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Used Toothbrushes: Microbial Evaluation and Antibiotic Susceptibility Profiles of Associated Bacteria

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Authors' contributions

This work was carried out in collaboration between both authors. Author OMO designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Author OTO carried out part of the bench work under author OMO's supervision. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Toothbrush has been universally accepted as the most effective tool for removing harmful plaque and bacteria from mouth. On average, colonization of bacteria is reduced by 88.8% as a result of brushing. Toothbrush if not properly taken care of before, during and after use may serve as a vector for the re-introduction of potential pathogens into the oral cavity.
This study therefore aimed at investigating the microbial quality of used toothbrushes among selected students of a Tertiary Institution in Ile-Ife, Nigeria as well as the antibiotic susceptibility profiles and adherence property of the associated bacteria.
Methodology: Fifty used toothbrushes were collected from students in sterile nylon and transported to Laboratory for processing within an hour of collection. The bacteria were isolated and characterized by conventional biochemical techniques. Antibiotic susceptibility test was carried out using the disk diffusion test according to the Clinical and Standard Laboratory Institute guidelines. Phenotypic adherence property of the isolates was investigated using the Congo Red Agar (CRA) method.

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Results: These revealed the presence of six genera of bacteria namely: *Bacillus* spp, *Staphylococcal* spp, *Klebsiella* spp, *Enterobacter spp*, *Citrobacter* spp and *Serratia* spp. *Bacillus flexus* was the predominant Gram-positive species accounting for 30.9% while *Klebsiella oxytoca* accounts for 26.5% as the predominant Gram-negative species. All the isolates were multidrug resistant. However, 22% of the isolates were adherent as they produced black crystalline colonies in Congo Red agar.
Conclusion: The study concluded that toothbrush should be adequately taken care of to prevent it from serving as vector for infection and re-infection of mouth.

Keywords: Toothbrush; colonization; infection; re-infection; vector.

1. INTRODUCTION

The mouth has the highest number of microorganisms compared to other parts of the human body. Billions of microorganisms from more than 400 different species live in the mouths of every adult [1]. These microorganisms, if not removed, have been shown to contribute to many disease processes. As part of the Global Burden of Disease Study in 2010, it was reported that approximately 35% of the world has untreated cavities; and of the 291 major diseases and injuries studied, dental cavities are by far the number one non-lethal communicable disease. Billions worldwide suffer from major tooth decay [2].

The primary etiological factor for dental diseases is dental plaque. The formation of plaque on the tooth surface is characterized by the progression from a limited number of pioneer microbial species to the complex flora of mature dental plaque. This progression involves initial adherence of bacteria to the salivary pellicle and subsequent accumulation by growth and inter-bacterial adherence. Ultimately, the tooth surface gets coated with a dense, complex micro-community that ends up in the destruction of hard enamel tissue [3].

Maintenance of good oral hygiene is therefore the key to the prevention of dental diseases. One of the ways by which good oral hygiene could be maintained is by the use of toothbrush and toothpastes. The toothbrush is the most effective tool for removing harmful plaque and bacteria from mouth. On average, colonization of bacteria is reduced by 88.8% as a result of brushing [4].

Tooth brushing has become a universally accepted first line of defense against illness. When used correctly, at least twice per day, quality toothbrushes remove pathogens from the mouth.

However, removal of pathogens from the mouth by toothbrush when used correctly leaves the toothbrush head contaminated as most people simply rinse the brush with plain tap water. As such, toothbrushes, however, are routinely reused for months and sometimes for more than a year. The head of a toothbrush contains up to 100 million germs [5] including *E. coli* that can cause diarrhea, yeasts such as *Candida albicans*, infective streptococcal bacteria as *Streptococcus mutans*, and staphylococci such as *Staphylococcus aureus* that can cause skin infections.

In addition to bacteria and other microorganisms from the mouth, toothbrushes are often kept near dirty toilets and sinks, further increasing the possibility of contamination. Aerosols released after flushing the toilet have been shown to deliver faecal matter up to 20 feet in the air, reaching toothbrushes that have been stored on bathroom counters [6]. It has been reported that toothbrushes could be a source of repeated oral infection.

This work therefore aimed at evaluating used toothbrushes by selected students of Obafemi Awolowo University, Ile-Ife, Nigeria for their microbial quality as well as investigating the adherence and antibiotic susceptibility profiles of the isolated bacteria.

