Research article

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Clinical Immunology and Infectious Diseases

2021
Vol.5 No.4:705

Gene Expression Profiling of Pseudomonas aeruginosa implicated in Otitis Media, Ile-Ife, South western, Nigeria

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Received date: August 06, 2020; **Accepted date:** August 12, 2021; **Published date:** August 22, 2021

Citation: Omololu AJ(2021) Gene Expression Profiling of Pseudomonas aeruginosa implicated in Otitis Media, Ile-Ife, South western, Nigeria. Clin Immunol Infect Dis Vol.5 No.4.

Abstract

Otitis media is an inflammatory disease of the mucosal lining of the middle ear, which includes a variety of medical conditions with different signs and symptoms. It has a worldwide distribution, particularly among children.

A total of 170 patients between the ages 0-75 years who presented with sign and symptoms of otitis media at Obafemi Awolowo University Teaching Hospitals Ear, Nose and Throat clinic were enrolled for this study. Middle ear discharge were collected, and cultured on MacConkey agar, Chocolate agar and Blood agar. It was incubated for 24 hours at 37oC and was observed for growth. The organisms were characterized and identified using conventional biochemical method. Disc susceptibility tests were performed on bacterial isolates using Gentamycin, ciprofloxacin, Augumentin, fusidic acid, rifampicin, clindamycin, Chloramphenicol, erythromycin and tetracycline. It was incubated for 24hours and zone of inhibition was measured and compared to CLSI, 2016. Gentamycin Resistant gene were determined in multiple antibiotics resistant isolates by Polymerase Chain Reaction using ermA, ermC, and mefA primers.

Results indicated Pseudomonas spp (49%) has a high frequency of occurrence incriminated in otitis media followed by Streptococcus spp (22.5%), Staphylococcus spp (16%), Proteus spp (6%), Escherichia coli (2.5%), Klebsiella spp (2.5%) while Citrobacter spp (1.5%) has the least occurence. In the 0-5 and 6-11 year age groups in the two sexes, female patients in the two age groups were more infected than the male counterparts.

Pseudomonas isolates harboured strong resistance against Erythromycin and it showed three resistance gene ErmA. ErmC and MefA were present as revealed by Polymerase chain reaction.

Changes in the DNA sequence constitution of P. aeruginosa can induce various disorder including otitis media.

Introduction

Global Otitis media (OM) is a group of complex infective and inflammatory conditions affect¬ing the middle ear, with a variety of subtypes differing in presentation, associated complications, and treatment. OM is a leading cause of health care visits worldwide, and its complications are important causes of preventable hearing loss, particularly in the developing world (Monasta et al., 2012).

Pseudomonas aeruginosa is seldom a member of the normal microbial flora in humans. Representative colonization rates for specific sites in humans are 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat, and 2.6 to 24% for fecal samples. However, colonization rates may exceed 50% during hospitalization (Pollack, 1995), especially among patients who have experienced trauma to or a breach in cutaneous or mucosal barriers by mechanical ventilation, tracheostomy, catheters, surgery, or severe burns (Blance et al.,1998, Erol et al., 2004,Ohara and itoh, 2003, Thuong et al., 2003, valles et al., 2004). Patients with impaired immunity have higher risks for colonization by this organism and disruption in the normal microbial flora as a result of antimicrobial therapy has also been shown to increase colonization by P. aeruginosa (Blance et al., 1998, Boutoille et al., 1999, Takesue et al., 2002).

The incidence is 1.2–6.0 in 100,000 and usually occurs in children under 2 years of age (Chesney et al., 2013). Patients usually present with the symptoms of AOM plus post-auricular swelling and mastoid tenderness. The condition is more serious than uncomplicated AOM, typi¬cally requiring hospital admission, intravenous antibiotics, and surgery if abscess has formed or mastoiditis has not responded to antibiotics.

The bacteria commonly implicated in upper respi¬ratory tract infections are also those most frequently isolated from middle ear effusions in AOM. These are Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, and to a lesser extent Staphylococcus aureus, and Streptococcus pyogenes; they are believed to enter the middle ear cleft via the eustachian tube. There is a variable incidence in the detection of these bacteria from effusions in AOM, but S. pneumoniae and H. influenzae have been detected most frequently in recent studies

(Pumarola et al.,2013; Chen et al., 2013). On the other hand, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus mirabilis, Klebsiella pneumoniae and Escherichia coli are the common organisms isolated from cases of OM according to Abera and Kibret (2011).

