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EVALUATION OF SEEPLEX PNEUMOBACTER MULTIPLEX PCR KIT FOR THE DETECTION OF COMMUNITY-ACQUIRED PNEUMONIA PATHOGENS AMONG OUT PATIENTS IN UNIVERSITY OF CALABAR TEACHING HOSPITAL, CALABAR

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ABSTRACT

Objectives: Identification of bacterial etiologic agents promptly and correctly is crucial in the management of community-acquired pneumonia (CAP). This study evaluates the analytical working effectiveness and clinical value of a PCR assay in identifying bacterial pathogens using Seeplex Pneumobacter multiplex PCR detection kit. **Materials and Methods:** Sputa from 120 clinically confirmed CAP out-patients aged between 5 and 75 years were analyzed by conventional culture. Fifty specimens were randomly selected from all the age groups and analyzed by multiplex PCR for the detection of *Streptococcus pneumoniae, Legionella pneumophila, Haemophilus influenzae, Chlamydophila pneumoniae, Bordetella pertussis and Mycoplasma pneumoniae*. Results: Forty two specimens (35%) were positive culturally, with *Moraxella catarrhalis* 20 (47.6%) being the most isolated organism. *S. pneumoniae* was the most detected CAP agent via PCR. Of the 50 specimens analyzed by multiplex PCR, 25 (50%) were positive and *S. pneumomiae*, 21 (42%) was the most detected organism. Comparing the 50 specimens evaluated using both methods: 21 *S. pneumoniae* and 14 *H. influenzae* were detected via multiplex-PCR while none of these two organisms was isolated via culture. **Conclusions:** Seeplex pneumobacter multiplex PCR kit could be of great value for detecting the etiologic agents of CAP quite promptly and accurately.

KEYWORDS: Community- acquired pneumonia, Culture, Microscopy, Multiplex- polymerase chain reaction, *Streptococcus pneumoniae.*

INTRODUCTION

Community-acquired pneumonia has not ceased to be a substantial cause of diseases and many deaths all over the world including Nigeria. The prompt knowledge of etiologic agent is required for early initiation of treatment to improve outcomes.[1] Unfortunately, this is not usually achieved due to delay in the isolation of a wide array of pathogens implicated in the disease.[2,1] Several community based studies have identified bacteria as the most common cause of CAP with *S. pneumoniae* isolated in nearly 50% of cases and *H. influenzae* in 20%.[3,4,5] Amongst the atypical bacterial pathogens, *Legionella pneumophila, Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*, have been indicated as frequent causes of CAP and acute bronchitis accounting for 15 to 50% of cases of atypical pneumonia.[6,7] The prompt and precise identification of the causative organisms of CAP provides access for the validation of an appropriate prognosis, facilitation of treatment without delay and possibly, relieving of public health concerns.[2] Nevertheless, the employment of traditional microbiological techniques of identification cannot provide a competent means of diagnosis, for reason that majority of the organisms exhibit slow growth or no growth culturally, bringing about late detection and diagnosis. As a result, serological diagnostic methods were employed[8,9,10] which also demonstrated their limitations such as lack of sensitivity and specificity and also difficulty in detection of increased titre in the acute phase of the disease.[11] Subsequently, the emergence of nucleic acid amplification based techniques was a boost for effective diagnosis of etiologic agents of CAP. The principle of these assays involves the amplification of either the DNA of a complete organism or an isolated nucleic acid by PCR using gene-specific primer. Though various PCR assays were created and adopted for several individual atypical organisms; however, for clinical usefulness and cost effectiveness, it was necessary to develop a multiplex-PCR test kit capable of accurately detecting multiple pathogens and at the same time exhibit outstanding sensitivity and specificity.[12] Recently, the Seeplex Pneumo Bacter ACE detection kit;

a single kit with multiple primers was developed to detect the six target pathogens of bacterial CAP: *Streptococcus pueumoniae, Mycoplasma pneumoniae, Haemophilus influenzae, Legionella pneumophila, Chlamydophila* pneumoniae and *Bordetella pertussis.*[13] This offers undeniable advantages when compared to separate primers of the different organisms in being sensitive, rapid and above all cost effective.

