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Age-related Distribution of Mycoplasma pneumoniae in Respiratory Tract Infection in a Developing Country

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Mycoplasma pneumoniae is an organism that belongs to the family mycoplasmataceae. Its role as a disease causing agent continues to draw interest especially with the advent of highly sensitive detection techniques. This bacterium poses a health problem to both animals and humans resulting in serious illnesses such as community-acquired pneumonia, lung damage and this work investigated the prevalence of *M. pneumoniae* as agent of respiratory tract infections using culture and molecular methods of identification, in patients attending Pulmonary Tuberculosis Clinic at Nnamdi Azikiwe Teaching, Hospital, Nnewi as well as detecting the most virulence gene of this organism. A total of 263 sputum samples were collected: 188 test subjects and 75 control subjects. These samples were examined bacteriologically using PPLO broth and agar, MacConkey, blood and chocolate agars. The overall prevalence rates of *M. pneumoniae* among the 263 subjects were 4.9% by culture. The prevalence rate of the organism was significantly higher among the test subjects 11(5.9%) by culture than the control subjects 2(2.7%) by culture. The colonization of the organism was significant among the age groups 31-40 years ($P<0.05$). The antibiotic sensitivity pattern of *M. pneumoniae* showed that the organism was susceptible to Lyntriaxone, Levofloxacin, Ciprofloxacin, Azithromycin and Doxycycline while it showed resistance to Septrin, Peflacine,

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Rifampicin, Erythromycin and Norbactin. *M. pneumoniae* is an additional bacterium that might contribute to respiratory tract infections and consequently to death when it disseminates to various organs of the body, hence their presence in the respiratory tract of children, adolescent and adults should not be treated with levity.

Keywords: Age; distribution; Mycoplasma pneumonia; respiratory tract infection; developing country.

1. INTRODUCTION

Mycoplasma pneumoniae (*M. pneumoniae*) is a common respiratory pathogen that produces diseases of varied severity ranging from mild upper respiratory tract infection to severe atypical pneumonia. *Mycoplasma pneumoniae* causes up to 40% or more of community acquired pneumonia (CAP) cases, than and as many as 18% of cases requiring hospitalization in children [1]. *Mycoplasma pneumoniae* causes both upper and lower respiratory tract infections. Respiratory tract infection can be defined as any infection of the respiratory tract. It refers to the pathological state resulting from the invasion of the body by pathogenic microorganism. Respiratory diseases are major cause of mortality and morbidity worldwide [2]. In most developing countries including Nigeria, the burden of respiratory disease is largely unknown. Respiratory tract infections are divided into two parts: the upper respiratory tract infection (URTI) and the lower respiratory tract infection (LRTI).

Upper respiratory tract infections are the illnesses caused by an acute infection which involves the upper respiratory tract: nose, sinuses, pharynx or larynx. This commonly include: Tonsilitis, pharyngitis, laryngitis, sinusitis, otitis media and the common cold [3]. Symptoms of URTI's commonly include cough, sore throat, running nose, nasal congestion, headache, low grade fever, facial pressure and sneezing. Onset of the symptoms usually begins 1-3 days after the exposure to a microbial pathogen.

Lower respiratory tract is the part of the respiratory tract below the vocal cords. This includes the lungs, bronchi, bronchioles etc. Symptoms of lower respiratory tract infections are shortness of breath, weakness, high fever, coughing and fatique. Lrti are more serious than upper respiratory infections. In 2012, lower respiratory tract infections were still the leading cause of deaths among all infectious diseases and they accounted for 3.9 million deaths world wide and 6.9% of all deaths that year [4]. *M. pneumoniae* affects the upper or lower respiratory tract or both. Symptoms commonly appear gradually, during a few days and can persist for weeks or months. Typical clinical features include an initial pharyngitis, sore throat and hoarseness; fever [5]. An intractable day and night cough characterises extension of the infection to lower airways. Initially, cough is non- productive but later may yield small to moderate amounts of non-bloody sputum [5]. Dyspnoea may be evident in more severe cases. Although most infections occur among out patients hence the colloquial term "walking pneumonia", *Mycoplasma pneumoniae* is a significant cause of bacterial infections in adults requiring hospitalization in USA. Marston *et al*., [6], reported that *M. pneumoniae* was definitively responsible for 5.4% and possible for 32.5% of 2776 cases of CAP in hospitalized adults based on complement fixation (CF) test for detection of infection.

Following the above incidences of *M. pneumoniae* infections, the role of the organism in respiratory tract infections in many parts of the world, including African will not be over emphasized. There is need for the study to be conducted to create awareness on the incidence and prevalence of this *M. pneumoniae* as a causative agent of respiratory tract infections. This will further save the lives of patients suffering from this and subsequently improve the public health.

