Afr J Med. Med. Sci. (2019) 48, 225-231

Molecular detection of *Mycoplasma pneumoniae* virulent gene from sputum samples of subjects attending Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State, Nigeria.

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Abstract

Background: Mycoplasma pneumoniae is a bacterium whose role as a disease causing agent has been continuously reported especially with the highly sensitive detection methods currently available. It has been implicated in serious illnesses such as community-acquired pneumonia and other lung diseases.

Aim: This work investigated the presence of glycerophosphodiesterase (GLPQ) gene of *M. pneumoniae* in patients attending the chest clinic at Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria.

Subjects and methods: A total of 263 sputum samples were collected, of which 188 were from test subjects while 75 were from the control subjects. Questionnaires were administered for all the subjects. The samples were examined molecularly for *M. pneumoniae* using specific primers in the polymerase chain reaction technique. The PCR positive samples were further tested for the presence of GLPQ gene using molecular technique.

Results: The *Mycoplasma pneumoniae* overall prevalence of 7.98% was obtained. A breakdown of the result into the two groups studied showed that the prevalence rate of the organism was more among the test subjects 18(9.6%) compared with the control subjects 3(4.0%). The prevalence of *M. pneumoniae* virulent gene (GLPQ) among the 18-PCR positive subjects was 5(27.8%) and they were detected only in symptomatic female subjects ($P<0.05$). No virulent gene was detected from the 3 positive control subjects.

Conclusion: This study reports the presence of *M. pneumoniae* in the sputum of symptomatic subjects and also the presence of the virulent gene (GLPQ gene) in some of the positive samples. This organism is thus an additional bacterium that may contribute to respiratory tract infections. It is recommended that the search for this organism be included in the routine analysis of samples of patients with respiratory tract infections using available diagnostic tools.

Keywords: *Mycoplasma pneumoniae, GLPQ gene, Prevalence.*

Abstrait

Contexte : Mycoplasma pneumoniae est une bactérie dont le rôle en tant qu'agent pathogène a été régulièrement rapporté, en particulier grâce aux méthodes de détection très sensibles actuellement disponibles. Elle a été impliquée dans des maladies graves telles que la pneumonie acquise dans la communauté et d'autres maladies pulmonaires.

But : Ce travail a examiné la présence du gène de la glycéro-phosphodiestérase (GLPQ) de *M. pneumoniae* chez des patients fréquentant la clinique de pneumologie de l'Hôpital d'Enseignement Universitaire Nnamdi Azikiwe, Nnewi, Nigeria.

Sujets et méthodes : Un total de 263 échantillons d'expectorations ont été recueillis, dont 188 provenaient de sujets à tester et 75 de sujets témoins. Des questionnaires ont été administrés pour tous les sujets. Les échantillons ont été examinés moléculairement pour *M. pneumoniae en* utilisant des amorces spécifiques dans la technique de la réaction en chaîne par polymérase. Les échantillons positifs à la PCR ont ensuite été testés pour la présence du gène GLPQ en utilisant une technique moléculaire.

Résultats : La prévalence totale de *Mycoplasma pneumoniae* de 7,98% a été obtenue. Une ventilation des résultats dans les deux groupes étudiés a montré que le taux de prévalence de l'organisme était plus fréquent chez les sujets testés 18 (9,6%) que chez les sujets témoins 3 (4,0%). La prévalence du gène virulent de *M. pneumoniae* (GLPQ) chez les 18 patients positifs pour la PCR était de 5 (27,8%) et ils ont été détectés uniquement chez les femmes

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symptomatiques (P <0,05). Aucun gène virulent n'a été détecté chez les 3 sujets témoins positifs.
Conclusion : Cette étude rapporte la présence de *M. pneumoniae* dans les expectorations de sujets symptomatiques ainsi que la présence du gène virulent (gène GLPQ) dans certains des échantillons positifs. Cet organisme est donc une bactérie supplémentaire pouvant contribuer aux infections des voies respiratoires. Il est recommandé d'inclure la recherche de cet organisme dans l'analyse de routine des échantillons de patients atteints d'infections des voies respiratoires à l'aide des outils de diagnostic disponibles.

