African Journal of
Clinical Medicine and Pharmacy Research

ISSN: 3434-2990

Index: Harvard, Boston, Sydney University, Dimensions, Lens, ResearchGet Scilit, Semantic, Google Scholar, Base etc

https://doi.org/10.58578/AJCMPR.v2i2.5121

Burkholderia Cepacia Complex: A Cause of Dental Caries in Uyo, Southern Nigeria State

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Article Info:

Submitted:	Revised:	Accepted:	Published:
Feb 5, 2025	Feb 18, 2025	Mar 2, 2025	Mar 7, 2025

Abstract

Background. Dental caries is a common oral disease worldwide, and it affects above 90% of the population. Several bacteria species are implicated in caries; many of which are the normal microbiota of the buccal cavity. These bacteria are often opportunistic. Caries process starts from proliferation of oral flora, establishment of non-oral bacteria, and production of biofilm leading to plaque formation. This serves as a shield for pathogenic bacteria from being eliminated by antibiotics. The aim of this study was to find out the dominant bacteria of caries in Uyo, and factors that influence their occurrence. **Methods**. One hundred and twenty plaque samples of participants were analyzed using standard laboratory methods; biochemical and antibiotics susceptibility tests results were obtained using Vitek 2 System (bioMe´rieux). CTX-M, TEM and OXA resistance genes were analyzed for, and were amplified on an ABI 9700 Applied Biosystems thermal cycler using pre-determined conditions set. **Results.** Nine (9 (33.3%) Gram-positive bacteria and 18 (66.7%) Gram-negative bacterial isolates were obtained. The dominant isolate was *Burkholderia cepacia complex* 7 (25.9%), while some of the least were; *Pediococcus pentosaceus* 1

(3.7%) and *Klebsiella pneumoniae* 1 (3.7%). Ceftazidime was the antibiotic *B. cepacia* were mostly resistant to, but greatly sensitive to Amikacin and Tobramycin. Six *B. cepacia* isolates (out of 7 identified) were further analyzed molecularly by 16s rRNA sequencing. *Burkholderia cenocepacia* (2 isolates) and *Burkholderia cepacia* (4 isolates) were the two species identified. **Conclusions.** *B. cepacia complex* is known to cause major complications in cystic fibrosis and immunocompromised patients. Therefore, it is a major health challenge for it to be implicated in caries in Uyo, as it can easily spread to different parts of the body (the lungs inclusive) through the carious tooth. It is also an established fact that *B. cepacia* resistance genes rapidly disseminate, making treatment difficult.

Keywords: Dental, Dominance, Plaque, Antibiotic, Resistance, Cystic, Fibrosis

INTRODUCTION

Background. Dental caries also known as caries, cavities and tooth decay. It is the gradual loss of minerals of the tooth leading to cavity (holes in the tooth). This disease affects more than 90% of a given population. This disease has also caused low self-esteem to some people due to foul breath or teeth discolouration and/or tooth breakage or tooth loss caused by the disease, thereby also making eating and drinking foods painful for many. This has a negative impact on the sufferer's health and nutrition. Caries is caused by several events that takes place in the mouth. They are; fermentable carbohydrate deposits on a susceptible tooth, bacteria colonisation, biofilm formation, loss of minerals, among others. It is an establish fact that the bacteria aetiology of caries is composed of oral microbial flora and several other opportunistic bacteria pathogens.

This is in line with several reports made by several researchers about bacteria of caries being opportunistic oral flora. According to Corby *et* al. (2005), earlier results from researchers had suggest that bacteria of a healthy buccal cavity was different from those that are implicated in caries, which is contrary to recent findings. Researchers such Maripandi *et al.*, 2011, Subramonian *et al.*, 2016 and Chandrabhan *et al.*, 2012 also added that bacteria species and quantity changes because of some modifying factors. Anejo-Okopi *et al.* (2015), had documented that advanced identification techniques help in identifying less predominant organisms. It is a fact that more bacteria aetiological agents of caries keeps emerging due to significant advancement in laboratory procedures, and

identification techniques. Worthy of note, is that this study identified *Burkholderia cepacia* (as dominant bacteria), among other less predominant bacteria, as bacterial cause of caries.

