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ORIGINAL ARTICLE

Neutrophil/lymphocyte Ratio to Predict *Mycoplasma pneumoniae* Infection in Children with Community Acquired Pneumonia

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ABSTRACT

Introduction: *Understanding the immunopathogenesis of bacterial infection has led to a marked improvement in the investigational diagnosis. M. pneumoniae is a common atypical bacterium that cause community acquired pneumonia (CAP). As many diverse microorganisms cause CAP, identification of the etiological cause is essential to guide antibiotic administration. Currently, laboratory diagnosis of M. pneumoniae is unsatisfactory, pediatricians around the word invented clinical scores to help in identifying the cause of CAP.* ***The aim of this work*** *was to introduce Neutrophil/ lymphocyte ratio as a simple, easy and reliable method to assist in the rapid diagnosis of M. pneumonia and to reevaluate the cut off value of M. pneumoniae clinical score (The CAF score suggested by Rodríguez and colleagues).* ***Methodology:*** *This prospective cross sectional study included 50 children from the age of 4 to 12 years old. Cases were selected according to the CAF clinical score. All patients were subjected to full examination and laboratory evaluation including C.B.C, CRP and Chest x-ray. Oropharyngeal swabs were used to isolate M. pneumoniae and diagnosis was made by PCR.* ***Results:*** *There were 6 cases positive for M. pneumoniae (12%) out of 50 patient cases. ROC curve of NLR reveals a statistically significant difference between cases infected with M. pneumoniae and other non infected cases (P value= 0.01). NLR≤ 2.1 is suggestive for M. pneumoniae infection with a sensitivity of 50% and specificity of 95%. ROC curve of CAF score reveals that scores above 10 are diagnostic M. pneumoniae infection with sensitivity of 100% and specificity of 44.19%.* ***Conclusion:*** *NLR is a valuable addition to the diagnosis of M. pneumoniae as an important cause of CAP. Also, we suggest raising the cut off value of CAF score from 5 to 10 to improve the sensitivity of the score with a mild decrease in the specificity. Using both diagnostic tools probably enhance the diagnosis of CAP caused by M. pneumoniae.*

INTRODUCTION

Community Acquired Pneumonia (CAP) in children is a worldwide problem; several microorganisms are known to cause CAP. *M. pneumoniae* is an atypical pathogen, which is a principle cause of CAP [1] that, although usually cause mild infection, may be presented with severe and fatal pneumonia[2,3,4] .

Currently, laboratory diagnosis of *M. pneumoniae* depends on either serial measurement of IgM titer or culture and sensitivity; both methods consume time, and do not aid rapid guiding of treatment decision; additionally they lake sensitivity and specificity. PCR, although rapid and sensitive technique, is not routinely used in the diagnosis of *M. pneumoniae* due to cost-effective consideration[5] .

These limitations in the diagnostic procedures for *M. pneumoniae* elucidate the need for simple, rapid, reliable and cost-effective method for diagnosis.

The use of the Neutrophil/lymphocyte ratio (NLR) rose as a well-established simple, easy marker for the diagnosis of different types of infections[6,7], autoimmune diseases and cancers[8]. NLR has been recently proposed by some clinician to stratify patients with CAP and could add to the performance of the well-accepted illness scores [7].

In this study we selected patients according to the Cough, Age and Fever (CAF) score that was proposed by Rodríguez de Ita and colleagues. It is a clinical score that recognizes CAP caused by *M. pneumonia* and differentiates it from other bacterial or viral causes. According to them, the most important variables were, the duration of cough, the age of the patient and the duration of fever, the score had a sensitivity of 85%, a specificity of 49%, a negative predictive value of 96%, and a positive predictive value of 17. It was considered positive if it is above 5 [9] .

The aims of this study were to evaluate both the NLR ratio as a marker used to differentiate CAP caused

by *M. pneumoniae* from other causes and to reevaluate the cut off value of CAF score.

METHODOLOGY

Study design:

A prospective cross-sectional study was carried out in the Pediatric department and Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, in the period from October 2016 till January 2017. It included 50 individuals from the age of 4 to 12 years old. Well-informed verbal and written consents were obtained from parents or caregivers. The study was approved by Zagazig University Institutional Review Board.

Inclusion criteria:

Children, clinically suggested to have community-acquired *M. pneumoniae* infection (Fever, persistent cough, tachypnea, chest retractions, abnormal auscultatory findings and/or radiologic evidence of lower respiratory tract infections (presence of consolidation, interstitial changes, pleural effusion or mediastinal lymphadenopathy) [10], and in whom *M. pneumoniae* CAF was above five as suggested by Rodríguez et al.[9] (Table 1)

Table 1: *M. pneumoniae* clinical score

.Age (years)	Points	Fever duration	Points	Cough duration	Points
<0.5	0	<1	0	<1	0
0.5–2	1	1–3	1	1–3	1
>2–4	2	>3–5	2	>3–5	2
>4–7	3	>5–7	3	>5–7	3
>7–10	4	>7–14	4	>7–14	4
>10	5	>14	5	>14	5

Exclusion criteria:

Children in whom CAF score was below five, patients on immunosuppressive drugs or steroids and refusal of consent.