Mots clés : *Mycoplasma pneumoniae, gène GLPQ, Prévalence.*

Introduction

Mycoplasmas belong to the family *Mycoplasmataceae* in the class of bacteria called Mollicutes. They are the smallest bacteria that are capable of growing on cell-free medium. Their characteristic cell wall-deficient nature made them highly pleomorphic in shape while conferring on them resistance to beta lactam and other antimicrobial agents that act on cell wall of bacteria and also prevent them from being stained by the Gram reagents. *Mycoplasma pneumoniae* is exclusively a human pathogen whose transmission is from person-to-person through air-borne droplets [1]. It is a pathogen of the respiratory tract and a common cause of community-acquired pneumonia (CAP). The infection, also called 'primary atypical pneumonia' usually has a prolonged, gradual onset [2] and may result in all degrees of respiratory involvement from in-apparent infection to pneumonia. Typical clinical features include an initial pharyngitis, sore throat and hoarseness, fever and cough [3].

Mycoplasma pneumoniae causes upper and lower respiratory tract infections in all age groups with the highest rate of infection found in the age group 5 to 20 years [4]. Children less than 5 years of age are less commonly affected [4]. The disease severity has been reported to be more in males than females [4]. Furthermore, it is observed that only 5 to 10% of infected individuals develop pneumonia while the larger percentages of infected persons remain asymptomatic [4].

Beside respiratory tract infection, extra-pulmonary infections caused by *M. pneumoniae* have been reported. They include acute hepatitis [5], immune thrombocytopenic purpura [6], severe autoimmune hemolytic anemia [7], Stevens-Johnson syndrome [8], arthritis [9], transverse myelitis [10] and dermatological manifestations [11] among others.

Pathogenic bacteria use different pathogenic mechanisms to achieve their aim and *M. pneumoniae* is not exempted. Several properties like adherence to cells (cytadherence), cytotoxic and inflammatory potential and the pathogenic role of community-acquired respiratory distress syndrome toxin (CARDS toxin) that activate the inflammatory agents and the genesis of extrapulmonary complications have been reported to play roles in the pathogenesis of *M. pneumoniae* [12]. Also two properties of *M. pneumoniae* seem to be responsible for its pathogenicity in humans. The first is its affinity for respiratory epithelial cells whereby it's remarkable gliding motility and specialized tip organelles allows it to burrow between cilia within the respiratory epithelium. The second is its ability to produce hydrogen peroxide (H_2O_2) which is believed to be the cause for most of the initial cell disruption in the respiratory tract [4]. Schmidl *et al.*, [13] also reported H_2O_2 as the major virulence determinant of the organism. They observed that H_2O_2 is generated during the utilization of glycerol-3-phosphate. The enzyme that generates glycerol-3-phosphate from glycerophosphocholine is called glycerophospho diesterase (GLPQ) formerly known as *Mycoplasma pneumoniae* 420 (MPN 420), and has been reported to be essential for the formation of H_2O_2 when the bacteria are incubated with glycerophosphocholine. *Mycoplasma pneumoniae* is unable to cause any detectable damage to the host cells in the absence of GLPQ [13].

Unlike the genital tract mycoplasmas that have been studied extensively in Nigeria [14-16], only limited work had been done on respiratory *M. pneumoniae* in the country [17]. *Mycoplasma pneumoniae* is still not being routinely sought for in respiratory tract specimens from patients in this environment. Therefore, this study was carried out to screen patients for this bacterium and subsequently search for the presence of virulent genes (GLPQ) from the positive samples of patients attending the chest clinic of Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria.

Subjects and methods

Subjects

A total of 263 subject aged 5 years and above attending chest/DOTS clinics and 75 control subjects who were hospital patients from clinics other than chest as well as some medical students were enrolled into the study. The test subjects had 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. The sputum production in the control subjects was induced using 5% saline mist as a stimulant. Each of the control subjects inhaled the 5% saline mist which stimulated coughing up of alveolar mucus material.
Questionnaires were administered to the subjects and were used to collect the demographic data as well as the complaints of the subjects.

Ethical consideration
Ethical approval of the study protocol was obtained from the ethics committee of the Nnamdi Azikiwe University Teaching Hospital, Nnewi. Written informed consent was obtained from adults and parents of the children included in the study.

Inclusion criteria
- Subjects aged 5 years and above.
- 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
Any subject treated with antibiotics in the preceding 7 days.None consenting patients

Collection of samples
A wide mouthed sterile universal container was given to each subject to collect sputum. Early morning sputum were collected once from each subject.

Molecular studies
The standard polymerase chain reaction (PCR) method was used to detect *M. pneumoniae* and it comprised of the following stages: Genomic DNA extraction stage (Extraction of DNA from samples), preparation of master mix, preparation of primer mix, PCR protocol optimization, PCR set up proper, running the PCR products on the gel electrophoresis and visualization with ultra violet (UV) light.
Genomic DNA extraction was carried out as described by Agbakoba *et al.*, (2008) using Gene JET Genomic DNA Kit.