This confirms that several other bacteria species of caries will continuously be discovered with more laboratory procedures, and identification techniques advancement. Caries bacteria are highly resistant to many antibiotics used in its treatment. This is because of their inherent composition of resistance genes in their genomes. Although some tend to acquire this resistance genes while in company of other bacteria that possesses them after a sometime. The bacteria of caries are known to show high level of resistance to beta-lactam medications, mostly due to their possession of beta-lactamases. Ogbolu *et al.* (2013), reported that the presence of beta-lactamases (β-lactamases) was the reason for resistance by these bacteria to beta-lactam antibiotics, especially with Gram-negative bacteria that possess them. *B. cepacia* in this study showed great resistance to beta-lactam medications just as it was documented by Ogbolu *et al.* (2013).

Although Gram-positive bacteria are known to secrete sufficient β-lactamase extracellularly, they are found in the periplasmic space in Gram-negative bacteria, between the inner and outer cell membranes. In a mixed population of Gram-positive and Gram-negative bacteria, the production of β-lactamases by some bacteria, is often enough to protect other bacteria in their association from antibiotic inhibition. This is because cell-wall synthesis enzymes are usually found at the outer surface membrane. Hence, β-lactamases being strategically positioned ensure survival of the bacteria. Beta-lactamases are known to be either chromosomal or plasmid encoded, or they may be inducible or constitutive as stated by Cesur and Demiröz (2013). Ensor *et* al. (2009), further added that antibiotic resistance genes harboured by *B. cepacia* are extended-spectrum β-lactamases (ESβL) progenitors. ESβL are known to change substrate profile swiftly due to possible substitution of amino-acid leading to the degradation of cephalosporins.

MATERIALS AND METHODS

Study design and bacterial isolates. This study was a descriptive cross-sectional hospital-based. 120 plaque samples of dental caries patients of dental clinics of; Saint Luke's General Hospital Anua, and University of Uyo Teaching Hospital (UUTH) were analyzed in UUTH Medical Microbiology and Parasitology Laboratory. Before embarking on the study, ethical approval was sought and obtained for the study from both authorities.

Samples were inoculated firstly on Brain Heart Infusion (BHI) broth, and incubated for 24hours at 37^0C. Bottles that showed turbidity were sub-cultured and incubated at 37 ^{0}C overnight on Nutrient Agar (NA), MacConkey agar (MAC), and Cystine Lysine Electrolyte Deficient (CLED) and Blood agar (BA) (Cheesbrough, 2006). Other standard laboratory procedures were done; Gram staining for classification of isolates, biochemical test and antibiotic susceptibility test were performed by Vitek 2 System (bioMe´rieux), for the identification of the several bacteria species, and antibiotic susceptibility pattern of the isolates respectively.

Antibiotic susceptibility testing interpretation. 12-18 hours after running the inoculated antibiotic susceptibility test kits in Vitek 2 System (bioMe´rieux), results were ready. Interpretation was done based on turbidity as reported by the machine as; 'Resistant' 'Intermediate' or 'Susceptible'. According to Magiorakos *et al*., (2012), resistant isolates "to at least one agent in three or more antimicrobial categories" are regarded as multiple-drug-resistance (MDR). *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 (for Gram-positive isolates and Gram-negative isolates respectively) were the control strains used to verify the methods used in this study (Cheesbrough, 2006).

Antibiotic resistance genes of dominant isolates (B. cepacia) molecular detection. Standard gel electrophoresis was carried out to detect their DNA by UV (ultra-violet) transilluminator, and antibiotic resistance gene for *B. cepacia* was detected by polymerase chain reaction (PCR) assay. Alpha DNA Company, Canada are designer of the primer used. 1% agarose gel electrophoresis was used to estimate the PCR products size, in order to confirm amplified products. Formed gel was stained with 1µl of 10mg/ml ethidium bromide (Sigma, USA), before running it at 80V for 1.5hr. UV transilluminator (Cleaver, UK), was used to detect a single band at the required point. Bands were photographed by gel documentation system (Cleaver, UK). Molecular weights of amplified products were measure on a 100bp ladder (Bioneer, Korea) (Saladin *et al*., 2002, Ensor *et al.* 2009 and Dallenne *et al.,* 2010). Other step-down procedures of gel electrophoresis amplification for resistance genes were done for CTX-M, TEM and OXA genes.

