing the important link between humans, animals and the environment in a bid to contain antibiotic resistance.

Key words: Drug resistance, humans, wild animals, *Escherichia coli*, *Proteus* spp., resistance plasmids.

INTRODUCTION

As human populations grow and transform landscapes, contact with wildlife concomitantly increases. Human modification of the environment is seen as the primary driver of the emergence of zoonotic diseases, through providing the opportunity for direct and indirect contact between humans and (sympatric) wildlife and increasing pathogen exposure and transmission potential (Mayer, 2000; Deem and Karesh, 2001; Pesapane et al., 2013). Disease emergence has been an important consequence of this escalation in interaction, with the majority of emerging infectious diseases in humans arising from wildlife reservoirs (Jones and Petel, 2008). These changes can induce immediate as well as long-term effects on pathogen transmission dynamics, modifying genetic and biological characteristics, biophysical elements, ecological dynamics, and socioeconomic, as well as host(s)-pathogen interactions (Smolinski and Hamburg, 2003).

Although controversy continues to surround the origin of many infectious diseases (Pearce-Duvet, 2006), many of humanity's most serious infections seem to have been the result of increasingly close and frequent contact with a new array of potentially zoonotic pathogens from animals intentionally domesticated for human use. Indeed, some of the most devastating and persistent human pathogens can be traced to zoonotic origins.

While environmental fecal waste may be an important source of pathogen exposure for both wildlife and humans, we still have a limited understanding of the complex process of pathogen spillover between wildlife and humans. The relative infrequency of pathogen spillover events limits our ability to evaluate the complexity of interacting and cascading factors driving this process (Goldberg et al., 2008).

Similarly, the genus *Proteus*, (an opportunistic infectious agent) which occur worldwide have been isolated from domestic dogs and cats around the world (Turkyilmaz, 2008; August, 1988). As opportunistic pathogens, they produce infections in humans only when they leave the intestinal tract. They are found in urinary tract infections, bacteremia, pneumonia and local lesions in debilitated patients or those receiving intravenous infusion. *Proteus* spp. are also alleged in food poisoning (Al-Mutairi, 2011).

E. coli and *Proteus* spp. have important roles in the increasing cases and dissemination of antibiotic resistance, potentially acquiring resistance determinants and acting as reservoirs for resistance genes (Reuben et al., 2013). Determining the resistance profile of these two organisms isolated from two different and diverse sources is the subject of this study. The study was aimed

at comparing the resistance patterns of these two organisms from humans (presumably exposed to antibiotic selective pressure) and those from wild animals (with at most conjectural exposure to antibiotics).

MATERIALS AND METHODS

Study population and sample collection

Human population aged 10 and above, and Wild animals (such as squirrels, farm lizards, grass cutters, rabbits, antelopes and rats) were analyzed for drug resistant bacterial load. The evaluation was carried out from January, 2011 to March, 2013. Stool samples were collected from human subjects after informed consents were obtained from both the individuals and the hospital authority. Rectal and intestinal swabs were collected from dead or sacrificed wild animals caught with traps or bought from hunters. The clinical human samples were collected from Bishop Shanahan Hospital, Nsukka and the wild life samples were obtained from Okutu, Okpuje, Ede-oballa, Allor uno, Opi, Opi agu and Obollo communities in Enugu state, Nigeria. Human samples were obtained from two human groups, designated as Human not on antibiotics and Human on antibiotics. Human not on antibiotics represented persons who had not used antibiotics for 3 months prior to sample collection while Human on antibiotics represented groups that have had antibiotics therapy within 3 months.

Isolation and identification of bacteria

Each sample was directly inoculated onto separate nutrient and MacConkey agar plates. The inoculated plates were incubated for 24 h at 37°C. The colonies obtained were isolated and further purified until pure cultures were obtained. Colonies obtained from MacConkey agar were classified as either lactose fermenters or non-lactose fermenters based on the pigmentation. All the isolates were Gram stained and examined microscopically. Biochemical tests were carried out based on the Gram reactions. Among the tests carried out were sugar fermentation, catalase, indole, oxidase, coagulase, H₂S and motility test. CHROMagar™ (orientation) was used to further confirm the identity of the isolates.

Antibiotic susceptibility testing and plasmid profiling

Resistance to commonly used antibiotics was determined using the Kirby-Bauer disc diffusion method with the following Gram negative susceptibility disc: ciprofloxacin (CPX) (10 µg/ml), gentamicin (GEN) (10 µg/ml), ofloxacin (OFX) (10 µg/ml), augmentin (AUG) (30 µg/ml), pefloxacin (PEF) (10 µg/ml), clarithromycin (CMN) (30 µg/ml), chloramphenicol (CMP) (10 µg/ml), ampicillin (AMP) (30 µg/ml), nitrofurantoin (NIT) (100 µg/ml) and ceftriaxone (CTN) (30 µg/ml) (Poltes Med. Lab. Enugu, Nigeria). Isolates were scored as susceptible or resistant based on the interpretive criteria of the Clinical and Laboratory Standards Institute (CLSI) 2008. *Escherichia coli* (ATCC 25922) was used as reference (control) strain. Antibiotic resistant strains were subjected to plasmid profiling using the agarose gel electrophoretic separation of DNA materials (Kraft et al., 1988; Kado and Liu, 1981).

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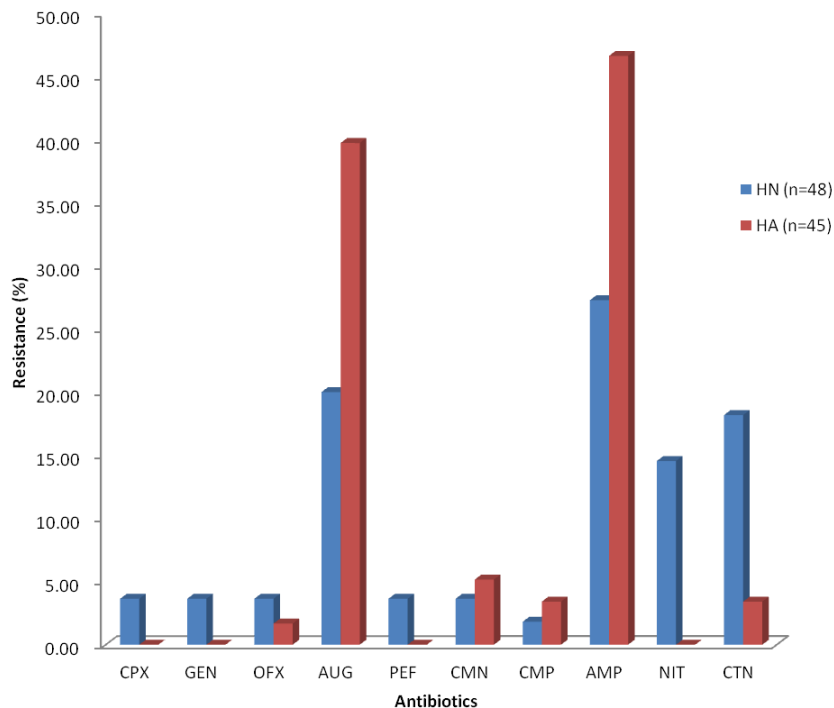


Figure 1. Antibiotic resistance pattern of *E. coli* isolated from the two human groups. CPX = Ciprofloxacin; GEN = Gentamycin; OFX = Ofloxacin; AUG = Augmentin; PEF = Pefloxacin; CMN = Clarithromycin; CMP = Chloramphenicol; AMP = Ampicillin; NIT = Nitrofurantoin , CTN = Ceftriaxone. HN=Human not on Antibiotics. HA= Human on Antibiotics.