2. MATERIALS AND METHODS

2.1 Isolation and Characterization

After ethical approval for the study was obtained from the OAUTHC Research and Ethical Committee, used toothbrush samples were collected from fifty students (29 males and 21 females) of Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria. Students that have suffered from any mouth infection as dental caries, plaque or periodontal disease in the last 3 months were excluded from the study. Similarly, students that have used any antibiotic in the last 3 months were excluded from the study.

Toothbrush of every person was rinsed in tap water and transported to the laboratory in sterile bag for processing. The method used for isolation was as described by Sammons et al. [7] with little modifications. Briefly, the handle of brush was cut off using a heat sterile scissors. The head of the brush was then soaked in 10 ml of sterile nutrient broth contained in McCartney bottle for 60 min followed by vortex mixing for 1 min. The head was swabbed to dislodge suspected adherent bacteria.

A loopful of the resulting suspension contained in the McCartney bottle was then streaked on sterile nutrient agar (Oxoid, UK) plate and incubated for 24 hours at 37℃. The isolated colonies were identified by conventional biochemical tests [8].

2.2 Phenotypic Adherence Test

Phenotypic adherence test (PAT) was done using the congo red method as described by Freeman et al. [9]. Briefly, the medium was made of Brain heart infusion broth (Lab M, UK) (37 g/L), sucrose (Qualikems Lab. Reagent) (5 g/L), agar number 1(Lab M, UK) (10 g/L) and Congo red dye (Kemlight Laboratories, PVT, Ltd) (0.8 g/L). Congo red stain was prepared as concentrated aqueous solution and autoclaved at 121℃ for 15 minutes. Then it was added to autoclaved brain heart infusion agar with sucrose at 55℃. Plates were inoculated with test organism and incubated at 37℃ for 24 hours aerobically. Black colonies with a dry crystalline consistency indicated slime production.

2.3 Antibiotic Susceptibility Test

Susceptibility of both the Gram-negative and Gram-positive isolates to eight antimicrobial agents each was tested by the disc diffusion technique according to the guidelines of the Clinical and Laboratory Standards Institute [10]. The Gram-negative antibiotic disc contained augmentin (30 µg); ofloxacin (5 µg); gentamycin (10 µg); ceftazidime (30 µg); nitrofurantoin (200 µg); cefuroxime (30 µg); cefixime (5 µg) and ciprofloxacin (25 µg) while the Gram-positive antibiotic disc contained cotrimoxazole (25 µg); cloxacillin (5 µg); erythromycin (5 µg); gentamicin (10 µg); augmentin (30 µg); streptomycin (10 µg); tetracycline (10 µg) and chloramphenicol (10 µg).

Four or five colonies of each test organism taken from a nutrient agar culture plate was inoculated into 10 ml of sterile distilled water using a sterile loop. The suspension was thoroughly mixed with a spin mixer. The resulting suspension was adjusted to a turbidity of 0.5 McFarland standard (A_{625nm} = 0.09). This was then applied to the surface of over-dried Mueller Hinton agar and spread evenly with a sterile swab stick. The inoculated plates were incubated at 37℃ for 20 minutes for acclimatization and growth of the inocula. Antibiotic discs (Abtek, Liverpool, UK) were then lightly but firmly pressed onto the surface of the plates using a pair of sterile forceps. The plates were then refrigerated at 4℃ for thirty minutes to ensure adequate diffusion of antibiotics. *E. coli* ATCC 25922 was used as control strain. All plates were incubated at 37℃ for 18 hours. The diameters of inhibition zones were measured in millimetres and interpreted according to CLSI manual.

3. RESULTS AND DISCUSSION

In this study, six genera of bacteria were isolated from used toothbrush collected for study. The distribution is as shown in Table 1. *Bacillus* spp was the predominant genera among the Gram-positive of which *Bacillus flexus* has the highest occurrence. *Klebsiella* spp was the predominant Gram –negative of which *Klebsiella oxytoca* has the highest occurrence.

The percentage distribution of the resistance patterns of Gram-negative and Gram-positive isolates to test antibiotics is as shown in Table 2 and Table 3 respectively. Streptomycin is the drug of choice against the Gram-positive bacterial isolates with least resistance especially against *Bacillus flexus* and *Bacillus licheniformis*. However, Ofloxacin and Ciprofloxacin, both quinolone derivatives, are the drugs of choice against the Gram-negative isolates with least resistance, although the only *Serratia marcescens* isolate in the study was resistant to all the antibiotics tested.