Statistical analysis. This was carried out using SPSS, version 22.0 (Chicago, IL, USA). P-values less than or equal to 0.05 ($p \leq 0.05$) of associations between variables were considered statistically significant.

RESULTS

Plaque samples of 120 participants cultured yielded; nine (9 (7.5%) Gram-positive bacterial isolates, 18 (15.0%) Gram-negative bacterial isolates, and 93 (77.5%) had scanty or no growth on culture (Table 1). The dominant Gram-positive species was *Coagulase positive Staphylococcus* 3 (2.6%), and one of the least dominant Gram-positive species was *Enterococcus faecalis* 1(0.8%). Whereas the dominant Gram-negative species was *Burkholderia cepacia complex* 7 (5.8%), and one of the least dominant Gram-negative species was *Klebsiella pnuemoniae* 1 (0.8%). Antibiotic susceptibility test results showed; Gram-positive isolates being most resistant to Tetracycline, Quinupristin/Dalfopristin at 77.8% each, while highest sensitivity was for Levofloxacin 7(77.8%). Gram-negative isolates were most resistant to Ceftazidime 12(66.7%) and highest sensitivity were for; Tobramycin, Nitrofurantoin and Trimethoprim/Sulfamethoxazole at 33.3% each.

The 16s gene electrophoresis was done for six of the dominant isolates (six out of the seven isolated *B. cepacia* complex isolates) using agarose gel for species identification. The 16s band (1500 bp) was represented by lanes 1- 6, and 100 bp molecular ladder by Lane M (Figure 1). There was percentage similarity to other species at 100 % for the 16S rRNA of the isolates analyzed. Using Jukes-Cantor method of evolutionary distances computed for *Burkholderia cepacia* complex (Bcc), two closely relatedness species were identified as; *Burkholderia cenocepacia* (2 Bcc isolates) and *Burkholderia cepacia* (4 Bcc isolates) (Figure 2).

Due to the high level of resistance showed by *Burkholderia cepacia* complex, it was further analyzed for resistance genes; CTX-M, TEM and OXA using agarose gel electrophoresis. Results showed all the 6 isolates having resistance genes for CTX-M gene, 1 isolate harboring the TEM gene, and none of the isolates had the OXA gene (Table 2).

Table 1: **Distribution of species of organisms isolated**

Organism Isolated	**Total (%)**
Gram negative	
Burkholderia cepacia complex	7 (5.8)
Proteus mirabilis	4 (3.3)
Serratia ficaria	2 (1.7)
Serratia marcescens	2 (1.7)
Klebsiella pnuemoniae	1 (0.8)
Acinetobacter spp	1 (0.8)
Enterobacter cloacae ssp dissolvens	1 (0.8)
Gram positive	
Coagulase Positive Staphylococcus	3 (2.6)
Coagulase Negative Staphylococcus	2 (1.7)
Enterococcus faecalis	1 (0.8)
Enterococcus spp (non *E. faecalis*)	1 (0.8)
Pediococcus pentosaceus	1 (0.8)
Kocuria kristinae	1 (0.8)
NO GROWTH	93 (77.5)
Total	**120**

Table 2: **Resistance genes found in the six dominant *B. cepacia complex* analysed**

Identification number given to isolates	**Species of *B. cepacia complex* identified**	**Resistance genes found**	**Position of resistance genes bands on 100 bp molecular ladder**
Isolate 1 (A1)	*B. cepacia*	CTX-M and TEM	550 bp and 400 bp respectively
Isolate 2 (A2)	*B. cepacia*	CTX-M	550 bp
Isolate 3 (A3)	*B. cenocepacia*	CTX-M	550 bp
Isolate 4 (A4)	*B. cepacia*	CTX-M	550 bp
Isolate 5 (A5)	*B. cepacia*	CTX-M	550 bp
Isolate 6 (A6)	*B. cenocepacia*	CTX-M	550 bp