Data analysis

Data obtained were subjected to analysis of variance (ANOVA) and Pearson Correlation Analysis using the Statistical Package for Social Sciences (SPSS 16.0) Inc. (444N Michigan USA). Correlation was carried out as a measure of the number of test isolates of a genus from a particular source that are resistant to the test drugs in relation to the isolates from same and the other sources.

RESULTS

Antibiotic sensitivity

E. coli isolated from persons on antibiotics showed resistance to ampicillin (46.62%), augmentin (39.72%), clarithromycin (5.16%); while resistance to antibiotics by *E. coli* isolated from those persons that are not on antibiotics were against ampicillin (27.27%), augmentin (20.00%), ceftriaxone (18.18%) and nitrofurantoin (14.55%) (Figure 1).

All members of the genus *Proteus* isolated were susceptible to all the test antibiotics, except the *Proteus* sp. isolated from one individual on antibiotics that exhibited resistance to ampicillin.

The microbial resistance pattern exhibited by each of

Table 1. Percentage distribution of antibiotic resistance among *E. coli* and *Proteus* spp. from wild life (WL) Percentage Resistance.

Antibiotics	<i>E. coli</i>	<i>Proteus</i> spp.
	WL(n=6)	WL(n=13)
CPX	0.00	0.00
GEN	0.00	0.00
OFX	0.00	4.17
AUG	0.00	4.17
PEF	0.00	4.17
CMN	14.29	8.33
CMP	0.00	16.67
AMP	85.71	54.17
NIT	0.00	8.33
CTN	0.00	0.00

CPX = Ciprofloxacin; GEN = gentamycin; OFX = ofloxacin; AUG = Augmentin; PEF = pefloxacin; CMN = clarithromycin; CMP = chloramphenicol; AMP = ampicillin; NIT = nitrofurantoin , CTN = ceftriaxone. 0.00 = No resistance that is all susceptible.

the microbial genera against the test antibiotics is shown in Table 1.

The organisms were most resistant to AMP, CMN and AUG, while the least resistance was demonstrated against

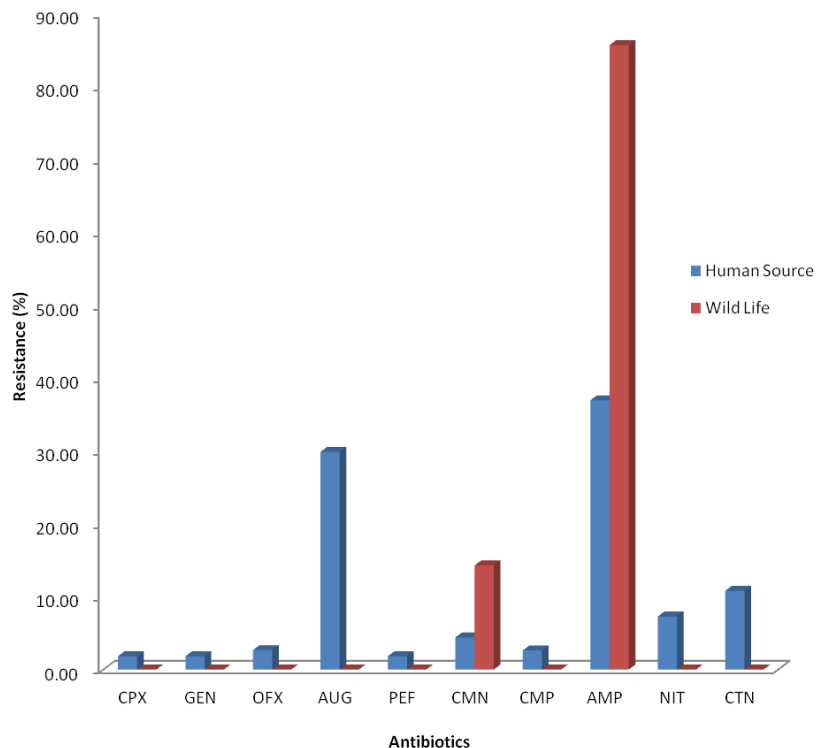


Figure 2. Antibiotic resistance pattern of *E. coli* isolated from the human and wild life sources. CPX = Ciprofloxacin; GEN = gentamycin; OFX = ofloxacin; AUG = Augmentin; PEF = pefloxacin; CMN = clarithromycin; CMP = chloramphenicol; AMP = ampicillin; NIT = nitrofurantoin, CTN = ceftriaxone. HN = Human not on antibiotics; HA = human on antibiotics.

PEF, CTN and CPX. Strains of *E. coli* isolated from wild life were more resistant to AMP (85.71%) than CMN (14.29%). They were susceptible to every other test antibiotic. In the same vein, *E. coli* isolated from Human sources also exhibited resistance against AMP (36.95%), AUG (29.86%) and CTN (10.80%). The comparative resistance pattern is shown in the Figure 2.

Species of *Proteus* isolated from human sources were susceptible to all antibiotics except AMP. Those isolated from wildlife were resistant to AMP (54.17%), CMP (16.67%), NIT (8.33%), CMN (8.33%), OFX (4.17%), AUG (4.17%) and PEF (4.17%) (Figure 3).

Correlation of resistance

A further analysis using correlation matrix showed that a strong direct relationship existed in the resistance pattern of the *E. coli* (0.704) regardless of the source of the isolate while the lowest correlate existed among *E. coli* and *Proteus* spp isolated from HN and HA (0.543) respectively.

In general, the analysis showed that there existed a high correlation in the resistance pattern of all the microorganisms obtained from both sources.

The correlates also examined the relationship between isolates from wildlife and human on antibiotics, as well as wild life and human not on antibiotics. Significance of correlation on the matrices was determined at $p \leq 0.05$.

From the correlation matrix, high correlation coefficient was observed among the different genera of bacteria isolated from one source to those from different sources. For instance, the correlation matrix between isolates from persons on antibiotics and those from wild life revealed a correlate of 0.986 between *Proteus* (from those on antibiotics) and *E. coli* (from wildlife). In the same vein, a correlation coefficient of 0.948 existed between wildlife isolates of *E. coli* and *Proteus*.

Plasmid profile

The plasmid isolation and electrophoretic separation revealed the presence of plasmid DNA in some of the isolates. The plasmid DNAs were isolated from both resistant and susceptible organisms. Agarose gel electrophoresis of plasmid DNA (plates not shown) revealed the presence of relatively large plasmids in few of the test organisms.