Clinical examination:

All patients were subjected to full history taking, full general examination and chest examination. Investigations included CBC, CRP and Chest X- ray

Specimen collection:

Oropharyngeal swabs were collected using sterile dacron swabs with plastic shafts according to CDC guidelines. Swabs were placed into a sterile, labeled vial and transported to the lab within 2 hours.

DNA extraction: was done using QIAamp DNA Minikits, (Qiagen, USA).

PCR: Three sets of allele-specific-PCR Primers were used[11]. The three primers were:

F 5′-AGAAGGAGGTTAGCGCAAGCG-3′,
S 5′-TATATTAGGCGCAACGGGACAGA-3′ and
R 5′-CTGGATAACAGTTACCAATTAGAACAGC-3′.

The 50-µl reaction mixture contained: 5 µl of 10 × PCR reaction buffer, 5 µl of $MgCl_2$ (25 mmol/L), 3 µl of dNTPs (2.5 mmol/L), 1.0 µl of forward (10 µmol/L) and reverse primers (10 µmol/L), 1.5 µl of specific primer (10 µmol/L), 1 µl of Taq DNA polymerase (2.5U), 10 µl of DNA template, and 22.5 µl of double-distilled water (Promega , Australia). PCR Thermal profile included 0.5 minute of denaturation at 94°C, 0.5 minute of annealing at 58°C and 1 minute of extension at 72°C.

PCR products were separated by 2.0% agarose gel electrophoresis with ethidium bromide. Bands were detected at 364 bp which is the diagnostic band for *M. pneumoniae* and 183 bp which is the diagnostic band for the presence of macrolide resistance gene. The absence of both bands means the absence of *M. pneumoniae* infection, while the presence of a band at 364 bp means that patient is infected with *M. pneumoniae* that is sensitive to macrolide. Presence of both bands means infection with *M. pneumoniae* that is resistant to macrolide.

Quality control:

To ensure reliability, *M. pneumoniae* FH reference strain (MyBioSource, USA) was used as a positive and a negative control was included in each reaction. Strict procedures (safety cabinet (II), sterile condition and multiple room procedures) were followed to avoid specimen contamination.

Statistical analysis:

Data obtained from the present study were computed using SPSS versions 16 under the platform of Microsoft Windows 7.

RESULTS

The study included 50 children with age range from 4 to 12 years old 30 (60%) of which were males and 20 (40%) were females.

Clinically, 96% of patients had low-grade fever and 92% had dry cough. The commonest physical findings were wheezes (26%) followed by pharyngitis (24%) and the least finding were cervical LNS enlargement and rhinorrhea (2%). The commonest chest X-ray findings were perihilar infiltrate (46%) followed by lower lobe

opacities (26%) and the least clinical finding was diffuse opacities (18%).

Analysis of PCR results showed that there were 6 positive cases of *M. pneumoniae* (12%) out of 50 patients cases; all those cases showed one band of 364 bp. Of these 6 cases, only 2 cases (33%) were sensitive to macrolide while, 4 cases (66%) were resistant; showed an additional band of macrolide resistance at 183 bp (Figure 1)

Fig. 1: Gel electrophoresis showing 100 bp DNA ladder at lane 1, 364 bp and 183 bands of macrolides resistant *M. pneumoniae* at lane 3 and 364 bp of macrolides sensitive *M. pneumoniae* band at lane 9.

When comparing cases that were proved to be infected with *M. pneumoniae* according to PCR results (no= 6 cases) to other cases (no=44), the absolute neutrophil count and absolute lymphocyte counts did not show a statistically significant difference between both groups (table 2)

Table 2: Neutrophil count and lymphocyte count in both groups

Variable	*Children infected with M. pneumoniae* *6 cases*	*Other children with CAP* 44 cases	Test	P value
Neutrophil count	5.3±0.8 (3.9-6) 5.3	5.3±1.2 (2.9-7.8) 5.9	0.2	0.8
Lymphocyte count	3.3±0.6 (2.6-3.9)	3.4±0.9 (2.1-5.8) 3.1	0.1	0.9

P-value <0.05 is significant

Analysis of ROC curve reveals a statistically significant difference in NLR ratio between cases infected with *M. pneumoniae* and other non infected cases (P value= 0.01). NLR≤ 2.1 is the suggestive cut off value for *M. pneumoniae* infection with a sensitivity of 50% and a specificity of 95.3%. Area under the curve (AUC) = 0.779 and (P value= 0.01). (Figure 2 & Table 3)