Polymerase Chain Reaction was carried out using the thermal cycler (2720 Applied Biosystems). Briefly, two sets of primers used were MP88F (5' CAAGCCAAACACGACCTCCGGC3') and MP88R(5'AGTGTCAGCTGGTTTGTC CTCCCC3'). The PCR amplification of the extracted DNA was performed using a final volume of 20µl of the PCR mix. Accordingly, each well of the PCR plate contained 8µl of primer mix; 10µl of PCR master mix and 2 µl of extracted DNA. The amplification conditions were as follows: Initial denaturation was at 95°C for 5 minutes and was followed by denaturation at 95°C for 30 seconds, annealing at 62°C for 45 seconds and extension (elongation) at 72°C for 1 minute. These three steps were repeated for 35 cycles and followed by final extension at 72°C for 5 minutes. The PCR products were analyzed electrophoretically on 1.5% Agarose gel stained with 10µl ethidium bromide. The expected bands for positivity were 172bp (base pair).

Controls
DNA–free distilled water and ATCC 29342D *Mycoplasma pneumoniae* genomic DNA were used as negative and positive controls respectively. Both were loaded in the DNA ladder and included in each run.

Detection of *Mycoplasma pneumoniae* virulent gene (GLPQ gene):
The extracted DNAs of all the samples positive for *M. pneumoniae* were subjected to further PCR tests to detect whether they contain GLPQ gene which is the most virulent gene in *M. pneumoniae.* The PCR was run on the same thermal cycler with similar conditions as for the first tests but with different primer pair and the expected band for positivity was at 90bp.

Results
Mycoplasma pneumoniae overall prevalence rate of 7.98% for *M. pneumoniae* was observed from this study. A breakdown of this result showed that 18 out of 188 test subjects and 3 out of 75 control subjects were positive for *M. pneumoniae*, thus giving prevalence rates of 9.6% and 4.0% respectively. *Mycoplasma pneumoniae* was predominantly detected from the test group and this result is statistically significant ($P<0.05$).

Glycerophosphodiesterase (GLPQ) gene was detected in 5 samples out of the 21 PCR-positive *Mycoplasma pneumoniae.* All the 5 positives were from the test subjects. No GLPQ gene was detected from the 3 PCR positive *M. pneumoniae* obtained from the control subjects (Figure 1). It was observed that all the five patients that were positive for *Mycoplasma pneumoniae* GLPQ gene were females (Figure 2). This result shows a significant relationship between occurrence of GLPQ gene and gender ($P<0.05$).

Table 1 shows the distribution of the GLPQ gene among the age groups. The virulent gene

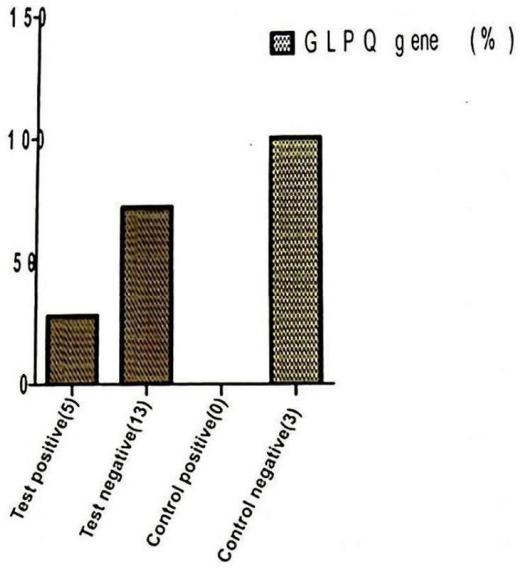

Fig. 1: Percentage occurrence of GLPQ virulent gene among the PCR positive *Mycoplasma pneumoniae*

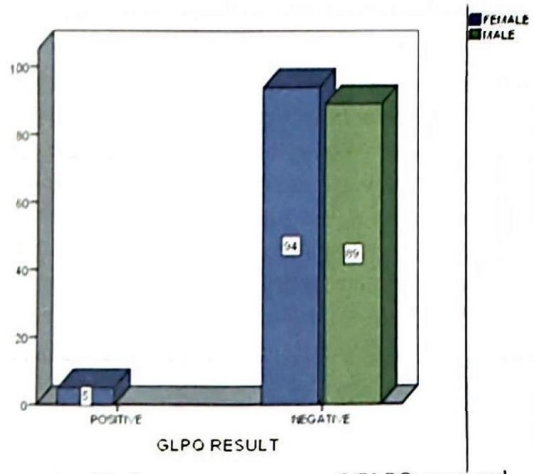

Fig. 2: Relationship between occurrence of GLPQ gene and gender.