The molecular weights of most of the plasmids were

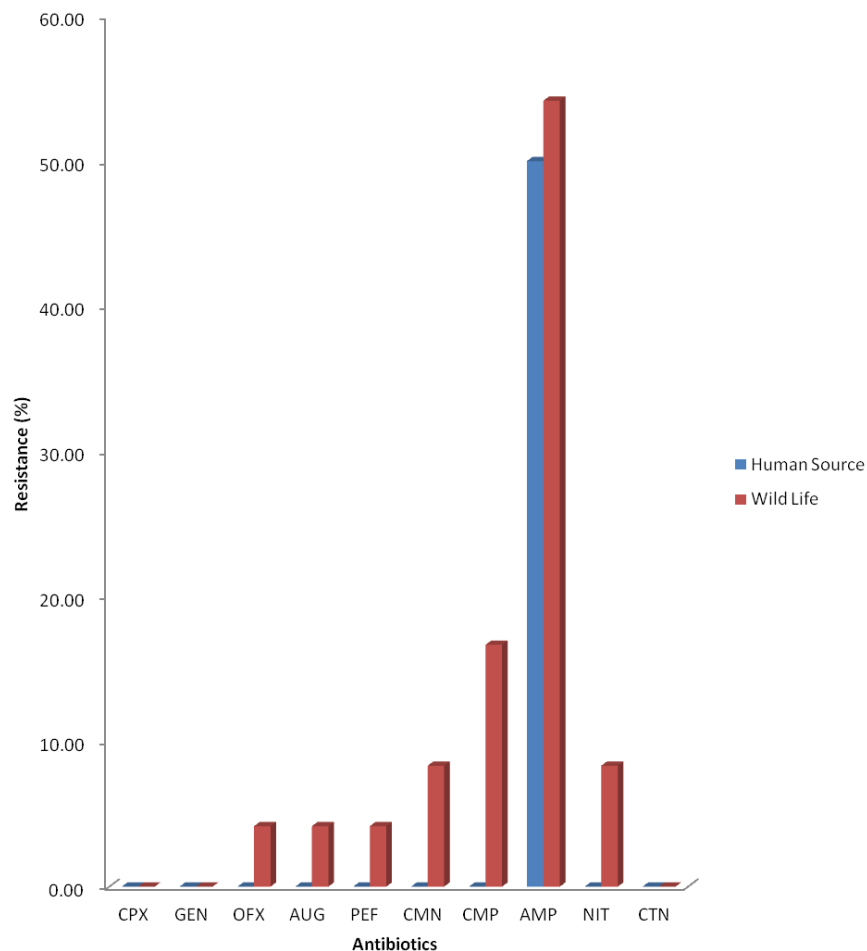


Figure 3. Antibiotic resistance pattern of *Proteus* isolated from human and wildlife sources. CPX = Ciprofloxacin; GEN = gentamycin; OFX = ofloxacin; AUG = Augumentin; PEF = pefloxacin; CMN = clarithromycin; CMP = chloramphenicol; AMP = ampicillin; NIT = nitrofurantoin, CTN = ceftriaxone. HN = Human not on antibiotics. HA = human on antibiotics.

Table 2. Distribution of the plasmid DNAs in *E. coli* and *Proteus* spp.

Bacteria Isolates	Number of Isolates tested	Number with plasmids	Antibiotic Profile	Mol. wt. of plasmid (kb)
<i>E. coli</i>	10	3	MDR & MDS	23.1
<i>Proteus</i>	10	4	MDR & MDS	23.1

MDR, Multi drug resistant; MDS, multi drug sensitive.

around 23.1 kb. The distribution of the plasmid DNA is shown in Table 2.

DISCUSSION

In this study, *E. coli* and *Proteus* were isolated and studied for the congruence in resistance pattern exhibited

by both against commonly used antibiotics. Human and Wildlife isolates of *E. coli* and *Proteus* were examined for resistance to some antibacterial agents and by extension their potentials as reservoirs of multidrug resistance traits. These enterics were selected in the study because of their ubiquity and the diversity of the ecological environments of their hosts. Particularly, wildlife samples were chosen because it is expected that they have

negligible, if any, previous exposure to antibiotics.

E. coli was predominantly isolated from all the samples examined in the study, while the recovery rate of *Proteus* was relatively low. Both genera of bacteria have been previously reported to be commensals in humans except where they become opportunistic pathogens as a result of immunological challenges on the host(s) or other factors (Costa et al., 2013). In some cases they are found in the blood system where they cause blood sepsis and in urinary and genital tracts where they cause varying degrees of urogenital disorders (Aibinu et al., 2003; Kruthi, 2006). Because these bacteria are found in large numbers in intestinal tract, they are transmitted most often via the faecal/oral route.

Tests of the ability of the *E. coli* and *Proteus* to resist commonly used antibiotics, using the disc diffusion test, revealed no observable significant difference in the resistance pattern between organisms isolated from persons on antibiotics and those that are not. This indicates that the ability of these organisms to resist some antibiotics is not only a function of previous exposure to antibiotics. It may also suggest a possible robust two-way transmission and colonization of these human groups by these organisms. Increasing microbiological and clinical evidences reveal that resistant bacteria or resistance determinants may be passed from animals to humans resulting in infections that are more difficult to treat (Sayah et al., 2004). The fact that ecological overlap increases the risks of microbial exchange between humans and wildlife gives credence to this. Infectious agents transmitted between humans and wildlife pose a risk to both human and animal. Human behavior such as hunting modifies and enhances this risk (Goldberg et al., 2007). Hunting (which made sample collection in this study easy) is a common feature in the study areas. This will definitely make it possible for wildlife borne organisms to be transferred to people through the food chain or direct contact and subsequent colonization, proliferation and development of difficult –to-treat or even untreatable diseases (Barbosa and Levy, 2000).

Typically, *E. coli* has been said to exhibit different resistance patterns (Reuben et al., 2013) without experiencing significant selection pressure (Goldberg et al., 2007). In Nigeria, data has shown that the prevalence of resistance to most drugs tested against *E. coli* isolates from apparently healthy students is within high range and has increased from 1986 to 1998. The observed increase in prevalence of resistance to beta-lactams and aminoglycosides were statistically significant (Okeke et al., 2000). The combined effects of fast growth rates to large densities of cells, genetic processes of mutation and selection, and the ability to exchange genes, account for the extraordinary rates of adaptation and evolution that can be observed in the bacteria. For these reasons *E. coli* adaptation (resistance) to the antibiotic environment seems to take place very rapidly.

The results of this study support this impression.

The difference in resistance between wildlife and human bacterial isolates was analyzed statistically to determine whether it was systematic or random. Being systematic implies that the mechanisms are similar if not the same (Mach and Grimes, 1982). High resistance correlations noticed among some bacteria of different genera isolated from the two sources are indicative of systematic rather than random variation. These results strongly suggest that the antibiotic resistance patterns in these bacterial groups are similar, perhaps showing similar mechanisms for the development of this resistance with a difference only in the rate. Specifically, this points to the presence of a common or closely related genetic trait as responsible for resistance among these bacteria. Support for this is found in the molecular weights of the plasmids isolated from the test organisms that were obtained from different ecological sources. Large plasmids in bacteria are important in public health perspective because they mediate more than one mechanism of resistance among their host bacteria. For example, Gram negative efflux pump genes are widely distributed and associated with large plasmids (Byarugaba, 2010). Their common presence in these isolates is indicative of congruence between the drug resistant pattern of *E. coli* clones and *Proteus* spp. isolated humans and wild animals.

In light of these, it is necessary to seriously consider strategies to prevent the emergence and dissemination of antimicrobial resistant bacteria and develop a more holistic and forward-looking approach that will take the complex interconnections among species into full account, recognizing the important link between humans, animals and the environment. This is our recommendation.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Carbon dioxide mitigation by microalga in a vertical tubular reactor with recycling of the culture medium

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Microalgae use photosynthesis as their principal metabolic mechanism to obtain organic carbon from the inorganic carbon contained in CO₂ using solar energy. This process releases oxygen into the atmosphere. The aim of this study was to determine the most appropriate biomass concentration for growing *Spirulina* sp. LEB 18 (Cyanobacteria) in a vertical tubular photobioreactor with biomass removal and recycling of the medium to maximize CO₂ biofixation. The maximum CO₂ biofixation was obtained when the culture was maintained at 600 mg L⁻¹ biomass concentration. Under this condition, the highest CO₂ biofixation value was 186.8 ± 73.1 mg L⁻¹ d⁻¹, and the maximum productivity was 85.9 ± 6.0 mg L⁻¹ d⁻¹.

Key words: Biofixation, carbon dioxide, microalgae, recycling, tubular photobioreactor.

INTRODUCTION

The increase in industrialization and urban population has led to a large demand by society for energy, which has increased the emission of atmospheric pollutants (CO₂, SO_x, NO_x, and other gases). According to NOAA (2013), atmospheric CO₂ has increased from 280 to 400 ppm over the last 215 years. The burning of fossil fuels for energy generation is the main source of the emissions of these pollutants.