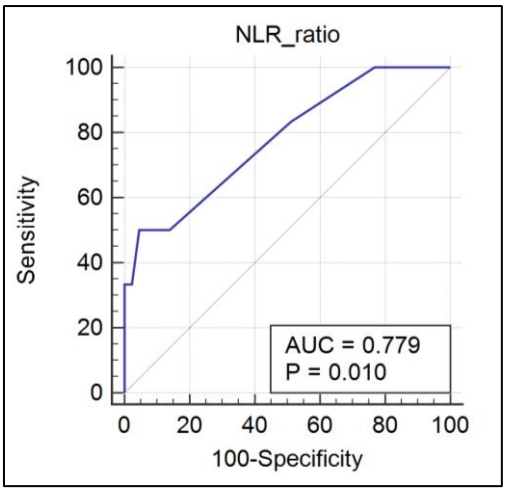

Fig. 2: ROC curve for NLR ratio

Table 3: Sensitivity and specificity of NLR

Variable	Sensitivity	Specificity	Area under the curve	P value
NLR	50%	95.3%	0.779	0.01*

* P-value <0.05 is significant

When comparing cases that were proved to be infected with *M. pneumoniae* according to PCR results (no= 6 cases) to other cases (no=44), there were statistically significant difference regarding age with higher age in positive cases ranged from 8 to 11 years (mean± SD = 9.7±1.3), while, age range was from 4 to12 years (mean± SD = 7.6± 2.1) (t-test= 4.7), (P value= 0.02). Cough duration was also significantly longer in positive cases (mean ± SD =12.2± 2.1). As compared with (9.1± 2.7) for negative cases (t-test 3.6 and P=0.01). Another statistically significant parameter was fever duration, that was longer (mean ±SD= 5.8±0.8) in positive cases compared with (4.7±1.9) in negative case (t-test=4.3), (P=0.02) (Table 4).

Table 4: Clinical parameters of CAF score between infected and non- infected children.

Variable	***Children infected with M. pneumoniae 6 cases***	***Other children with CAP* 44 cases**	**Test**	**P value**
Age mean ± SD	9.7± 1.3	7.6± 2.1	4.7	0.02*
Cough duration mean ± SD	12.2± 2.1	9.1± 2.7	3.6	0.01*
Fever duration mean ± SD	5.8± 0.8	4.7± 1.9	4.3	0.02*

* P-value <0.05 is significant

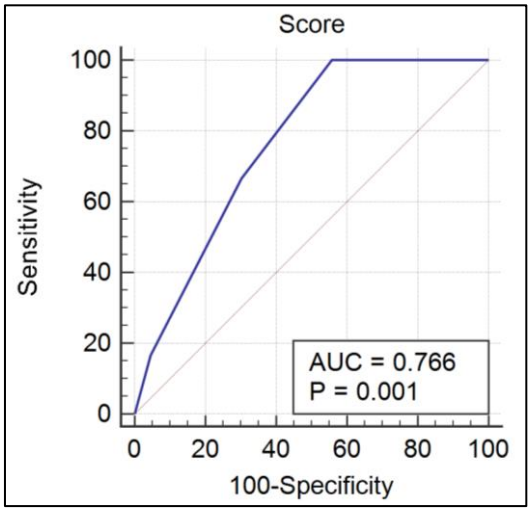

Fig. (3): ROC Curve of CAF score

Analysis of ROC curve reveals that there is a high statistically significant difference in the CAF score between infected and non-infected cases (P value= 0.001). CAF score above 10 diagnose *M. pneumoniae* infection with a sensitivity of 100% and a specificity of 44.19%, a positive predictive value of 10.71% and a negative predictive value of 100%. (Table 5)

Table 5: Sensitivity, Specificity, Positive predictive value and Negative predictive value of CAF score

Characteristic	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Area under the curve (AUC)	P value
CAF score	100.00%	44.19 %	10.71%	100%	0.746	0.001*

DISCUSSION

Community-acquired pneumonia (CAP) is one of the most common infectious diseases worldwide, and an important cause of mortality and morbidity [12]. Many pathogens can cause CAP. Classically, pneumonia is classified into typical and atypical types. Typical lobar pneumonia is usually caused by *S. pneumoniae*, and atypical pneumonia may be caused by *C. pneumoniae*, *Legionella* species, *M .pneumoniae*, respiratory viruses [13] and *M. tuberculosis* (MTB) [13].

It is often difficult to differentiate typical from atypical causes of pneumonia using clinical bases only [13]. However, this differentiation is essential to guide treatment for CAP. For example, typical pneumonia frequently responds well to β-lactam antibiotics whereas atypical pathogens do not respond to them but respond better to tetracyclines, macrolides, and some quinolones. Therefore, there is a great need for an adjunct rapid diagnostic method that can determine the causative agent of CAP[14].