MATERIALS AND METHODS

This study was conducted at the University of Calabar Teaching Hospital (UCTH), a tertiary federal institution in Nigeria. One hundred and twenty patients aged between 5 and 75 years having a temporary diagnosis of pneumonia were enrolled from two outpatient clinics before they were admitted. The diagnosis includes patients presenting severely with at least 2 consistent clinical features including fever, cough, difficulty in breathing, coughing up of blood, production of sputum, breathlessness, chest pain, headache and also, signs that are compatible with pneumonia. Furthermore, a confirmatory diagnosis which involves finding of new infiltrates on chest X-ray was established. Approval for the study was obtained from the Ethics and Research Committee of the hospital prior to commencement of the study. Informed consent was obtained from the patient/patient's parent or guardian. Exclusion criteria included unwillingness to give a written informed consent, and CAP patients that cannot produce sputum.

Specimen collection

The CAP patients enrolled in this study were given proper instructions by a nurse on improved specimen collection methods in an attempt to enhance the adequacy and duplicability of sputum specimens. These included: non ingestion of food 1 to 2 hours prior to expectoration, rinsing of the mouth with saline and the patient being encouraged to cough deeply and expectorate sputum (and not saliva) directly into a sterile wide – mouthed container and lastly sputum specimen should get to the laboratory within 30 minutes of expectoration for immediate processing.

Microscopic sputum screening by gram stain

Smears were made from the most purulent, mucoid or bloody portion of the specimens on a clean grease-free slides and the smears gram stained. All sputum gram stains were viewed under low power (x100) and evaluated according to the standard criteria described by Bartlett (1974). One hundred and twenty sputa were suitable according to the criteria.

Sputum culture

All sputum specimens were cultured alongside smears made for sputum microscopy. Sputa were cultured on 5% blood agar (BA), chocolate blood agar (CBA) and cystein lactose electrolyte deficiency (CLED) agar. Incubation was done at 37^0c for 24 – 48 hours while BA and CBA were incubated under 5 – 10% co_2 in a canister jar. Suspect colonies were gram stained and subcultured on appropriate media from where they are identified.

PCR procedure

Multiplex – PCR was carried out on 50 samples randomly selected from all age groups. This was intended to compare result with those from traditional cultural method for the two organisms: *Streptococcus pneumoniae* and *Haemophilus influenzae*.

DNA was extracted directly from sputum samples using Quick – gDNA Mini Prep isolation kit. Seeplex pneumobacter ACE detection kit designed for the simultaneous detection of six target pneumonia organisms: *Streptococcus pneumoniae, Bordetella pertussis, Mycoplasma pneumoniae, Haemophilus influenzae, Chlamydophila pneumoniae* and *Legionella pneumophila* was used for the PCR assay. An aliquot of 3µL of template DNA was added to 17µL of PCR mastermix made up of: 4µL 5x PneumoBacter ACE primer, 3µL methoxypsoralen (8 – MOP) solution and 10µL 2x Multiplex master mix. The samples were subjected to 40 PCR cycles, consisting of 0.5 minutes of annealing at 60^0c and 1.5 minutes of elongation at 72^0c. The last lap of the elongation process was extended to 10 minutes at 72^0c and the reactions were carried out in a Thermocycler PEQLAB Biotechnologie GmbH.

The approximated sizes of amplicon sizes characteristic of the organisms *Streptococcus pneumoniae, Bordetella pertussis, Mycoplasma pneumoniae, Haemophilus influenzae, Chlamydophila pneumoniae* and *Legionella pneumophila* were 349, 3, 583, 259, 154 and 472 base pairs (bp) respectively. Every run of the amplification reaction included an internal control, a negative control and a positive control composed of plasmids of the 6 pneumonia pathogens.

5 µL of the amplicon (and marker) were subjected to a gel electrophoresis containing 2% agarose and 0.5x TBE, 150v. The size of the amplicon was estimated by comparing it with a standard ladder of 100 bp molecular size. 0.5µl/ml of ethidium bromide stain was added to the gel to aid in the visualization of the amplicons under an ultraviolent transiluminator. Finally, this action was photographed using a digital camera.