Many alternatives have been studied to reduce CO₂ emissions into the atmosphere. One of the methods that is most suitable for reducing CO₂ emissions is the

cultivation of microalgae. The photoautotrophic capability of these microorganisms converts CO₂ in biomass efficiently, minimizing environmental problems and the cost of carbon in the culture. Microalgae develop rapidly and can be grown in engineering systems, such as photobioreactors (Chiu et al., 2009). In addition to the ability to fix CO₂, the biomass of microalgae is rich in minerals, vitamins, lipids, pigments and proteins, with industrial and/or commercial applicability (Khan et al., 2009).

Microalgae are photosynthetic microorganisms that use

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inorganic carbon for growth and can be used for CO₂ mitigation. The process of mitigation by microalgae has many purposes: the capture of fossil carbon dioxide; the production of renewable energy with additional services to the environment (water treatment); and the generation of bioproducts (animal feed and fertilizers) (Kumar et al., 2010).

Spirulina is a filamentous cyanobacterium with a spiral format. Cyanobacteria are photosynthetic prokaryotic microorganisms that appeared more than 3 million years ago, forming the current atmosphere with oxygen, and since then have regulated the biosphere of the planet by removing CO₂ and releasing O₂ (Romano et al., 2000).

The photobioreactor configuration is one of the most important factors in controlling the biomass yield from photosynthetic cultures (Carvalho et al., 2011). The efficiency of light transmission per unit volume of culture, photosynthetic capacity, gas exchange and use of substrates are affected by the photobioreactor geometry and the homogenization of the culture medium with the added microalgae and gases, which are controlled by design and operation. To design a photobioreactor with an appropriate gas transfer system, the substrate must be effectively dissolved in the liquid medium to create a non-limitation situation for the cells (Pandey et al., 2014).

The use of a vertical tubular photobioreactor increases the time that the gas remains in the medium, the area of contact between the light and culture, the photosynthetic rate, and consequently, the CO₂-use efficiency (Morais and Costa, 2007a). With microalgae cultures, recycling the medium allows the microalgae to use the nutrients until they are exhausted, with less exposure to the environment of the residues after biomass harvesting.

This study aimed to assess the most suitable biomass concentration in which to cultivate the Cyanobacteria *Spirulina* sp. in a vertical tubular photobioreactor, with biomass removal and the recycling of the medium, to obtain the maximum CO₂ mitigation.

MATERIALS AND METHODS

Microorganisms and culture medium

This study used the microalgae *Spirulina* sp. LEB 18 isolated from Mangueira Lagoon (33°30'12"S, 53°08'58"W). Zarrouk culture medium (Zarrouk, 1966) (Table 1) modified by Morais and Costa (2007b) was used to maintain the inoculum without the original carbon source of the culture medium (NaHCO₃). The inoculum was acclimatized with air mixed with 1% (w/w) CO₂ for 168 h with a 0.3 vvm flow rate.

Culture conditions

Spirulina sp. LEB 18 was grown in a 2 L (net volume 1.8 L, $\phi = 0.07$ m) vertical tubular photobioreactor (VTPB) (Figure 1) at 30°C with a 12 h light/dark photoperiod. The 1200 Lux illuminance was provided by daylight-type fluorescent lamps (40 W).

Aeration was carried out by mixing compressed air with CO₂ through an industrial cylinder (White Martins - Brazil) with a flow

Table 1. Zarrouk medium composition.

Reagents	Amount (g L ⁻¹)
NaHCO ₃	16.8
K ₂ HPO ₄	0.50
NaNO ₃	2.5
K ₂ SO ₄	1.00
NaCl	1.00
MgSO ₄ ·7H ₂ O	0.20
CaCl ₂	0.04
FeSO ₄ ·7H ₂ O	0.01
EDTA	0.08
Solution A5	1 mL
Solution B6	1 mL

Solution A5: (g L⁻¹): H₃BO₃, 2.86; MnCl₂·4H₂O, 1.81; ZnSO₄·7H₂O, 0.222; Na₂MoO₄·2H₂O, 0.390; CuSO₄·5H₂O, 0.079. Solution B6: (mg L⁻¹): NH₄VO₃: 22.86; KCr(SO₄)₂·12 H₂O: 192; NiSO₄·6H₂O: 44.8; Na₂WO₄·2H₂O: 17.94; TiOSO₄·8H₂O: 61.1; CO(NO₃)₂·6H₂O: 43.98.

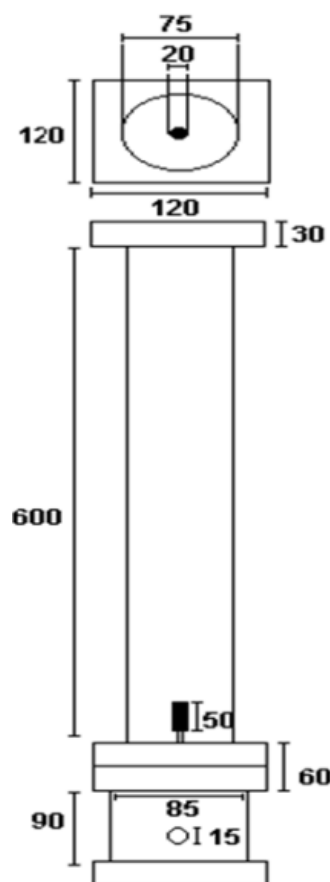


Figure 1. VTPB dimensions (mm).

rate of 0.3 vvm and a concentration of 12% (v/v) CO₂. The gas was added to cultures for 15 min every h during the light period (Morais and Costa, 2008).

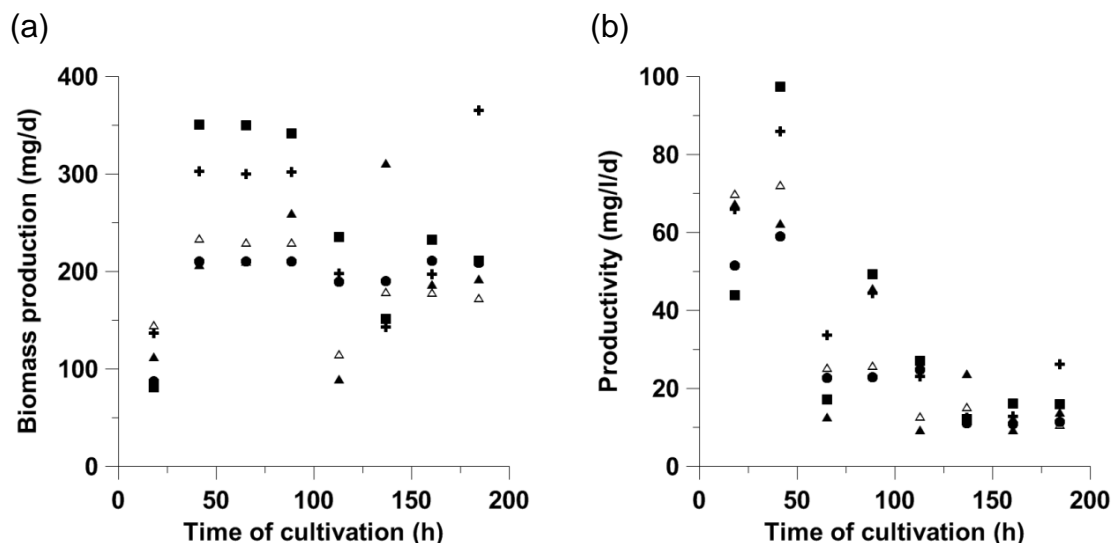


Figure 2. (a) Production of biomass and (b) productivity as a function of time for the cultivation of *Spirulina* sp. LEB 18 with the removal of biomass to return the cell concentration to 200 mg L⁻¹ (●), 400 mg L⁻¹ (■), 600 mg L⁻¹ (+), 800 mg L⁻¹ (▲) and 1000 mg L⁻¹ (△).