All the isolates are multidrug resistant. The multiple antibiotic resistance pattern of the isolates is as shown in Table 4. While 85.7% of *Staphylococcal* spp were resistant to all the eight antibiotics used for the study, 34.6% of *Bacillus* spp and 60% of *Klebsiella* spp were resistant to all the antibiotics tested. Table 5 shows the distribution of adherence of the isolates as detected by the Congo red method. Of the *Klebsiella oxytoca* isolated, 38.9% were adherent while 28.6% of the *Staphylococcus aureus* were adherent.

Table 1. Percentage distribution of the isolates associated with used toothbrush

S/N	Bacterial isolates	Composition	Percentage	Total number	Total percentage
1	*Staphylococcal* spp	*S. aureus*	10.3% (N = 7)	7	10.3%
2	*Klebsiella* spp	*Klebsiella pneumoniae*	2.9% (N = 2)	20	29.4%
		Klebsiella oxytoca	26.5% (N = 18)		
3	*Bacillus* spp	*B. flexus*	30.9% (N = 21)	26	38.3%
		B. licheniformis	7.4% (N = 5)		
4	*Citrobacter* spp	*Citrobacter freundi*	5.9% (N = 4)	4	5.9%
		Citrobacter kosere	1.5% (N = 1)	1	1.5%
5	*Enterobacter spp*	*Enterobacter cloacae*	13.2% (N = 9)	9	13.2%
6	*Serratia* spp	*Serratia marcescens*	1.5% (N = 1)	1	1.5%

Table 2. Percentage distribution of resistance pattern for each gram-negative isolates to commonly used antibiotics

Antibiotics	*Citrobacter freundii* % resistance N =4	*Citrobacter kosere* % resistance N = 1	*Klebsiella pneumoniae* % resistance. N = 2	*Klebsiella oxytoca* % resistance. N = 18	*Enterobacter cloacae* % resistance N =9	*Serratia marcescens* % resistance N = 1
Gentamicin	100	0	50	100	44.4	100
Ofloxacin	25	0	0	66.7%	22.2	100
Ciprofloxacin	75	0	50	66.7%	44.4	100
Augmentin	100	100	100	100	100	100
Ceftazidime	100	100	100	100	100	100
Cefuroxime	100	100	100	100	100	100
Cefixime	100	100	50	100	88.9	100
Nitrofurantoin	100	100	50	100	77.8	100

Table 3. Percentage distribution of resistance pattern of each gram-positive isolates to commonly used antibiotics

Antibiotics	*S. aureus* N = 7	*B. flexus* N = 21	*B. licheniformis N = 5*
Gentamicin	100	100	100
Augmentin	100	100	100
Streptomycin	100	38.1	60
Tetracycline	100	100	80
Chloramphenicol	85.7	95.2	100
Cotrimoxazole	85.7	90.5	100
Cloxacillin	100	100	100
Erythromycin	100	100	100

Table 4. Multiple Antibiotic Resistance (MAR) patterns of isolates associated with used toothbrush

S/N	Isolates	Total no	Resistance pattern (N = no of antibiotics to which the isolates were resistant)					
			N = 8	N = 7	N = 6	N = 5	N = 4	N = 3
1	*Bacillus spp*	26	9	15	2	-	-	-
2	*Staphylococcus spp*	7	6	-	1	-	-	-
3	*Klebsiella spp*	20	12	2	5	-	-	1
4	*Enterobacter cloacae*	9	2	2	-	2	2	-
5	*Citrobacter spp*	5	1	2	1	1	-	-
6	*Serratia marcescens*	1	1	-	-	-	-	-

Table 5. Distribution of the adherent bacteria associated with used toothbrush

S/N	Isolate	No of adherent organism	% adherence
1	*Klebsiella oxytoca* (n = 18)	7	38.9%
2	*Bacillus flexus* (n = 21)	4	19.0%
3	*Staphylococcus aureus* (n = 7)	2	28.6%
4	*Bacillus licheniformis* (n = 5)	1	20.0%
5	*Enterobacter cloacae* (n = 9)	1	11.1%
6	*Citrobacter kosere*	Nil	Nil
7	*Citrobacter freundii*	Nil	Nil
8	*Serratia marcescens*	Nil	Nil
9	*Klebsiella pneumoniae*	Nil	Nil

The human oral cavity is colonized by a larger variety of bacteria flora than any other anatomic area. More than 700 species of bacteria have already been identified of which 400 were found in the periodontal pocket adjacent to teeth [11]. The oral microflora is divided into two groups as (i). saprophytic which are permanent micro flora of the oral cavity whose presence is necessary for normal functioning of the dental system, as well as the entire body. Saprophytic micro flora affects the conditions of local immune system, prevents the development of pathological conditions and support the bacterial equilibrium; and (ii). pathogenic micro flora which affect the organs and tissues of the mouth and the entire body causing the emergence and development of various diseases. This micro flora ideally should not be there, or perhaps should be present in very limited quantities that do not substantially affect the oral cavity and the body.