Statistical analysis

Statistical analyses were done using the statistical package, Epi Info (CDC, Atlanta, GA, USA) Version 3.5.4. Frequency table was produced. The results were shown as frequency and percentage notation. Differences between two proportions were also investigated. P values ≤ 0.05 were evaluated as statistically significant.

RESULTS

Sputa from a total of 120 clinically diagnosed community-acquired pneumonia (CAP) patients of age range between 5 – 75 years were studied. The patients were 61 (51%) males and 59 (49%) females (mean age

of 36 years). Table 1 showed that 42 (35%) sputum cultures were positive for 9 (21.4%) classes of bacteria, including 20 (47.6%) *Moraxella catarrhalis*, 6 (14.3%) each for *Escherichia coli* and *Pseudomonas aeruginosa*, 3 (7.1%) each for *Enterococci* specie and *Staphylococcus aureus*, 1 (2.4%) each for *Klebsiella* pneumoniae and Salmonella specie and 1 (2.4%) each for cultures that had mixed infection.

TABLE 1: Distribution of isolates by culture

Pathogens isolated	Number of isolates (%)
Moraxella catarrhalis	20 (47.6)
Escherichia coli	6 (14.3)
Pseudomonas aeruginosa	6 (14.3)
Enterococci sp.	3 (7.1)
Staphylococcus aureus	3 (7.1)
Klebsiella pneumoniae	1 (2.4)
Salmonella sp.	1 (2.4)
Mixed infections : *Escherichia coli* and *S. pyogenes* *Escherichia coli* and *Serratia marcescens*	2 (4.8)
Total	42 (100)

Figure 1 showed that in the age distribution of subjects enrolled in the study, the peak age group was between 26–35 years with a prevalence of 32 (26.7%). Of the 42 positive cases for pneumonia via culture as shown in Figure 2, 19 (45.2%) were females, while 23 (54.8%) were males. Figure 3 showed the (cultural) distribution of pneumonia positive cases by gender and age range. Though the highest prevalence of cases by gender 7 (16.67%) occurred in males aged between 36-45 years and also in the same age range the highest number of cases by age range (31%) was found; there exists no statistical significant difference between the prevalence of pneumonia and the two variables: gender and age.

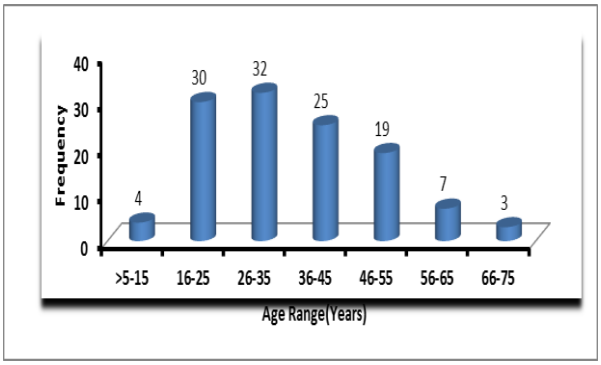

Figure 1. Age distribution of subjects

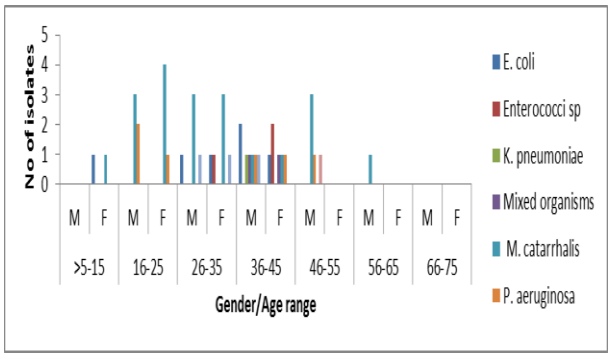

Figure 3. Gender and age distribution of pneumonia positive cases by culture

The 50 cases analyzed by multiplex PCR had 25 (50%) positive cases including, 11(22%) *S. pneumoniae*, 4(8%) *H. influenzae*, 9(18%) cases for both organisms *S. pneumoniae* and *H. influenzae* and 1 (2%) for the three organisms: *S. pneumoniae*, *H. influenzae* and *M. pneumoniae* as shown in Figure 2. *M. pneumoniae* was the only atypical bacteria detected in one case (2%) of multiple infections.