Distribution of the five GLPQ genes according to patients' complaints showed that the virulent gene was detected more in patients with symptoms of cough, 2 (40%). One patient each with complaints of chest pain alone, chest pain and cough, and sore throat and cough had 1(20%) of the gene detected in them (Figure 3)\

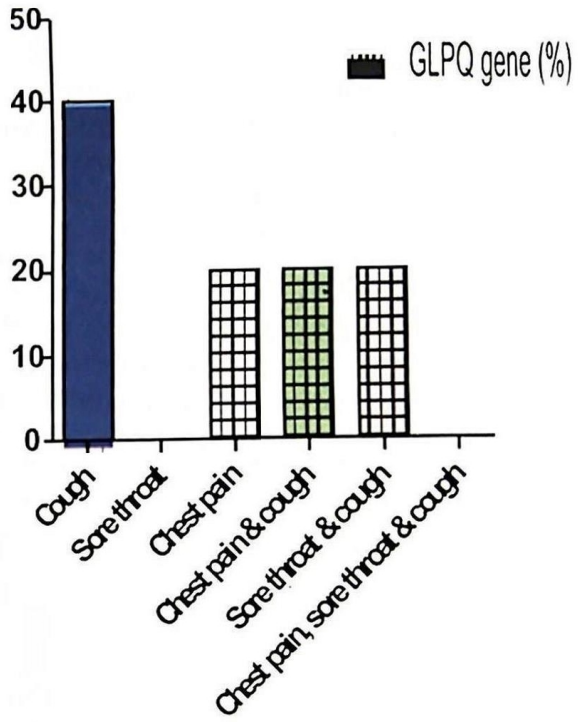

Fig. 3: Distribution of the GLPQ gene according to the patients' symptoms

Table 1: Distribution of the GLPQ gene among the age groups.

Age group (years)	No. of test subject	No. (%) of subjects positive for M. pneumoniae	No. positive for GPLQ gene	% positive for GLPQ
1-10	14	2 (11.1)	1	5.6
11-20	30	2 (11.1)	2	11.1
21-30	64	4 (22.2)	2	11.1
31-40	27	5 (27.8	0	0
41-50	27	2 (11.1)	0	0
50	26	3 (16.7)	0	0
Totatal	188	18 (100)	5	27.8

occurred more between the ages of 11 and 30 years with the rate of 40% each among age groups 11-20 years and 21-30 years followed by those less than 10 years with 20% rate. The virulent gene GLPQ was not found in age groups 31-40 years and above. The statistical analysis showed the result was not significant (P >0.05).

Discussion

The prevalence rate of *M. pneumoniae* (7.98%) obtained in this study is higher than that obtained by Ghotaslou *et al.* [18] who reported a rate of 6.15% from Iran. This rate is lower than that obtained in a study done in Zaria, Northern Nigeria which showed an overall prevalence of 16.2% [17]. The rate in this

result is also lower than the 16% reported by Bhattacharjee *et al.*[19], 19.5% by Tsutomu *et al.*,[20], 27% by Anna *et al.*[21], 32% by Fang-chang *et al.*[22] and 52% by Maczynska *et al.*[23]. However, the result from this study is comparable with the findings of Dorigo-Zetsma *et al.*[24] who reported 8% prevalence rate for *M. pneumoniae;* Csango *et al.* [25] with overall prevalence of 9.8% and Fang-Chang *et al,*[26] who reported an overall prevalence rate of 6.9% for *M. pneumoniae* infection in children in Taiwan Republic of China. These varying isolation/detection rates could be attributable to the differences in the categories of subjects studied, types of specimen collected and processed, detection methods employed and the overall sensitivities of the culture media and serological kits used by the various researchers.

A breakdown of the result into 2 groups of subjects studied (test and control) showed that the prevalence rate of *M. pneumoniae* was more among the test subjects 18(9.6%) than the control 3(4.0%) and this difference is statistically significant (P<0.05). This result contradicts that of Dorigo-Zetsma *et al.*[24], who reported that none of the 74 control subjects sampled were positive for *M. pneumoniae,* although throat swabs were used as against sputum samples used in our study. Sore throat subjects from our study did not yield *M. pneumoniae.*