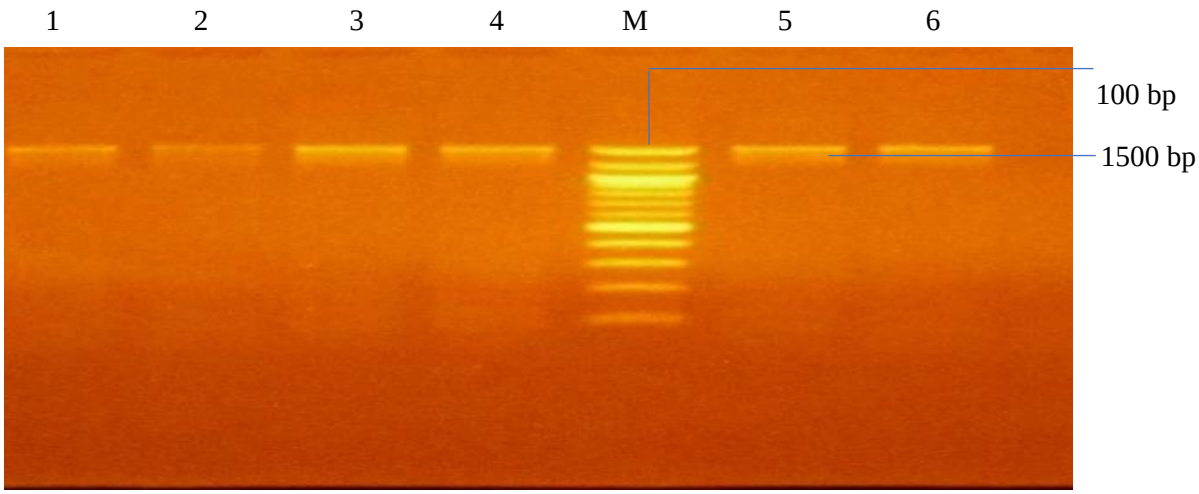

Figure 1: Detection of the 16s band at 1500 bp on 100 bp molecular ladder

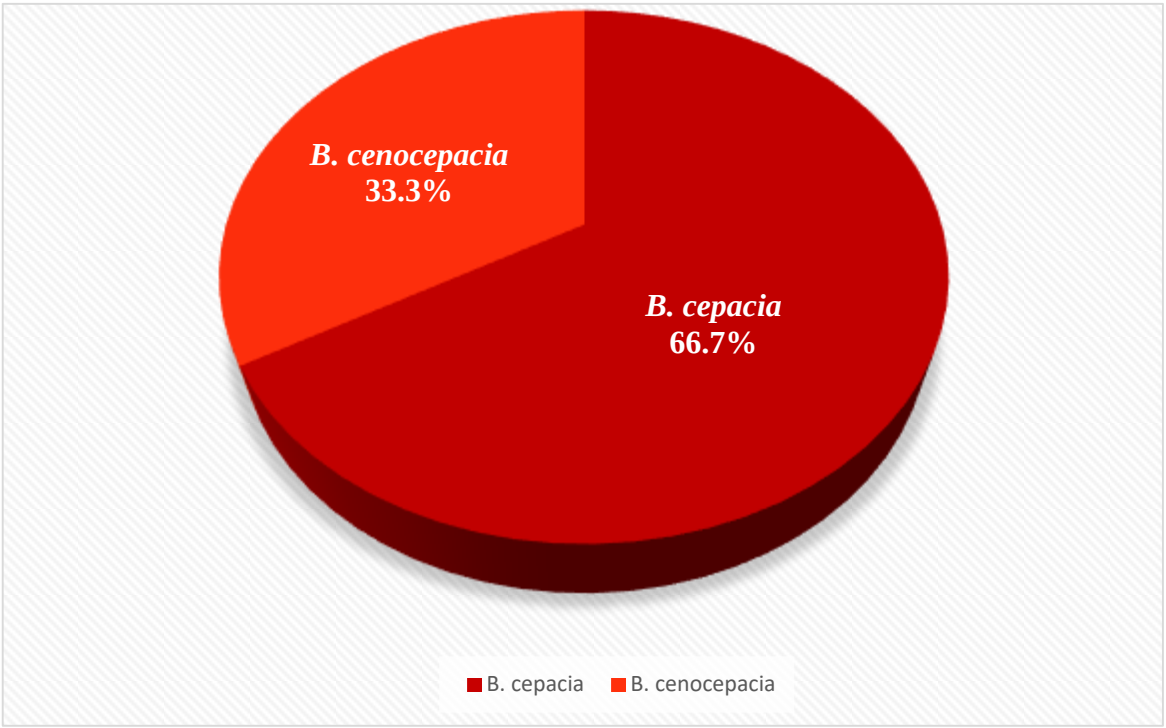

Figure 2: Distribution of the six genotypically identified dominant *B. cepacia complex* species