The species composition of permanent oral micro flora is normally quite stable and includes representatives of various microorganisms (bacteria, fungi, protozoa, and viruses', etc.) [7]. Predominant are anaerobic bacteria, e.g., streptococcus, lactic acid bacteria (lactobacilli) bacteroids, fuziforms, prevotelly, vellonella, spirochete and actinomycetes. When personal hygiene is at a low level or is missing altogether, the qualitative composition of bacterial flora changes. Pathogenic micro flora prevails, its quality increases by the tens or hundreds of times within a very short time.

However, both saprophytic and pathogenic microflora of the mouth have been isolated from used toothbrushes and reported. For instance, isolation of Enterobacter, Pseudomonas, Klebsiella, *Escherichia coli*, Staphylococcus, Lactobacilli, *S. mutans*, Candida, Proteus and Leuconostoc had been reported [7,12,13].

In this study, six genera of bacteria comprising of *Bacillus* spp, *Staphylococcal* spp, *Klebsiella* spp, *Enterobacter spp*, *Citrobacter* spp and *Serratia* spp have been isolated. *Bacillus* spp, mainly *Bacillus flexus* and *Bacillus licheniformis*, is the predominant of all the isolates. *Bacillus* species are aerobic, sporulating, rod-shaped bacteria that are ubiquitous in nature. Some *Bacillus* species are pathogenic but the large majority of *Bacillus* species are harmless saprophytes. Several other *Bacillus* spp, such as *B licheniformis*, are periodically associated with bacteremia/septicemia, endocarditis, meningitis, and infections of wounds, the ears, eyes, respiratory tract, urinary tract, and gastrointestinal tract [14]. Because *Bacillus* spp are sporulated, their spores are usually suspended in air and may settle on used tooth brush if not properly kept after use. However, the isolation of *Bacillus* spp, *Staphylococcal* spp and *Enterobacter spp* in this study corroborates the findings as earlier reported [13]. Isolation of infectious microorganisms from used brush signifies that brush can reinfect mouth teeth again with some of them spreading to the rest of the body and cause serious health problems, including heart disease, stroke, arthritis, haematogenous, and bacteremia [7].

Although it belongs to the oral microbiota, *Staphylococcus aureus* as isolated in this study deserves greater attention because it is capable of producing many oral infectious diseases.

On the other hand, the presence of Enterobacter as found in this study can be attributed to incorrect storage of brushes, most likely out of a closet and over the bathroom sink, where it is a target of aerosols from the toilet [15].

However, isolation of *Serratia marcescens* made this study different from other reported studies. *Serratia marcescens* is a Gram-negative bacterium that belongs to the Enterobacteriaceae family and can be found as intestinal microbiota of humans and animals and in enviromments with poor nutriment conditions [16]. This microorganism is capable of producing pigments and its various strains are generally found in water, soil, plants and insects, but not in hospitalized patients [17]. However, because the organism readily adheres to invasive hospital instrumentation, such as catheters, endoscopes and intravenous tubing and has relative resistance to standard sterilization and disinfection protocols, it has always been a source of nosocomial infection and can also function as an opportunistic pathogen in imunocompromissed hosts [17]. The bacteria can cause infections at various sites, including the urinary and respiratory tract, septicaemia, meningitis and wound infections. Eye infections are also common and the bacteria frequently cause keratitis. *S. marcescens* infections induce inflammation and fever, but fatal bacteremia can develop in patients weakened by previous infections, surgery and imunosupression [17].

However, relationship has been established between how toothbrushes are kept and the nature of the contaminating microorganisms. It has been reported that brushes that were kept inside the bathroom cabinet showed no growth of enterobacteria whereas the degree of contamination with the presence of two major sorts of feacal coliforms (*Enterobacter spp* and *Citrobacter spp*) in brushes kept on the bathroom sink was 70% [18]. Nonetheless, while the cabinet seems to be the safest place in the bathroom to prevent bristles contamination, controversies have continued to trail the storage of toothbrushes in the bathroom cabinets. While some authors have reported that bathroom cabinets, boxes and bristles protectors are not the most appropriate location for the storage of toothbrushes because those places maintain a moist environment and warm around the bristles and it may promote the microbial growth [19], others submitted such places can encourage cross contamination [20,21].