All of the tests were duplicated, and the initial biomass concentrations were 200, 400, 600, 800 and 1000 mg L⁻¹. Daily, the produced biomass was removed from cultures, and the cells were returned to their initial concentration. The produced biomass and the cultivation medium were separated by filtration, and the medium without microalgae was returned to the culture (medium recycling). The duration of cultures was 185 h.

Analytical assessments

Every 24 h, samples were collected aseptically to monitor cell concentration, which was calculated by measuring the optical density at 670 nm in a spectrophotometer (Femto 700 Plus, Brazil). The calibration curve was used to relate the optical density and dry weight of the biomass. The pH of the cultures was measured every 24 h with a digital pH meter (Quimis Q400H, Brazil) (Bailey and Ollis, 1986).

Kinetic parameters and carbon dioxide mitigation (CO₂)

The kinetic parameters and CO₂ mitigation were calculated using the mean of the duplicate tests. The cellular concentration was measured daily, before and after biomass removal, and then used to calculate the concentration of produced biomass (X_R , mg), the productivity (P , mg L⁻¹ d⁻¹) and the mean CO₂ fixation (BF , mg L⁻¹ d⁻¹). The produced biomass was determined according to the equation $X_R = X_t V_R$, where X_t (mg L⁻¹) is the cell concentration at time (t), and V_R (L) is the volume of the medium that was withdrawn for the cultivation to return to the initial cell concentration value. The total biomass removal (X_{t_i} , mg L⁻¹) was calculated by totaling the daily removal of biomass. The productivity (P , mg L⁻¹ d⁻¹) was obtained according to the equation $P = (X_o - X_t) t^{-1}$, where X_t is the cellular concentration (mg L⁻¹) at time t (d), and X_o (mg L⁻¹) is the cellular concentration at time t_o (d) (Bailey and Ollis, 1986).

A CHNS elemental analyzer (Perkin Elmer 2400, USA) was used to calculate the carbon content. The equipment was calibrated using the default cystine certificate (Perkin Elmer, USA). The results

of cystine recovery, taken as a sample, were 100% for carbon (Baumgarten, 2010). The mean CO₂ fixation during cultivation was determined by the ratio between the sums of daily CO₂ fixation and the total number of days, according to the equation $BF = (X_R V_{FBR} m_{CB} m_{CO_2} m_C^{-1} t^{-1})$, where X_R (mg) is the concentration of the produced biomass, V_{FBR} (L) is the useful volume of the photobioreactor (PBR), m_{CB} (mg mg⁻¹) is the mass fraction of carbon as determined in microalgal biomass, m_{CO_2} (mg mol⁻¹) is the molar mass of CO₂, m_C (g mol⁻¹) is the molar mass of carbon, and time (t) is the time of cultivation.

RESULTS AND DISCUSSION

The maximum biomass production (365.4 ± 97 mg d⁻¹) was obtained when the culture was kept at a 600 mg L⁻¹ cellular concentration, followed by the culture being kept at 400 mg L⁻¹ cells (350.9 ± 43 mg d⁻¹) (Figure 2a).

Figure 2b and 3b show that maximum productivity rates (P) and maximum CO₂ fixation (B_{CO_2}) were obtained in tests at cellular concentrations of 400 mg L⁻¹ ($P = 97.3 \pm 7.6$ mg L⁻¹ d⁻¹, $B_{CO_2} = 167.0 \pm 18.5$ mg L⁻¹ d⁻¹) and 600 mg L⁻¹ ($P = 85.9 \pm 6.0$ mg L⁻¹ d⁻¹, $B_{CO_2} = 186.8 \pm 73.1$ mg L⁻¹ d⁻¹), respectively. The total biomass removal was 1954 and 1945 mg L⁻¹ for cultures that kept at cellular concentrations of 400 and 600 mg L⁻¹, respectively.

The experiments that were performed at 200, 800 and 1000 mg L⁻¹ had total biomass removal values of 1517.9, 1567.9 and 1485.1 mg L⁻¹, respectively. According to Travieso et al. (2001), the ideal cell concentration in discontinuous processes for the maximum microalgal productivity is between 500 and 700 mg L⁻¹. In our study, the concentration of 600 mg L⁻¹ had the best results because it was the compensation, or saturation, point of photosynthesis. At this point, there is the maximum

photosynthetic efficiency because of the balance between the photosynthetic rate and respiration (Vonshak, 1997). Pelizer et al. (2003) obtained a maximum productivity of $76 \text{ mg L}^{-1} \text{ d}^{-1}$ in discontinuous cultivation that took place in a Raceway with an initial concentration of 100 mg L^{-1} .

The photosynthesis process is the transformation of light energy into chemical energy in the form of ATP, NADPH, carbohydrates, proteins and lipids. The process is called photosynthesis because the coenzymes ATP and NADPH are used to add CO_2 to organic molecules, featuring carbohydrate synthesis, where for each glucose molecule six fixed CO_2 are generated (Alberts, 2010).

In cultures that were grown in an Erlenmeyer flask in semi-continuous mode with an initial concentration of 150 mg L^{-1} , Reichert et al. (2006) obtained a maximum productivity ($42.3 \text{ mg L}^{-1} \text{ d}^{-1}$) in a test with a blended concentration of 500 mg L^{-1} and a renewal rate of 25%. No recycling of the medium took place in the Reichert et al. (2006) cultures; the amount removed was replaced by new medium, which provided rapid cell development. What differed with the tests that were presented in this study, which allowed the generation of higher yields compared to the aforementioned authors, was probably the use of a tubular photobioreactor. According to Morais and Costa (2007b), vertical tubular photobioreactors resulted in better fixation and kinetic results of CO_2 compared to Erlenmeyer-type photobioreactors for *Spirulina* sp., *S. obliquus* and *Chlorella vulgaris* (Chlorophyta) when these microorganisms were supplemented with 0.038, 6, 12 and 18% CO_2 .

The advantage with recycling the culture medium is that it reduces costs because the nutrients are used until they are exhausted. However, some microalgae do not develop when this process is adopted. *Nannochloropsis* (Eustigmatophyceae) microalgae release auto-inhibitors during cultivation, affecting the culture's development and limiting the recycling of the culture medium (Rodolfi et al., 2003).

Under all of the initial cell concentration conditions, the productivity of the tests decreased over time (Figure 2b). The cultures with cell removal provided the microalgae with the increased capacity to utilize the medium nutrients compared to cultures without removal because the microalgal concentration is daily reduced to the initial concentration, thereby decreasing competition for nutrients. However, as new culture medium was not added, there was a reduction in the medium nutrients, which reduced the productivity over time. The consumption of nutrients decreases the osmotic pressure of the medium, which potentially affects the productivity (Poza-Carrion et al., 2001).

The cultures in which the biomass was removed until concentrations of 200, 800 and 1000 mg L^{-1} were reached had lower CO_2 productivity and mitigation rates than did the cultures with concentrations of 400 and 600 mg L^{-1} . The lower responses that were obtained for the

yields and rates of mitigation of CO_2 may have been caused by the phenomenon of photoinhibition in cultures that were maintained at the cellular concentration of 200 mg L^{-1} and by photolimitation in cultures with concentrations of 800 and 1000 mg L^{-1} .

Photoinhibition is a phenomenon that occurs in the cultivation of microalgae because of a low cellular concentration in the culture, which allows a high incidence of light in the cells (Vonshak, 1997). In photolimitation, dense cultures of cells can block the penetration of light in the culture, limiting growth (Carvalho et al., 2011). In the dark, the rate of CO_2 mitigation is negative because of cell respiration, which interferes with the process of photosynthesis and consequently the rates of mitigation of CO_2 and microalgal growth. The flow of light that is emitted to cause photoinhibition or photolimitation depends on the genus and species of the studied microalgae.