The 27.8% prevalence rate of the virulent gene, GLPQ, detected from *M. pnemoniae* positive samples in this study though not statistically significant (p>0.05), indicates that not all the positive samples possess this enzyme. This enzyme was totally lacking in the control subjects that were *M. pneumoniae* positive. This fact probably shows the importance of the enzyme in disease causation. The finding is supported by the work of Schmidl *et al,* [13] who stated that GLPQ gene is the only active glycerophosphodiesterase in *M. pneumoniae* and the active glycerophosphodiesterase is required for full gliding velocity of the bacteria. These investigators also reported that *M. pneumoniae* is unable to cause any detectable damage to the host cells in the absence of GLPQ. They further observed that in addition to the enzymatic activity of GLPQ, it is also involved in the control of expression of several genes, among them the glycerol transporter. Consequently, they concluded that GLPQ is central to the normal physiology and pathogenicity of the pathogen, *M. pneumoniae.*

Distribution of *M. pneumoniae* by gender showed that the overall prevalence rate of the organism was more among the females 13 (9.4%) than males 8(6.5%). Ghotaslou *et al.*[18] also had similar findings of 55.5% for females and 44.5% for males. These results were not statistically significant (P>0.05) and the finding contradicts that of Bhattacharjee *et al.*, [19] who reported 17.6% prevalence in males and 12.6% in females. However, the relationship between the presence of GLPQ gene and gender in this study was found to be statistically significant, as all the 5 GLPQ genes detected were from female subjects (P<0.05). This shows that the gene probably has an affinity for female gender thus suggesting that females could be more susceptible to the *M. pneumoniae* containing GLPQ gene than their male counterparts.

Age distribution of *Mycoplasma pneumoniae* in this study showed that *M. pneumoniae* was isolated more in adults than in children (P <0.05). This result is in agreement with the findings of Marston *et al.*[27] who reported that *M. pneumoniae* was responsible for 32.5% of 2,776 cases of community-acquired pneumonia in hospitalized adults in Ohio, USA. Interestingly, the virulent gene of *M. pneumoniae* detected in this study was in age groups less than 30 years, children less than 10years had 5.6% while 11.1% each was found in age groups 11-20years and 21-30years respectively. None was detected in patients above 30 years of age. Contrarily, other workers reported higher prevalence rates of *M. pneumoniae* among children, 24% by Shenoy *et al.*[28] and 27.4% by Chaudhry *et al.*[29]. Our low finding could be due to the low number of children enrolled in this study.

The *M. pneumoniae* detected from this study were observed to be more predominant from symptomatic subjects with various complaints (86%) than asymptomatic cases (14%). This result agrees with the findings of some other workers who reported that those infected with *M. pneumoniae* develop symptoms gradually over a period of several days, often persisting from weeks to months [30]. On the contrary, *M. pneumoniae* from the control subjects were observed from those without any complaints and the absence of the virulent gene (GLPQ) in any of the control samples helps in buttressing this fact. Dorigo-Zetsma *et al.* [30] also reported that 15% of asymptomatic house-hold contacts of 30 index cases with acute respiratory tract infection due to *M. pneumoniae* harboured the organism. When left undetected, it could result to serious disease sequelae such as: arthritis, transverse myelitis and dermatological manifestations.

The most common complaints (symptoms) observed among the patients who had respiratory tract infection due to *M. pneumoniae* in this study were cough, followed by chest pain and cough. Other

complaints included sore throat, sore throat and cough, chest-pain, sore-throat and cough concurrently. It was observed that *M. pneumoniae* respiratory tract infection in this study had relationship with symptoms as almost all those who were positive to *M. pneumoniae* had one symptom or more. This is statistically significant ($P < 0.05$) and agrees with the work of Steven *et al.*[31]; Ferwerda *et al.*[32] and Talkington *et al.*[33] who reported that most common manifestation of patients with *M. pneumoniae* respiratory tract infection include sore throat, hoarseness, fever, cough which is initially nonproductive but later may yield small to moderate amounts of non-bloody sputum, headache, chills, coryza, myalgias, ear ache and general malaise. The occurrence of the virulent gene, GLPQ, was more in those who complained of only cough while none was detected from those who had complaints of sore throat alone.

Conclusion

Detection of *Mycoplasma pneumoniae* from sputum samples and the further detection of the virulent gene, GPLQ, from only the test subjects show that this bacterium actually contributes to the etiology of respiratory tract infection. To the best of our knowledge, this is the first report of the detection of *M. pneumoniae* virulent gene (GLPQ gene) in Nigeria. *Mycoplasma pneumoniae* is not among the organisms being sought for in routine microbiological analysis of respiratory tract specimens. This is due to its fastidious nature and high cultural requirements. Thus, it is recommended that the search for this organism be included in the routine analysis of samples of patients with respiratory tract infections using available diagnostic tools.

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