Suffice it to say that there has not been a consensus as to where best to store toothbrushes but there have been recommendations on how to store toothbrushes to avoid contaminations. Some authors recommended that brush should be kept clean without waste (food or toothpaste) and should be stored where it can dry and without direct contact with other brushes [22]. Other authors submitted that brushes should be washed with running water and the excess water removed by tapping the edge on the sink. The bristles should then be sprayed with mouthwash and stored in an open and airy place [23].

It has also been submitted that the decontamination process of brushes must have a longer range and should be evaluated by disinfection methods that are effective and easy to perform, such as the use of sodium hypochlorite 1% and 0.05% acetic acid [24].

All the isolates in this study are multidrug resistant with majority resistant to all the eight antibiotics used either for Gram-positive or Gram-negative bacteria. This may be attributed to possibility of the presence of genes that code for multiple antibiotics resistance in the isolates. Some of the ways by which bacteria can develop resistance to antibiotics include: (i) Antibiotic inactivation –direct inactivation of the active antibiotic molecule [25]; (ii) Target modification – alteration of the sensitivity to the antibiotic by modification of the target [26]; (iii) Efflux pumps and outer membrane (OM) permeability changes – reduction of the concentration of drug without modification of the compound itself [27]; or (iv) Target bypass – some bacteria become refractory to specific antibiotics by bypassing the inactivation of a given enzyme. This mode of resistance is observed in many trimethoprim- and sulfonamide-resistant bacteria.

Ofloxacin, a fluoroquinolone, and streptomycin, an aminoglycoside, are the drugs of choice with the least resistance by Gram-negative and Gram-positive isolates respectively in this study. Ofloxacin exerts its antibacterial effect by disrupting DNA synthesis through interference with type II topoisomerases DNA gyrase and topoisomerase IV during replication and by causing double strand breaks [28]. Resistance could be developed to ofloxacin by alterations in drug target enzymes and alterations that limit the permeability of the drug to the target [29].

The bactericidal activity of streptomycin is attributed to the irreversible binding to the ribosomes. Resistance to streptomycin is through modification of the target sites by enzymes as aminoglycoside acetyltransferases (AAC), aminoglycoside adenyltransferases (also named aminoglycoside nucleotidyltransferases [ANT]), and aminoglycoside phosphotransferases (APH) [30]. The activity of these enzymes is not restricted to streptomycin alone but other members of the aminoglycosides group.

Aminoglycosides modified at amino groups by AAC enzymes or at hydroxyl groups by ANT or APH enzymes lose their ribosome-binding ability and thus no longer inhibit protein synthesis. Besides aminoglycoside-modifying enzymes, efflux systems and rRNA mutations have been described [31].

Bacterial adhesion to surfaces is an important step in the development of many infections such as dental plaque. The formation of plaque on the tooth surface is characterized by the progression from a limited number of pioneer microbial species to the complex flora of mature dental plaque. This progression involves initial adherence of bacteria to the salivary pellicle and subsequent accumulation by growth and inter-bacterial adherence [32]. One of the ways by which bacterial adhesion can be detected is the use of Congo Red Agar (CRA) method. This method allows for the direct analysis of the colonies and the identification of slime-forming strains (which appear as black colonies on the red agar) and non-slime-forming strains (red-coloured colonies). This is not a quantitative assay because it is based on a subjective chromatic evaluation. The strains that score positive during the test have black spikes on red colonies which remain unchanged in colour [9]. Polysaccharides, which are polymers that significantly impact bacterial virulence [33], are the target of the congo red dye [9]. Relationship between ability of bacterial to produce polysaccharides and their adhesion capability, hence virulence, has been established.

In this study, 38.9% of *Klebsiella oxytoca* and 19% of *Bacillus flexus* isolated were found to be Congo red positive, hence adherent. All these adherent isolates were however resistant to all the eight antibiotics used in the study. This finding corroborates the report of direct relationship between adherence and antibiotic resistance [34]. Adherent bacteria, often called biofilm can develop resistance to antibiotics by trapping of antibacterial in the exopolysaccharides matrix [35], escape of host immune system [36] and by quorum sensing and genotyping adaptation [37].

4. CONCLUSION

The study concludes that toothbrush should be adequately taken care of before, during and after use to prevent it from serving as a vector for the re-introduction of potential pathogens into the oral cavity, and also for the introduction of other microbial species originating from the bathroom environment.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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