Aim of the Study:

The study was done to determine the prevalence of *Mycoplasma pneumoniae* in respiratory tract infection using culture method and determine the antibiotic sensitivity pattern of the organism.

2. MATERIALS AND METHODS

Study Design:

In an attempt to evaluate cultural and molecular identification methods of *Mycoplasma pneumomae*, from sputum samples of subjects

attending a Tuberculosis Clinic, this case controlled study was carried out. The patients were recruited from the Directly Observed Treatment Short Course Clinic (DOTS Clinic) of the Nnamdi Azikiwe University Teaching Hospital, Nnewi. Informed consent was obtained and questionnaire administered. Sputum samples were collected and part of each sample was cultured using appropriate media while the other part transported in an ice-park to Safety Molecular Laboratory, University of Nigeria, Enugu Campus where molecular diagnosis was performed.

Study Setting:

This study was carried out at the Tuberculosis Clinic now known as DirectlyObserved Treatment Short Course Clinic (DOTS Clinic), a subunit of Microbiology Department of Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nnewi North LGA, Anambra State.

Selection Criteria:

Entry criteria were Age between 1 year and above and those having one or more of the following symptoms: a productive cough associated with (a) signs of upper or lower respiratory tract infection for at least 3 days or (b) a sore throat or chest pain.

Exclusion Criteria:

Patients treated with antibiotics especially non beta-lactam antibiotics in the preceding 7 days were excluded, likewise those who objected to the screening.

Study Population:

This comprised two hundred and sixty three male and female subjects aged between 1 - 70 years attending Directly Observed Treatment Short Course (DOTS) in Nnamdi Azikiwe University Teaching Hospital, Nnewi in Anambra State for a 17 month period. May 2013 through September, 2014. The 263 subjects selected for this study were distributed as follows: 188 having signs and symptoms of respiratory tract infection served as test subjects and 75 apparently healthy individuals without any signs and symptoms of respiratory tract infection served as controls. Of these, females were 139; 99 test and 40 control subjects. Males were 124; 89 test and 35 control subjects.

Sample Size:

Sample size was determined using Naing *et al.* [7] formular. The prevalence of *Mycoplasma pneumoniae* respiratory tract infection in all age groups carried out in Nigeria was 9% [8]. Using the formular by Naing and co-workers, the sample size was calculated thus.

$$\frac{n = z2\,pq}{d2}$$

Where n = minimum sample size required.

P = expected prevalence rate in % = (9.0) q = 1 – p
d - Degree of accuracy desired set at 0.05
Z = the standard normal deviate set at 1.96 which corresponds to the 95% confidence level.

$$\frac{N = (1.96)2 \text{ x } 0.09 \text{ x } (1 - 0.09)}{(0.05)2}$$

$$\frac{= 3.8416 \text{ x } 0.09 \text{ x } 0.91}{0.0025}$$

= 125.8508
= 126

Computation with the values above gave a sample size of 126. However, the minimum sample size was increased to 263 to increase the chances of isolating this aetiologic agent from the study population.

Therefore, 188 subjects having symptoms of respiratory tract infection were selected with 75 other individuals not having symptoms of respiratory tract infection as controls.

Controls:

These were patients who visited the hospital for diseases other than respiratory tract infection and students on laboratory posting in all the laboratory units of the Nnamdi Azikiwe Teaching Hospital, Nnewi and they were seventy five (75) in number. The sputum production was induced in the control subjects using 5% saline mist (Appendix IV) as a stimulant. Each of the control subjects inhaled the 5% saline mist which stimulated coughing up of alveolar mucus material. Entry criteria for them were:

i. No antibiotic usage in the 7 days before entry in the study.

ii. No signs of upper or lower respiratory tract infection.

Specimen Collection:

A wide mouth sterile universal container was given to each subject to produce sputum. The container was labeled assigned identification number and each subject was also given a questionnaire to fill.

Sample Preparation for Molucular Studies:

Part of the sputum specimens were stored at a refrigerator temperature of 4^oC before transporting in an ice-pack to Safety molecular laboratory, University of Nigeria Enugu Campus (UNEC).

Laboratory Analysis:

Media

The media used were Pleuro pneumonia like organism (PPLO) Broth, PPLO Agar, mycoplasma supplement, for the isolation of the *Mycoplasma pneumoniae* and Tryptose soy broth, blood agar, MacConkey agar and chocolate agar for the isolation of other pathogens.