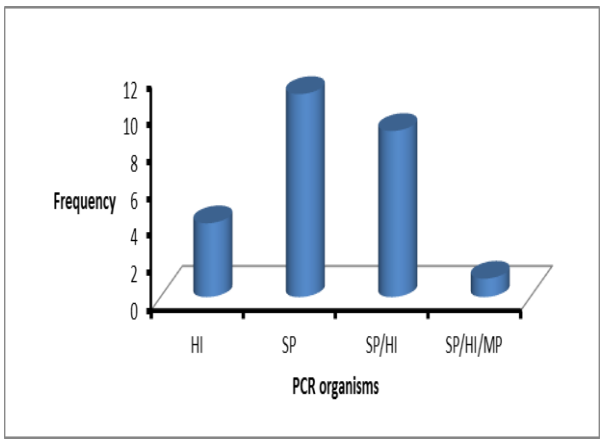

Figure 2. Distribution of organisms detected via multiplex-PCR.
HI, SP and MP represents the organisms: *H. influenzae*, *S. pneumoniae* and *M. pneumoniae* respectively.

The comparison of the results of the 50 specimens evaluated using both cultural and Multiplex-PCR methods (Figure 4) showed that 21 *S. pneumoniae* and 14 *H. influenzae* were detected via Multiplex-PCR while none of these two organisms were isolated via conventional culture methods.

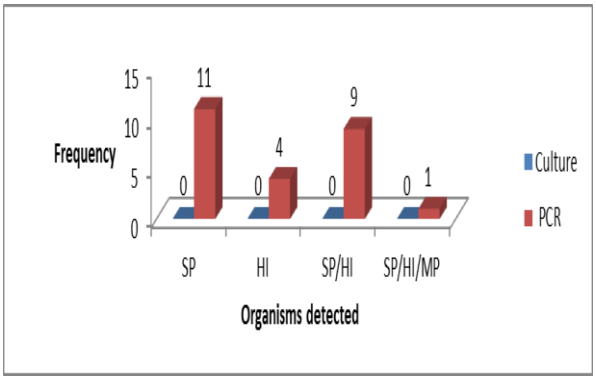

Figure 4. Distribution of organisms detected via multiplex-PCR.
HI, SP and MP represents the organisms: *H. influenzae*, *S. pneumoniae* and *M. pneumoniae* respectively.

All 25 isolates were correctly identified and confirmed as either *S. pneumoniae*, *H. influenzae*, or *M. pneumonia* with the presence of a single band of approximately 349 bp, 259 bp and 583 bp respectively by Multiplex-PCR (Plate 1) using each organism's – specific primers.

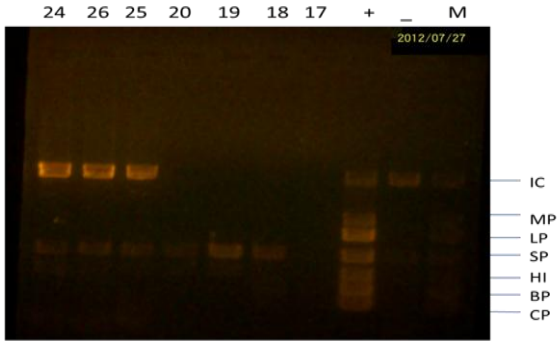

Plate 1: Results of the Multiplex-PCR Seeplex pneumo Bacter ACE Detection Assay. To show the Agarose gel bands containing representative amplicons of the six target pneumonia pathogens: *M. pneumoniae, L. pneumophila, S. pneumoniae, H. influenzae, B. pertussis* and *C. pneumoniae* abbreviated as MP, LP, SP, HI, BP and CP respectively; Lane M, molecular size marker; band of IC, Internal control; lane –ve, negative control; lane +ve, positive control; lanes 17-20 and 24-26, sputum samples. Lanes 19 and 24, sputum samples from a *S. pneumoniae* and *H.influenzae* positive patients; lanes 18, 20, 25, and 26, sample from *S. pneumoniae* positive patients; lane 17, sputum sample from a patient negative for the six target pneumonia organisms.