More recently, atopy (MacIntyre et al., 2012) and specific gene abnormali¬ties (TLR421 and FBX01122) have also been implicated as host risk factors for Otitis media. A cross-sectional study of bacterial microbiota in middle ear, adenoid and tonsil specimens from a paediatric patient with chronic serous OM utilizing 16S rRNA gene-based pyrosequencing analysis revealed Pseudomonas spp. as the most common pathogen present in the middle ear, whereas Streptococcus spp. dominated the tonsil microbiota at relative abundance rates of 82.7 and 69.2 %, respectively (Liu et al., 2011).

Despite the wide distribution of P. aeruginosa in nature and the potential for community-acquired infections, serious infections with P. aeruginosa are predominantly hospital acquired.

A review of surveillance data collected by the CDC National Nosocomial Infections Surveillance System from 1986 to 1998 shows that P. aeruginosa was identified as the fifth most frequently isolated nosocomial pathogen, accounting for 9% of all hospital-acquired infections in the United States (Emori, 1993, NNIS, 1998). P. aeruginosa was also the second leading cause of nosocomial pneumonia (14 to 16%), third most common cause of urinary tract infections (7 to 11%), fourth most frequently isolated pathogen in surgical site infections (8%), and seventh leading contributor to bloodstream infections (2 to 6%). Data from more recent studies continue to show P.

aeruginosa as the second most common cause of nosocomial pneumonia, health care-associated pneumonia, and ventilator-associated pneumonia (kollef et al., 2005) and the leading cause of pneumonia among pediatric patients in the intensive care unit (ICU) (kollef et al., 2005). P. aeruginosa can develop resistance to antibacterial either through the acquisition of resistance genes on mobile genetic elements (i.e., plasmids) or through mutational processes that alter the expression and/or function of chromosomally encoded mechanisms. Both strategies for developing drug resistance can severely limit the therapeutic options for treatment of serious infections. The main objective of the study is to detect multiple antibiotics resistance genes in P. aeruginosa bacteria isolates associated with Otitis media.

MATERIALS AND METHODS

Study Area

Obafemi Awolowo University Teaching Hospital is a tertiary Health Care Institution located in Ile-Ife , Osun State ,which has its referrals from neighboring states including Ekiti State and Ondo state. The samples was collected from the Ear, Nose and Throat Clinic of the hospital.

Study Population

All out –patients with clinically suspected cases of ear infection (Otitis media) arrived the unit of ENT during the period of the study.

Inclusion Criteria

Patients who presented with clinical diagnosis of ear infection with ear discharge (Otitis media).

Exclusion Criteria

Patients with other type of ear infections other than otitis media and patients under antibiotic therapy were excluded.

Ethical Clearance (Protocol No Iphoau/12/683)

Permission of this study was obtained from local authority in the area of the study, the objectives of the study clearly and simply explained to all individuals participating in the study, verbal consent was obtained.

Sample Size

The total number of ear discharge samples aseptically obtained was 170 samples using Leslie and Kish formula and analyzed microbiologically. A sample here is defined as any swab specimen obtained from a patient, hence one sample from one patient. The prevalence rate was obtained from Akinpelu et al., 2002 which estimated it to be 11%.

The Leslie and Kish formula is given below as:

$$n = \frac{z^2 pq}{d^2}$$

where,

n= desired sample size, z =Standard normal deviation of 1.96 which corresponds to 99% confidence level, p= prevalence of Otitis media from previous studies to 11%, d= degree of accuracy set as 0.05

q= 1-p

Sample Collection

Ear swabs were collected from patients by aid of sterile cotton tipped swabs with the help of an otologist and kept in thioglycolate broth and were immediately transferred to the Laboratory for further analysis.

Inoculation

The swabs were cultured onto blood agar (HiMedia laboratories Pvt, Ltd, India chocolate agar (HiMedia laboratories Pvt, Ltd, India and MacConkey agar (HiMedia laboratories Pvt, Ltd, India), Then incubated at 370C for 24hrs.