Methods:

Inoculation and incubation of media

The sputum specimen from each subject was inoculated into PPLO broth (Biotech, USA) and incubated at 37^0C for up to 4 days. After incubation, subcultures were made from the PPLO broth onto PPLO Agar (Biotech, USA). The Agar plates were incubated under increased carbon dioxide atmosphere for up to 2 weeks. These were examined daily for growth and the sterile plates were re-incubated and any plate that did not show any growth after 14 days was discarded. The plates that showed growth were examined with the use of dissecting microscope for the presence of “fried egg” colonies, which if present, indicates suspected *Mycoplasma pneumoniae.* Isolates were identified as described by Cruickshank *et al.* [9] and Waites *et al.,* [10,11]. The sputum from each subject was also inoculated into Tryptose soy broth (Appendix V) and incubated aerobically at 37^0C for 24 hours and subcultures were made onto blood agar, chocolate agar and MacConkey agar plates (Appendix V) for isolation of other respiratory pathogens that might have been in the sputum co-existing with *Mycoplasma pneumoniae* or which might have singly colonized the respiratory tract. The Blood agar and MacConkey agar plates were incubated in air at 37^oC for 24-48hours while the chocolate agar plates were incubated under increased carbon dioxide atmosphere at 37^oC for 24-48 hours. The isolates were identified according to the methods described by Cheesbrough [12].

Identification of *Mycoplasma pneumonia:*

Haemolysis test

Isolates of mycoplasma were inoculated onto PPLO medium to give well dispersed colonies and incubated until colonies are grown, about 5-8 days. The plate was then overlayed with a thin layer of saline-agar containing 1 percent V/V sheep erythrocytes (Appendix VI) and re-incubated aerobically overnight. *M. pneumoniae* produces a maximum clearing resembling β-haemolysis.

Tetrazoluim reduction test

Plates of PPLO agar were prepared with the addition of 2.0ml of 1 percent W/V stock solution of 2-3-5 triphenyltetrazolium chloride per 100ml of medium. The stock solution was sterilized in the autoclave.
A block of agar containing numerous colonies was placed, colony-side down, on the tetrazolium plate and the plate reincubated aerobically. The plates were examined after 3 days and the colony-containing block became pink in colour indicating the presence of *Mycoplasma pneumoniae*.

Sputum lysis stage

Sample: 500µl of sputum. Reagents, Materials and equipment.

The sputum specimens were arranged serially with 10 tubes labeled 1-10. Appropriate numbers of 10ml tubes were arranged and labeled to correspond with the number on sputum samples. Five hundred microlitres (500 µl) of each of the sputum was transferred into a clean 10ml tube.

About 500µl of sputum lysis buffer (ATL buffer) was added and mixed gently by vortexing using vortex machine. The mixture was incubated at room temperature for 25 minutes, with shaking using a rocking platform. The volume was then

adjusted to 10ml with sterile distilled water after the incubation. This content was centrifuged at 6000 rpm for 30 minutes. At this stage, the sample was separated into two layers. The aqueous supernatant layer and the pellet (deposit or lysate).The pellet were resuspended in 500µl of ATL tissue lysis buffer and vortexed gently. This was transferred to 2ml Eppendoff tube for onward procedure to DNA extraction.

Statistical Analysis:

The Data Obtained was described as numbers and percentages when appropriate. The calculations between variables were explored with the chi-square (χ^2) test using fisher's exact test. P-values less than 0.05 (P<0.05) were considered significant. Cross tabulations were equally used to establish the prevalence of the above infection.

All calculations were performed using statistical package for social sciences (SPSS) version 21. The significant level was set at 95% confidence interval and P-value less than 0.05 (P<0.05) were considered significant.

3. RESULTS

Table 1. shows that *Mycoplasma pneumoniae* was obtained among the age groups 21-30 years and 31-40 years with a rate of 66.7% and 33.3% respectively by PCR method while a rate of 50% each was obtained by culture in age groups 21-30 and 31-40 years. The result is statistically significant showing that there is a significant relationship between *Mycoplasma pneumoniae* infection and age (P<0.05).

The antibiogram of *M. pneumoniae* isolates showed that Ciprofloxacin, levofloxacin, Lyntriaxone and Azithromycin showed excellent clearance of *M. pneumoniae* isolates indicating that *M. pneumoniae* was highly susceptible to them while Doxycycline showed intermediate clearance (Table 2). Other antibiotics like Rifampicin, Erythromycin, Septrin, peflacine and Norbactin were not effective against *M. pneumoniae* (Table 2).

Table 1. Distribution of Mycoplasma pneumoniae among the age groups investigated *Mycoplasma pneumoniae* n (%)

Age range (Years)	Test group by culture	Control group by culture
1-10	1 (9.1)	0 (0)
11-20	1 (9.1)	0(0)
21-30	3 (27.3)	1 (50)
31-40	4 (36.4)	1 (50)
41-50	1 (9.1)	0(0)
> 50	1 (9.1)	0(0)
Total	11 (100.0)	2(100)
χ2	4.58	6.483
P-value	0.03*	0.011*