M. pneumoniae is an important cause for the atypical CAP, in developing countries, it was found as a causal agent in 30% of 452 CAP. In Argentina, *M. pneumoniae* was found in 15.2% of 197 children aged from 3mo to 10 years [15]. In Egypt, in a study done at Ain Shams University, *M. pneumonia* was found to be the most common atypical bacterium that causes CAP. It was responsible for 11.1% of the cases of CAP [16]; this result is very near to ours, where *M. pneumonia* was responsible for 12 % of cases of CAP in this study.

Understanding the immunopathogenesis of *M. pneumonia* infection led to the use of IgM measuring as the gold standard for diagnosing *M. pneumoniae* infection. However, IgM antibodies are preferred to be measured in paired serum samples 2 or 3 weeks apart as single assay has a sensitivity of only 31.8% [17]. Cold agglutinins are another diagnostic marker for *M. pneumoniae*. Nevertheless, it is nonspecific and insensitive in children younger than 12 years old [18].

Although culture and sensitivity testing are usually sufficient to detect offending organism and guide the antibiotic use in most of the clinical settings, they are of limited benefit in *M. pneumoniae* due to the fastidious nature of the organism and its slow rate of reproduction [19]. Meanwhile, PCR which is both a specific and a sensitive test, is not cost-effective to be used in the diagnosis of the etiologic cause of CAP [5].

As a result, Searching for other diagnostic tools for *M. pneumonaie* is an interesting area for research. Understanding the immunopathogenesis of bacterial infection is essential to improve diagnostic and management strategies.

Neutrophilia and lymphocytopenia are well-established markers of severe bacterial infection [6]. Neutrophil lymphocyte ratio (NLR) has been discovered to be a simple marker to distinguish between viral and bacterial infections [7,20]. Also, NLR has been used by other researchers to predict the survival in patients with various conditions ranging from cancer to cardiovascular diseases [21,22]. In a retrospective study, the NLR proved to be a simple and even better marker in predicting bacteremia than routine parameters, like white blood cell (WBC) count and C-reactive protein (CRP) level, in infectious emergency admissions [23]. As CAP is an important reason for emergency department admission, the use of NLR may allow the clinician to stratify patients with CAP into different categories and could possibly improve patient's management.

In this study, we aimed to assess the potential use of NLR as relatively cheap and easily measurable laboratory parameters to be added to the CAF score to discriminate between different types of CAP.

Multiple previous works differentiated between NLR in bacterial and viral infection, It has been found that NLR ≥ 2.7 (1.1-5.3) indicates bacterial infection, while NLR as low as 0.6 is diagnostic for viral infection (P< 0.001)[24,25,26]. According to our results, we found that cases infected with *M. Pneumonia* have a statistically significant lower NLR ratio mean±SD is 2.100±0.290, while it is 2.366±0.127 for bacterial, non-*M. pneumonia* cases, this difference is highly significant (P value=0.0002). ROC curve suggests (NLR ≤ 2.1) is a cut off value to differentiate *M. pneumonia* cases from other cases of CAP included in the study. This result is correlated with the result obtained by El-Emshaty et al. 2017 who demonstrated a statically significantly higher median value of NLR among patients with bacterial pneumonia than those with atypical pneumonia[14]. This is also similar to the findings of de Jager et al, who agreed with the same result [23]. In this study, the specificity of NLR was 95% while the sensitivity was 50%. These results match those of Naeess and colleagues who found NLR to be a convenient marker for infection, with high specificity (83.9%) but a moderate sensitivity [27].

Regarding the CAF score that was proposed by Rodríguez de Ita and colleagues and used to select cases in this study, as a clinical score to recognize CAP

caused by *M. Pneumonia*, from those caused by other etiological agents, according to them, the most important variables were, the duration of cough, the age of the patient and the duration of fever, the score had a sensitivity of 85%, a specificity of 49%, a negative predictive value of 96%, and a positive predictive value of 17 and was considered positive if it is above 5. In this study, we diagnose *M. pneumoniae* using PCR which is a sensitive and specific test, after analysis of data using the ROC curve we suggest to raise the cut off value of the scoring system to 10, under this new consideration the score sensitivity will increase from 85% to 100% and the negative predictive value will also improve from 96% to 100%. However, the specificity will mildly decrease from 49% to 44.19.

CONCLUSION

NLR is a valuable addition to the diagnosis of *M. pneumoniae* as an important cause of CAP. Also, raise the cut off value of CAF score from 5 to 10 improve the sensitivity of the score with a mild decrease in the specificity. Using both diagnostic tools probably enhance the diagnosis of CAP caused by *M. pneumonia.*

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