DISCUSSION

Dental caries is a chronic oral disease that affects almost all members of a population. Its processes and manifestations are usually controlled mainly by presence of; cariogenic bacteria, susceptible tooth (due to high net loss of minerals), degradable carbohydrates, and/or poor oral hygiene. Among the 120 dental plaque samples that were cultured, 27

plates had significant growth of various species of microbes. *B. cepacia* complex was the predominant species with 7 isolates. The less common groups were; *Enterococcus faecalis* 1 (3.7%), *Enterococcus sp* (non *E.* faecalis) 1 (3.7%)*, Pediococcus pentosaceus* 1 (3.7%), *Kocuria kristinae* 1 (3.7%), *Klebsiella pnuemoniae* 1 (3.7%), *Acinetobacter spp* 1 (3.7%) and *Enterobacter cloacae ssp dissolvens* 1 (3.7%). The less predominant organisms reported by Chhour *et al.* (2005), to be implicated in caries were; *Lactobacillus, Prevotella, Elenomonas, Dialister, Fusobacterium, Eubacterium, Olsenella, Bifidobacterium, Propionibacterium, and Pseudoramibacter*.

Six of the seven isolated *B. cepacia* complex were molecularly analyzed, and 16S bands were detected at 1500 bp on the molecular ladder. *B. cepacia* (4 isolates) and *B. ceno*cepacia (2 isolates), were the two species of *B. cepacia* complex identified. B. *cepacia* complex is usually not pathogenic in healthy individuals. Although, *Burkholderia cepacia* in the last twenty years has been frequently linked with microbial proliferations and cystic fibrosis (CF) pulmonary infections (Siddiqui *et al.,* 2001 and Ramsey *et al.,* 2001, Ku *et al.,*2011 and Liao *et al.,*2011). *Burkholderia cenocepacia* and *Burkholderia cepacia* are the two species that have been linked with human infections. *B. cepacia* is also known to cause serious diseases in healthy hosts (Hobson *et al.,* 1995 and Wong *et al.,* 1991). It is further implicated in various infections, example; bacteremia, pneumonia, skin infection, soft tissue infection, as well as genitourinary tract infection (Aygencel *et al.,* 2008).

The *B. cepacia* is often implicated in nosocomial infections, especially in intensive care units of hospitals, these bacteria are usually found in the environment in abundance. The bacteria have also seen in disinfectants, antiseptics, dextrose solution and nebulizer solution, thereby contaminating them (Jones *et al.,* 2001). *Burkholderia cepacia complex* (Bcc) in this study, were mostly isolated from patients with stage 5 dental caries. This is alarming, due to the fact that it could be taken to other parts of the body, like the lungs, from the decayed tooth via the blood capillaries. This is a potential risk for individuals with cystic fibrosis and immune depressed persons. Bcc have been observed to be made up of wide intricate genomes, made up of two to four replicons, whose insertion sequences can rapidly change. This result in high genomic plasticity of *B. cepacia*. Hence, the bacteria possess high levels of adaptability in different environments, like in humans (Sousa *et al.* 2017).

The *B. cepacia* are noted to harbor extended spectrum beta-lactamases (EsβL). These help them to resist several antibiotics, such as; penicillins, ceftazidime, cefotaxime, ceftriaxone (third-generation cephalosporins). The observed resistance mechanism to penicillins or

cephalosporins is the hydrolysis of the β-lactam ring by β-lactamase, resulting to the antibiotic inactivation. β-lactamase are plasmid-encoded, and it help confers resistance to; penicillins, as well as; first, second and third generation cephalosporins, like ceftazidime, ceftriaxone and cefotaxime for bacteria that possesses it. Amongst the major genetic groups of ESBLs of medical importance for Gram-negative bacteria are; SHV, TEM, and CTX-M. Typically, β-lactamases are found in *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*), but are noted to be harbored by several other clinical isolates, such as *Pseudomonas* species and *Enterobacteriaceae* (Al-Muhanna and Al-Ammar, 2020).