DISCUSSION

Globally, CAP has continually being an important cause of disease and excess death.[14] The prompt knowledge of etiologic agent is required for early initiation of treatment to improve outcomes.[15] The prompt and precise identification of causative organisms using PCR assay is a major means of achieving the right prognosis that will aid in early administration of treatment that invariably reduces morbidity and mortality.[16] Although few studies had been carried out to ascertain the prevalence and etiology of CAP by culture in Nigeria[17, 18, 19], literature is silent on the determination of etiologic agents of CAP by PCR methods.

Male gender has been identified as a risk factor for pneumonia.[20] This study demonstrated that male patients have a higher prevalence of pneumonia than the female patients by culture though this was not statistically significant. This was similar to previous findings[19,15], suggesting that gender has a strong influence on the prevalence of bacterial pneumonia.

Pneumonia has been considered a disease of young adults in sub-Saharan Africa.[21] This study corroborates this consideration having a highest age group enrolled at 26-35 years (26.7%). However the occurrence of pneumonia positive cases by culture was prevalent at the age range 36 – 45 years. This is contrary to the reports from previous studies.[15,21,19] This may be due to differences in the study design, population evaluated and the characteristics of patients involved in the different studies.

In this study, pathogens were identified in 42(35%) of the 120 patients by culture and 25(50%) of 50 patients by multiplex-PCR. Combining culture and PCR results, *S. pneumoniae* remained the predominant pathogen in the study as in other studies. By extrapolation, we agreed that if the 120 samples were evaluated by PCR methods, twice the 21 detected or even more may have been the result of the number of cases with *S. pneumoniae.* It was observed in this study that gram negative pathogens were the most common group of organisms implicated in pneumonia (by culture), as reported by Fiberesima and Onwuchekwa in South South Nigeria. Gram-negative pathogens are isolated predominantly in healthy young adults in Africa unlike in the western countries[4] and these organisms as causes of pneumonia had been associated with certain risk factors such as prior antimicrobial therapy, comorbid respiratory disorders, use of sputum examination, severe disease, alcoholism, debilitation and intravenous drug abuse.[23,24]

PCR as a current diagnostic tool offers many advantages including yield of positive result at the initial time of disease presentation even when test sample contains low number of causative organism. Also, it is unaffected by prior administration of antibiotics and by host response. In addition, samples can be stored and tested or retested months or years later. From this study, 21 cases for *S. pneumoniae*, 14 cases for *H. influenzae* and one case of *M. pneumoniae* including multiple infection cases were detected. These PCR results are lower than observations from previous studies.[22,13] This may be attributed to variations in sample sizes assessed via multiplex PCR. This has shown that PCR is more sensitive, reliable and beneficial than conventional culture by which none of the two fastidious organisms: *S. pneumonia* and *H.*

influenzae were isolated. None isolation of *S. pneumoniae* and *H. influenzae* may be due to prior antibiotic therapy, death of the two fastidious organisms due to delay in sample delivery to the laboratory. Another reason may be that conditions such as attainment of the optimum incubation temperature and sustaining of the 5-10% co_2 in the candle jar canister needed for the optimum growth of these organisms were not achieved in the working environment due to epileptic power supply. In addition, past literature has it that most of those pneumonia cases of unknown etiology may actually be due to *S. pneumonia*.[4] The detection of *M. pneumoniae* as the only atypical bacteria causing pneumonia in this study affirms the fact that *M. pneumoniae* is an etiology of pneumonia in the tropics as it is a common respiratory pathogen causing pneumonia in temperate climates.[25] The 2% prevalence rate of *M. pneumoniae* detected in this study corroborates with that from a study by Mizra *et al.* (1991)[26] in children with pneumonia in Northern Nigeria.

CONCLUSION

This study demonstrates the usefulness of applying a more sensitive diagnostic method; the PCR alongside the conventional culture method in determining the causative agents of CAP. Pneumonia is a disease of young adults and gender influences its prevalence. *Streptococcus pneumoniae* is the predominant pathogen responsible for CAP, while gram-negative bacilli (including *Serratia marcescens* and *Salmonella* sp) form the commonest group of bacteria isolated. *M. pneumoniae* (2%) was the only atypical bacteria detected in this study via multiplex-PCR.

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