Biochemical Identification of the Isolates using Conventional Methods

The biochemical attributes of the isolates were investigated and these include: the ability to ferment sugars, produce

catalase, coagulase, motility utilize citrate, produce indole, methyl red (MR), Voges Proskauer (VP) test and oxidative-fermentation characteristics.

Polymerase Chain Reaction (Pcr) Amplification and Detection of Pcr Products

Twelve representative multiple antibiotic resistant Pseudomonas isolates were further screened for the detection of resistance genes (ERM A, ERM C, MEF A) using Polymerase Chain Reaction (PCR). The selection of the isolates was based on the antibiotic reaction profiles.

DNA Extraction in Pseudomonas Isolates

A colony of the Pseudomonas bacteria harbouring the DNA of interest was picked and an overnight broth of the colony was prepared at 370C for 24 h. The tube containing an overnight broth culture of the Pseudomonas isolates were vortexed at high speed to re-suspend the cells. One milliliter of the vortexed broth culture of the Pseudomonas isolate was then transferred into an already labelled eppendorf tube and centrifuged at 14,000 rpm for 5 min. The supernatant in the tube was then discarded and blotted on paper towel. One milliliter of sterile distilled water was then added into the tube. The tubes were then vortexed and centrifuged at 13,000 rpm for 10 min. The supernatant was again discarded and blotted on the paper towel. After this, 200 µl of sterile distilled water was added and vortexed to homogenize the pellets. The tube was then boiled at 1000C for 10 mins. After boiling, the tube was again vortexed and centrifuged at 13,000 rpm for 10 mins. The supernatant obtained was then transferred into another pre- labelled eppendorf tube by gently aspiration using a micropipette and kept in the refrigerator 40C until needed for Polymerase Chain Reaction (PCR).

Molecular Detection of Resistance genes in the Pseudomonas Isolates

Twenty five representative multiple antibiotics resistant Pseudomonas and isolates that were resistance to (Tetracycline, Rifampicin, Clindamycin, erythromycin and fusisdic acid) and aminoglycoside (gentamycin) antibiotics were selected for the detection of ERM A, ERM C and MEF A resistance genes.

Amplification reaction were carried out in a volume of 25 µl of a Polymerase Chain Reaction (PCR) mixture containing 1.5 mM MgCl2, 200 µM each of dATP, dCTP, GTP and dTTP, 0.2 µl primer 2,1.5 µl of genomic DNA and 0.1 µl of Taq polymerase. The thermocycle (PRIME, UK) was programmed for optimum condition. The PCR mixture was poured in micro centrifuge tube and vortexed for proper mixing before loading them into the thermocycles. The PCR reaction was preformed as follows: an initial denaturation at 95oC for 3 min, 35 cycles at 95 oC for 30 sec, annealing temperature at 54oC 60 sec, elongation at 72oC for 60 sec and final extension period at 72oC for 10 min.

Agarose gel Electrophoresis

A 0.8 % (w/v) agarose gel (Promega, Madison, USA) was used to resolve plasmid DNA fragments, while 1.5 % (w/v) agarose gel was used to resolve the amplified Polymerase Chain Reaction (PCR) products of the isolated Pseudomonas sp. The 0.8 % (w/v) agarose gel was prepared by combining 0.8 g agarose in ten times concentration of Tris acetate ethylene tetraacetate (10 ml 10XTAF) buffer and 90 ml distillation water in 250 ml beaker flask, while the 1.5 % (w/v) agarose gel was prepared by combining 1.5 g agarose in ten times concentration of Tris acetate ethylene tetraacetate (10 ml 10XTAF) buffer and 90 ml distillation water in 250 ml beaker flask and heated in a microwave for 2 minutes until the agarose was dissolved. The agarose solution was allowed to cool to about 60 oC. After cooling, 2.5 ml ethidium bromide (5.0 mg/ml) (Promega, Madison, USA) was added to the dissolved agarose solution with swirling to mix. The gel was poured into a gel electrophoresis tank and the casting combs were inserted. It was allowed to gel for 30 min. The casting comb was then carefully removed after the gel had completely solidified. One times concentration (1X) TAE electrophoresis buffer was then added to the reservoir until the buffer just covered the agarose gel. Two microliter of gel tracking dye (bromophenol blue) was added to 20 µl of each sample with gentle mixing. Twenty microliter each of the samples were carefully loaded into the wells of the gel created by the combs (the marker was loaded on lane 1 followed by the samples). The electrophoresis tank was then covered and the electrodes were connected to the power pack in such a way that the negative terminal is at the end where the samples have been loaded. Electrophoresis was carried out at 80 volts until the loading dye has migrated about three-quarter of the gel. At the completion of the electrophoresis, the electrodes were then disconnected and the power pack was turned-off. After this, the gel was removed from the buffer and viewed under an Ultra violet-transilluminatior. The band pattern of the DNA fragments were then photographed with a Polaroid camera and documented using an electrophoresis gel documentation system.