Nikaido and Pages (2012), attributed the difficulties faced in *B. cepacia* complex infections treatments to their various antibiotics and disinfectants intrinsic resistance mechanisms. A clinical *E. coli* strain, in 1965, was the first reported TEM-type β-lactamase produced. According to Roschanski *et al.,* (2014), TEM and SHV ESBLs developed the CTX-M-type ESBLs, and have five subgroups depending on the relatedness of their amino acid sequence. They are; CTX-M-I, CTX-M-II, CTX-M-III, CTX-M-IV, and CTX-M-V. Carbapenemases are another class of enzymes noted to confer resistance to carbapenems antibiotic. They are found in three β-lactamase classes; A, B and D. Worthy of note, is the fact that OXA-β-lactamases are found in carbapenemases Class D (Antunes *et al.,* 2014).

Manenzhe *et al.,* (2015), documented that Class B carbapenemases are the metallo-β-lactamases (MBLs), often encoded by integron borne mobile gene cassettes, whereas Class A are chromosomal or plasmid encoded, example being *Klebsiella pneumoniae* carbapenemase (KPC) family. Worthy of note according to Tseng *et al.* (2014), is the fact that *B. cepacia* molecular assay shows resistance to beta-lactam drugs to be multiple, usually due to actions of beta-lactamases which may be inducibly or constitutively oriented or brought about by efflux pumps mechanisms.

CONCLUSION

Dental caries has been linked to have a negative economic impact on the society, due to its morbidity, which may cause complications leading to mortality. The treatment cost for caries are usually expensive, especially for average or poor individuals. These treatments sometimes fail, due to some factors such as; delayed treatment and/or use of wrong medications. It was of great concern for us to find out the most potent antibiotic for the disease treatment, and also detect the genes linked to antibiotic resistance of the bacteria in

Uyo. Hence, this study concludes that there is a significant rate of antibiotic resistance attributed to bla-CTX-M gene, whereas bla-TEM gene confer very little antibiotic resistance, while bla-OXA gene was not implicated in antibiotic resistance in Uyo. Therefore, prompt and effective treatment/management of the disease is paramount to reduce the morbidity and/or mortality rates, and economic burden of the disease.

Acknowledgements

We are thankful to the ethical board members of; Akwa Ibom State Ministry of Health and UUTH for authorizing this research. To our families and friends, we are grateful for your support, as well as the patients and members of staff of Dental clinics of; Saint Luke's Hospital (especially Dr. Aziken Prince Uche, Dr. (Mrs) Eunice Cletus Umeh and Mrs Margaret Okon Otu) and UUTH (especially Dr. Sylvester Ogbode), Uyo were samples were obtained, and members of staff of Medical Microbiology Laboratory, where the study was carried-out.

Author's roles/participation in the authorship of the manuscript:

1. **Mary Athanasius Udoh**: substantial contributions to the conception and design of the work, patients' selection, acquisition of data, analysis and interpretation of data, drafting the work, final approval of the version to be published
2. **Ifeanyi Abraham Onwuezobe**: substantial contributions to the conception and design of the work, patients' selection, revising the work critically for important intellectual content, overall supervision, final approval of the version to be published
3. **Anthony Nathaniel Umo:** substantial contributions to the design of the work, revising the work critically for important intellectual content, final approval of the version to be published
4. **Victoria Intimate Kingsley:** substantial contributions to the design of the work, revising the work critically for important intellectual content, final approval of the version to be published
5. **Amina Jummai Shehu:** substantial contributions to the design of the work, revising the work critically for important intellectual content, final approval of the version to be published

Declarations

Funding: None (No external funding)

Conflict of Interests: None declared

Ethical Issues/Approval: This research study was approved by the Ethical Review Board of; Akwa Ibom State Ministry of Health and University of Uyo Teaching Hospital, and informed consent were given by participants of this study.

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