Key: = Significant at P < 0.05*

Table 2. Antibiogram of *Mycoplasma pneumoniae*

Antibiotic	Disc Potency	Average Diameter of zone of inhibition (mm)	Sensitivity
Azithromycin	15µg	19	S
Doxycycline	5µg	15	I
Ciprofloxacin	10µg	18	S
Lyntriaxone	30µg	25	S
Levofloxacin	5µg	21	S
Erythromycin	10µg	13	R
Peflacine	10µg	10	R
Septrin	30µg	10	R
Rifampicin	5µg	11	R
Norbactin	30µg	9	R

Key: S-Sensitivity, I-Intermediate, R -Resistance

4. DISCUSSION

In this study, it was observed that 13 out of 263 patients visiting the DOTS' clinic (Directly Observed Treatment, Short Course clinic) at the Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State were positive for *Mycoplasma pneumoniae* by culture methods, thus giving overall prevalence rates of 5.9% and 9.6% respectively. The result is higher than that obtained by Naoyuki and Atsushi [13], who reported a prevalence rate of 4.8% for *M. pneumoniae* and 5.1% obtained in a study in Nigeria by Oluwa *et al.,* [14], also 1.6% reported in a study in South African by Carrim *et al*., [15] but is lower than that obtained in a study done in Zaria, Northern Nigeria which showed an overall prevalence of 16.2% [16]. This result is also lower than the 19.5% reported by Tsutomu *et al.,* [17], 27% by Anna *et al*., [18].

However, our result is comparable with the findings of some other workers: Dorigo-Zetsma *et al.,* [19] who reported prevalence rate for *M. pneumoniae* by 7% by culture. This notwithstanding, work done by different researchers on different categories of subjects showed varying rate of isolation/detection which may be attributed to different detection methods, cultural techniques, sensitivities of isolation media and categories of subjects used. Considering the prolong period of incubation and fastidious nature of Mycoplasma, Krause and Taylor-Robinson (1992) reported that failure to meet their nutritional needs could limit successful culture.

Age distribution of *Mycoplasma pneumoniae* in this study showed that *M. pneumoniae* was isolated more in adults and elderly than in children. The result is statistically significant ($P<0.05$). This shows that there is a significant relationship between *Mycoplasma pneumoniae* infection and age. This result is in agreement with the findings of Marston *et al;* [6] who reported that *M. pneumoniae* was definitely responsible for 32.5% of 2,776 cases of community-acquired pneumonia in hospitalized adults in a two-county region of Ohio, USA. They also had an additional striking finding that the incidence of pneumonias due to *M. pneumoniae* in hospitalized adults increased with age and it was second only to *Streptococcus pneumoniae* in elderly people. Our result is also in agreement with another study of hospitalized adults with community-acquired pneumonias performed in Isreal [20] and that performed in South Africa [15]. On the contrary, this result is in total disagreement with older studies which relied upon serology and culture and reported *M. pneumoniae* pneumonia to be somewhat uncommon in children aged less than 5 years and greatest among school-aged children 5 to 15 years of age with a decline after adolescence and on into adult hood [21,22], Alexander *et al*., 1996). Our result is also in disagreement with the work of Kim *et al*., [23] who reported that *M. pneumoniae* pneumonia had been more in older children including adolescents.

The antibiotic sensitivity test conducted on the isolates of *M. pneumoniae* in this study revealed that *M. pneumoniae* was highly susceptible to Lyntriaxone, Ciprofloxacin, Levofloxacin and Azithromycin. The organism showed moderate susceptibility to Doxycycline. However, *M. pneumoniae* showed resistance to Rifampicin, Septrin, Peflacine, Erythromycin and Norbactin. This is in agreement with the finding of Waites *et al.,* [10] and [11] who reported that *M. pneumoniae* is inhibited by tetracyclines, macrolides, ketolides and fluoroquinolones. Macrolides include Azithromycin, Clarythromycin etc. Fluoroquinolones include Levofloxacin, Ciprofloxacin, Ofloxacin, gatifloxacin, gemifloxacin, garenoxacin, ketolides eg cethromycin. Our discovering is in line with the work of recent studies that evaluated macrolides and reported that tetracyclines and fluoroquinolones indicated comparable in-vitro activities against isolates of *M. pneumoniae* [24,25,26].

5. CONCLUSION

M. pneumoniae was successfully isolated from the respiratory tract of the subjects. In conclusion, *M. pneumoniae* is an additional bacterium that might contribute to respiratory tract infections and consequently to death when it disseminates to various organs of the body, hence their presence in the respiratory tract of children, adolescent and adults should not be treated with levity.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement

of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

A written consent was obtained from the adult test subjects and controls and from the parents of the children who were under-aged and could not fill the consent form. Also, questionnaire was given to every participant which assisted in providing the demographic data of the subjects as well as the selection and exclusion of the subjects.

ETHICAL APPROVAL

This was obtained from Nnamdi Azikiwe University Teaching Hospital Ethics Committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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