Photosynthesis occurs in two distinct phases: a light phase (photochemical step) and a dark phase (chemical step). In the photochemical phase, radiant energy excites the photosynthetic pigments, and this state of excitement (energy) is transferred with the aid of water until the molecules NADP and ATP (chemical energy) are produced. The primary products of the photochemical step are ATP and NADPH. In this step also the release of oxygen occurs, as a byproduct of dissociation of the water molecule. In the chemical stage, the carbon from a molecule of CO_2 is absorbed by a series of enzymatic reactions using the energy that is stored in the ATP and NADPH molecules, eventually forming the first product of photosynthesis, carbohydrate (CH_2O) for carbon skeletons (Cox and Nelson, 2014).

The Zarrouk cultivation medium for *Spirulina* microalgae in its original composition had a pH between 10.5 and 11.0. At the beginning of cultivation, the pH of all of the cultures was approximately 7.0 because the carbon source of Zarrouk medium (NaHCO_3), which is responsible for the alkaline pH, was replaced by 12% CO_2 . When Westerhoff et al. (2010) replaced the standard carbon source of the culture medium by CO_2 , they also found a reduction in the pH in microalgae cultivation. To avoid this reduction in pH Kumar et al. (2011), suggest the use of buffered systems. The highest-concentration nutrient in Zarrouk medium is sodium bicarbonate (16.8 g L^{-1}), constituting 40 to 50% of the total nutrient cost (Doucha et al., 2005), which means that if microalgae are used to fix CO_2 , there will be a reduction in the cost of nutrients in the culture and in the environmental problems that are caused by this gas.

According to Vonshak (1997), the optimal pH for the development of *Spirulina* is between 9.5 and 10.5. In cultures of *Spirulina* sp. LEB 18, with the removal of biomass and the recycling of the medium, the pH of the cultures varied between 6.83 and 9.11. Although the pH of the culture was within the optimum pH levels that were suggested by Vonshak (1997), there was no cell death.

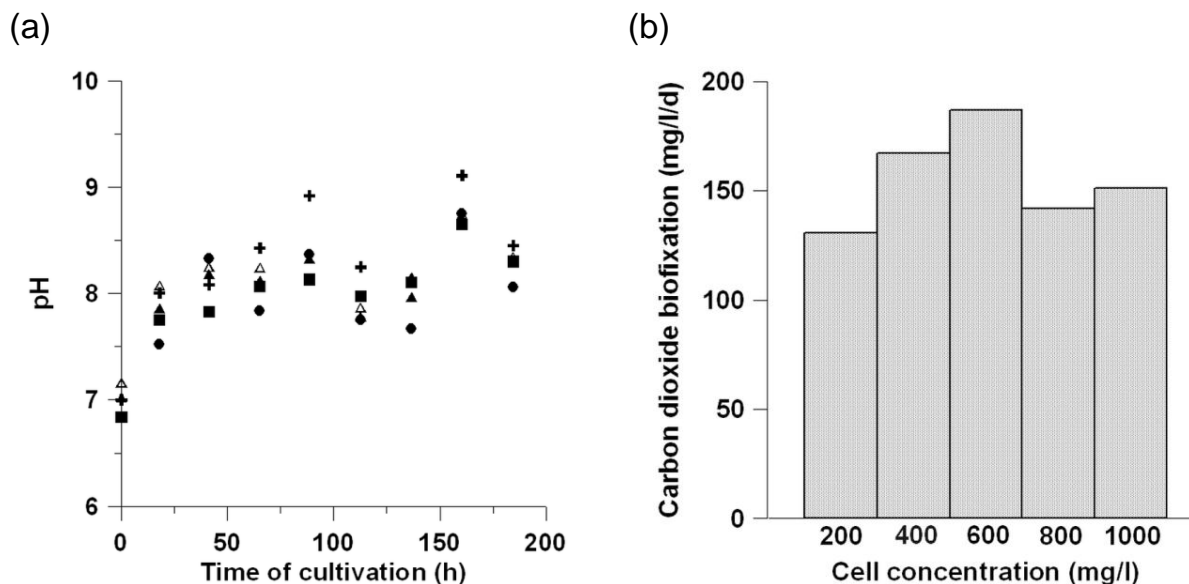


Figure 3. (a) The pH as a function of time for the cultivation of *Spirulina* sp. LEB 18 with the removal of biomass to return the cell concentration to 200 mg L⁻¹ (●), 400 mg L⁻¹ (■), 600 mg L⁻¹ (+), 800 mg L⁻¹ (▲) and 1000 mg L⁻¹ (Δ). (b) The mitigation of carbon dioxide as a function of the cellular concentration.

The experiments under different conditions studied showed daily increases in pH until 88.8 h, followed by decreases at 112.8 and 136.8 h, peaking at 160.8 h and mildly decreasing in the last h of cultivation (Figure 3a). This pH behavior was consistent with the daily production of biomass, which increased until 88.8 h, followed by a decrease in the consecutive 48 h (Figure 2a and 3a). The culture with a cell concentration of 600 mg L⁻¹ had the maximum production of biomass and a higher pH than that of the other cultures. The change in pH affects the solubility and bioavailability of nutrients, the transport of substrates through the cytoplasmic membrane, the enzyme activity and the transport of electrons for photosynthesis and respiration (Poza-Carrion et al., 2001).

Conclusion

When the microalga *Spirulina* sp. LEB 18 was cultivated in a vertical tubular photobioreactor with cell removal and medium recycling at a cellular concentration of 600 mg L⁻¹, the maximum CO₂ mitigation was reached. Under these conditions, the values that were obtained were 85.9 ± 6.0 mg L⁻¹ d⁻¹ biomass productivity and 186.8 ± 73.1 mg L⁻¹ d⁻¹ CO₂ fixation. Therefore, combining the cultivation of microalgae, the fixation of CO₂ and the recycling of the medium can lead to a reduction in the problems caused by the emission of this gas and in the expenses with culture medium and to the generation of biomass that can be used to obtain different products.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Plasmid profile of *Staphylococcus aureus* from orthopaedic patients in Ahmadu Bello University Teaching Hospital Zaria, Nigeria

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The aim of this work was to determine the antibiotic susceptibility pattern of the *Staphylococcus aureus* isolates from orthopaedic patients and their plasmid carriage. A total number of 39 *S. aureus* were isolated from wound, skin and bed of orthopaedic patients in Ahmadu Bello University Teaching Hospital Zaria, Nigeria with the use of API STAPH identification kit. Antibiotics susceptibility test was done using disc agar diffusion test, plasmid analysis was also carried out. A high percentage (97.4%) of the *S. aureus* isolates were susceptible to both vancomycin and gentamicin followed by ciprofloxacin (94.9%) and pefloxacin (84.6%). The *S. aureus* isolates were highly resistant to the following antibiotics: ampicillin (94.9%), ceftriaxone (79.5%), cefoxitin (64.1%) and amoxicillin - clavulanic acid (59%). The antibiotic susceptibility tests showed that 29.4% of *S. aureus* with plasmids were multi-drug resistant being resistant to three or more classes of antibiotics. In the plasmid analysis 60.7% of the *S. aureus* isolates had plasmids with size range 9.2 to 13.3 kb. Plasmid-coded antibiotic resistance encompasses most classes of antibiotics commonly used at the forefront of clinical antibiotic therapy.

Key words: *Staphylococcus aureus*, plasmid, orthopaedic, susceptibility.