Primer	Sequence (5'-3')	size
ermA forward	GAAAAACCCTAAAGA CACGCAAAA	458bp
reverse	AGTGACATTTGCATG CTTCAAAG	
ermC Forward	TATTAAATAATTTATAG CTATTGAAAA	700bp
Reverse	TGAACATGATAATATC TTTGAAAT	
mefA forward	TGTGCATATTTCTATT ACG	300bp
Reverse	CCAATTGGCATAGCA AG	

Table 1: Primers Used for the Detection of Resistant Gene`

Statistical Analysis

Significant differences and relationship between various data obtained were compared using differential and inferential statistics.

Result And Discussion

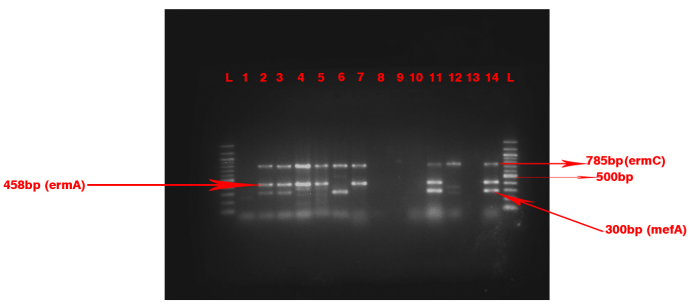
Bacteria isolated	Percentage (%) occurrence
Pseudomonas aeruginosa	(83)49%
Streptococcus spp	(39)22.5%
Staphylococcus spp	(27)16%
Proteus spp	(11)6%
Escherichia coli	(5)2.5%
Klebsiella spp	(5)2.5%
Citrobacter spp	(3)1.5%

Table 1.1: Percentage Frequency of Bacteria Isolated from Ear Discharge of Patients

In this study 2% culture were sterile with no growth after incubation while 97% has growth after 24hours of incubation. Enoz et al., (2008) recorded similarly 5% sterile culture from ear discharge associated with P. aeruginosa.

The results of this present work showed that P. aeruginosa was the most commonly isolated pathogen (49%) followed by Streptococcus spp (22.5%).Similar findings have been observed in Ireland, Pakistan and Greece which reported that P. aeruginosa and Streptococcus spp are the most common organisms isolated from the cases of otitis media (Mukassabi, 2007; Arshad et al., 2004; Bardanis et al., 2003).

The study provides information on the type of resistant gene that is present in Pseudomonas spp. Pseudomonas spp harboured three different resistant gene ermA, ermC and efflux gene mefA. Luna et al., (2000) reported the detection of mefA efflux gene in Pseudomonas spp which described the use of efflux mechanism as a means of resistance to chemotherapeutic agents. The high resistance showed by Pseudomonas spp may be due to the fact that the organism is ubiquitous and it proliferates easily at an available habitat and it's also implicated greatly in nosocomial infections.



Agarose gel electrophoresis of the amplification product coding ermC(785bp) and ermA (458bp) and mefA(300 bp) genes in selected MAR isolates.
Ladder L: DNA marker 100bp, 2,3,4,5,6,7,11,12,14 were positive to the three genes, 8 and 9 is the negative control.

Recommendation

Molecular methods analyzes biological marker in a microbial genome to detect how cells express their genes as proteins. The techniques overlap with clinical chemistry for it analyzes how genes and protein are interacting inside a cell. A change in the DNA sequence constitution of P. aeruginosa can induce various disorder including otitis media. With the advancement of molecular biotechnology various molecular diagnostic methods are now applied in the diagnosis and also treatment of diseases.

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