INTRODUCTION

Staphylococcus aureus is an important pathogen both in the hospital and in the community. About 35-50% of normal adults carry it in the anterior nares (Arora, 2006); it is skin normal flora, and other sites of colonization include the perineum, axillae and vagina (Arora, 2006). It

is still one of the five most common causes of nosocomial infections and is often the cause of post surgical wound infections (Bowersox, 2007). Its remarkable ability to acquire antibiotic resistance has contributed to its emergence as an important pathogen in a variety of

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Table 1. Distribution of *S. aureus* isolates.

Source	Number of sample	Number of <i>S. aureus</i> identified from the sample
Wound	22	6
Skin	43	16
Bed	35	17
Total	100	39 (39.0%)

settings (David et al., 2006; Sampathukumar, 2007; Hotu et al., 2007).

Multidrug-resistant strains of staphylococci have been reported with increasing frequency worldwide, including isolates that are resistant to methicillin, lincosamides, macrolides, aminoglycosides, fluoroquinolones, or combinations of these antibiotics (Von Eiff et al., 2001). Infections by *S. aureus* are often difficult to treat because of frequency of multiple antibiotic resistance of strains (Alghaithy et al., 2000). *S. aureus* has a proven ability to adapt to the selective pressure of antibiotics (Chang et al., 2003).

Different patterns of antibiotic resistance and plasmid profiles among strains of *S. aureus* have been reported (Akinyemi et al., 1997; Bhakta et al., 2003; Diep et al., 2006; Daini and Akano, 2009). Plasmid genes for antimicrobial resistance often control the formation of enzymes capable of destroying antimicrobial drugs. Thus plasmids determine resistance to penicillins and cephalosporin by carrying genes for the formation of β -lactamases.

In orthopaedics, *S. aureus* has been implicated in surgical site infection, painful infection of joint fluid known as septic or infective arthritis, post operative infection, prosthetic joint infections, implant devices, infection following trauma, chronic osteomyelitis subsequent to an open fracture, meningitis following skull fracture (Donatto, 1998; Goldenberg, 1998; Green, 1991; Zimmerli, 2006).

This study was aimed at determining the antibiotic susceptibility pattern of *S. aureus* isolates from orthopaedic patients in a tertiary institution in North-western Nigeria and their plasmid profile.

MATERIALS AND METHODS

Sample collection

One hundred clinical samples were collected aseptically from the wound, skin and bed of orthopaedic patients in Ahmadu Bello University Teaching Hospital Zaria, Nigeria over a period of 5 months. Ethical approval obtained was obtained from the ethical committee of the institution while individual patients' consent was obtained before sample collection.

Purification and preliminary identification of Staphylococcal isolates

A loopful of overnight nutrient broth cultures of the isolates were streaked on previously prepared plates of mannitol salt agar and

incubated at 37°C for 24 h. Isolates that produced colonies exhibiting characteristic deep golden yellow colouration were selected and subcultured into nutrient broth incubated at 37°C for 18 h. This was then streaked on nutrient agar slants and later stored in the refrigerator until required for further tests.

Identification of *S. aureus* isolates

API STAPH identification kit (bioMerieux, Inc, Durham, USA) was used to identify the *S. aureus* isolates; the procedures were carried out according to the manufacturer's instructions.

Antibiotic susceptibility test

Disk diffusion tests was performed for each of the isolates identified as *S. aureus* by following the method recommended by the Clinical Laboratory Standard Institute (CLSI, 2007). List of antibiotics used were: Cefoxitin, 30 μ g; Ceftriaxone, 30 μ g; Vancomycin, 30 μ g; Ampicillin, 10 μ g; Gentamicin 10, μ g; Pefloxacin, 5 μ g; Ciprofloxacin, 5 μ g; Amoxicillin-clavulanic acid, 30 μ g; Erythromycin, 15 μ g and Clindamycin, 2 μ g (Oxoid Ltd. Basingstoke, London).

Plasmid profile analysis

Lysate preparation was done according to manufacturer instructions (Plasmid MiniPrep Kit (Norgen Biotek Corporation, Canada): Plasmid DNA were separated by horizontal electrophoresis in 1.5% agarose gel in a Tris- acetic acid- EDTA (TAE) buffer containing 10 μ l Green nucleic acid stain at room temperature at 80 volts (50 mA) for 30 min. Briefly, 20 μ l of plasmid DNA solution were loaded into the individual well of the gel. DNA bands were visualized and photograph was taken using the gel imaging system. The molecular weight of the unknown plasmid DNA was determined on the basis of its mobility through agarose gel in comparison with molecular weight marker.

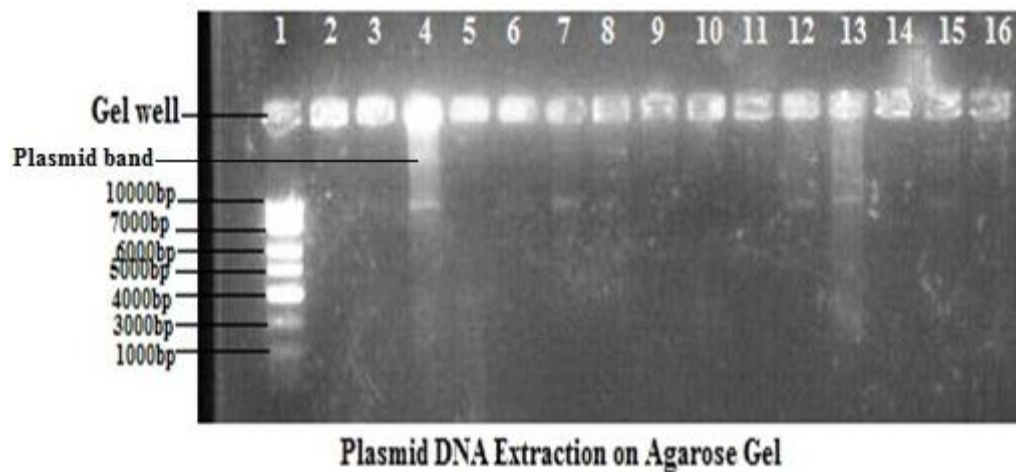
RESULTS AND DISCUSSION

A total number of 100 samples were collected from the wound, skin and bed of the orthopaedic ward and the details are presented in Table 1. Out of these clinical samples, 39% were identified as *S. aureus* being 6, 16 and 17 isolates from wound, skin and bed respectively (Table 1).

The isolation of *S. aureus* from the patients' beddings in this study is an indication that *S. aureus* is a cause of nosocomial infection. The majority of nosocomial infection is caused by a patient's own endogenous microbial flora present upon admission to the hospital

Table 2. Percentage susceptibility of *S. aureus* isolates.

Antibiotics	No of sample (%) n=39		
	Sensitive	Intermediate	Resistant
Ampicillin (10 µg)	2 (5.1)	0	37 (94.9)
Ceftriaxone (30 µg)	8(20.5)	13 (33.3)	18 (46.2)
Cefoxitin (30 µg)	14 (35.9)	0	25 (64.1)
Amoxicillin-clavulanic acid (30 µg)	16 (41.0)	0	23(59.0)
Clindamycin (2 µg)	12(30.8)	13 (33.3)	14 (35.9)
Erythromycin (15 µg)	19 (48.7)	10 (25.6)	10 (25.6)
Pefloxacin (5 µg)	33 (84.6)	0	6 (15.4)
Vancomycin (30 µg)	38 (97.4)	0	1 (2.6)
Ciprofloxacin (5 µg)	37 (94.9)	2 (5.1)	0
Gentamicin (10 µg)	38 (97.4)	0	1 (2.6)

**Figure 1.** Plasmid extraction on 1.5% agarose gel electrophoresis. Lane 1, Mass ruler ladder; 2, W7b; 3, W51; 4, W39 (2 bands); 5, S46; 6, S1; lane 7, B22; 8, S12; 9, B55; 10, W20; 11, S8; 12, S41; lane 13, B26; 14, B20; 15, B62;16, B16.

(Arif et al., 2007).

Antibiotic susceptibility pattern of isolates

A high percentage (97.4%) of isolates were susceptible to both vancomycin and gentamicin followed by ciprofloxacin (94.9%) and pefloxacin (84.6%). The level of resistance of the *S. aureus* isolates to antibiotics is as follows: ampicillin (94.9%), ceftriaxone (79.5%), cefoxitin (64.1%) and amoxicillin - clavulanic acid (59%) (Table 2).

The resistance to cefoxitin observed indicated that 64.1% of the *S. aureus* isolates were resistant to methicillin. Clinical Laboratory Standards Institute (CLSI) has recommended cefoxitin disc diffusion method for the detection of methicillin resistant *S. aureus* (MRSA) due to its ability to enhance induction of PBP2a (CLSI, 2013). The implication of MRSA in orthopaedic patients include extended length of stay, infection and wound breakdown;

loss of alignment of fractured bone, failure of internal fixation, delay or non-union of bone, loss of earnings, pain, anxiety and depression (John and David, 1991; Makoni, 2002).

The image of the plasmid profile on agarose gel from this study is presented in Figures 1 and 2. Seventeen (17) out of the 28 *S. aureus* isolates tested had plasmid bands and their individual plasmid size is presented in Table 3.

In this study, the encountered plasmids sizes were between 9.2 and 13.3 kilobase, this is similar to previous reports (Diep et al., 2006; Uchechi and Erinma, 2007; Adeleke et al., 2010; Akinjogunla and Enabulele, 2010; Tula et al., 2013). The molecular weight of the plasmids observed in this study falls into the category of small multicopy plasmids that carry single resistance (Berg et al., 1998). They can also be described as mobilizable resistant plasmids which are relatively small (often less

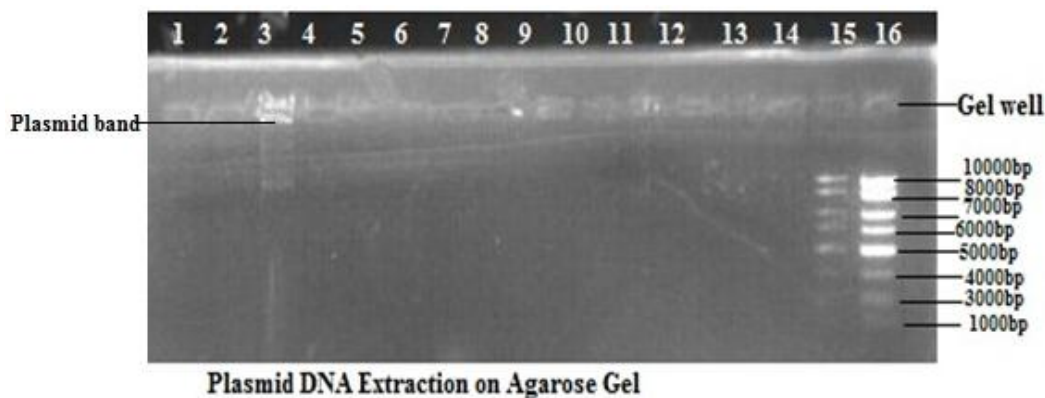


Figure 2. Plasmid extraction on gel electrophoresis. Lane 1: B35; 2: B69; 3: S55 (2 bands); 4: B47; 5: W4; lane 6: S27; 7: ATCC 25923; 8: S72; 9: B49; lane10: W7a; 11: B13; 13: S24; 14: S46s; 15&16: mass ruler ladder.

Table 3. Identification of *S. aureus* isolates and their plasmid profile.

Isolates (represented with code)	Number of plasmids	Plasmid size (kb)
W7b	1	12.06
W51	0	No plasmid
W39	2	11.65 and 9.23
W20	0	No plasmid
W4	1	12.06
W7a	1	12.06
S27	0	No plasmid
S55	2	13.27 and 10.04
S72	0	No plasmid
ATCC 25923	0	No plasmid
S24	1	12.46
S46s	0	No plasmid
S8	0	No plasmid
S41	1	9.23
S46	0	No plasmid
S1	1	9.23
S12	1	12.06
B13	0	No plasmid
B35	1	11.65
B16	0	No plasmid
B69	0	No plasmid
B22	1	9.23
B55	1	12.06
B26	1	9.23
B20	0	No plasmid
B62	1	9.23
B47	0	No plasmid
B49	0	No plasmid
B77	0	No plasmid

than 10 kb in size) encoding only a handful of genes including the resistance genes (Esimone et al., 2010).

The resistant plasmids observed in this study could be due to indiscriminate use of antibiotics in the hospital and

Table 4. Resistogram of plasmid DNA containing isolates.

Isolates(represented with code)	Resistance Pattern	Number of class of antibiotics
W39	FOX, AMP, CRO, VA, DA	3
W7a	FOX, AMP, CRO, ERY, DA, AMC	3
W7b	FOX, AMP, CRO, AMC	1
S12	FOX, AMP, CRO, AMC	1
S41	FOX,AMP, CRO, CN, PEF, AMC, ERY	4
S24	FOX, AMP, CRO, AMC, ERY	2
B69	FOX, AMP, CRO, ERY, AMC, DA	3
S55	FOX, AMP, CRO	1
B13	FOX, AMP, CRO, CIP, AMC, ERY, DA	4
B16	FOX, AMP, CRO, AMC, ERY	2
B22	FOX, AMP, CRO, AMC	1
B35	FOX, AMP, CRO, AMC	1
B26	FOX, AMP, CRO, AMC	1
B55	FOX, AMP, CRO, PEF, AMC	2
B62	AMP	1
S1	DA	1
W4	AMP, CRO	1

AMP, Ampicillin; CRO, ceftriaxone; FOX, ceftioxin; ERY, erythromycin; CN, gentamicin; CIP, ciprofloxacin; AMC, amoxicillin-clavulanate; DA, clindamycin; PEF, pefloxacin; VA, vancomycin.

to the over the counter availability of antibiotics.

The resistance pattern of plasmid DNA containing isolates are presented in Table 4. Out of the 17 *S. aureus* with plasmid five (29.4%) were resistant to three or more classes of antibiotics.

In this study, the *S. aureus* isolates were generally resistant to beta lactam antibiotics, this resistance to beta lactam antimicrobials often come concurrently with resistance to other antimicrobial agents such as macrolides (erythromycin), quinolone (pefloxacin) and clindamycin as observed in this study. This poses a great challenge to the prevention and treatment of *S. aureus* infections. The resistance pattern of the plasmid borne *S. aureus* isolates to antibiotics showed that 29.4% of the isolates were multi-drug resistant. Multidrug resistant organisms are defined as organisms which had acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Nikaido, 2009; Magiorakos et al., 2012; CDC, 2013; WHO, 2014). Previous studies have supported the important role plasmids play in staphylococcal multi-drug resistance (Paulsen et al., 1998; O'Brien et al., 2002; Olowe et al., 2007).

Conclusion

The result of this study shows that the plasmid-coded antibiotic resistance encompasses most classes of antibiotics commonly used at the forefront of clinical antibiotic therapy including the cephalosporins (beta lactams), fluoroquinolones and macrolides. Increasing

rate of development of resistance to antibiotics by bacteria is worrisome especially in the developing countries where antibiotics are usually misused due to their presence over the counter and irrational prescriptions. It is therefore suggested that appropriate regulatory policies be put in place by the government and duly enforced to control the use of antibiotics in order to reduce the spread of the resistant strains between individuals in the community.

Conflict of interests

The authors did not declare any conflict of interest.

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