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CLINICAL MICROBIOLOGY - RESEARCH PAPER

High frequency of *Chlamydia pneumoniae* and risk factors in children with acute respiratory infection

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Abstract

This study was performed as a contribution for a better understanding of *Chlamydia pneumoniae* frequency in children with respiratory infections. A total of 416 children were recruited from two clinical centers in Sao Luis, Brazil. Of these patients, 165 children had upper respiratory tract infections (URTI), 150 had community-acquired pneumonia (CAP), and 101 were asymptomatic volunteer children. Clinical and epidemiological data from the participants were recorded. Nasopharyngeal swab samples were collected to extract DNA. *C. pneumoniae* DNA positivity and copy numbers were obtained by an absolute quantitative real-time PCR method.

Results Positivity for *C. pneumoniae* DNA was higher in samples from URTI children (38.2%) and from CAP children (18.0%) than in those from the control group (7.9%; $p < 0.001$). Moreover, *C. pneumoniae* DNA was denser in children with URTI than in asymptomatic children. Considering the cutoff, the highest value of *C. pneumoniae* DNA found in asymptomatic children of the 3.98 log10 copies/mL, 8.5% (14/165) of the children with URTI, and 3.3% (5/150) with CAP presented high copy numbers of *C. pneumoniae* DNA.

Conclusion Taken together, these results revealed a high frequency of *C. pneumoniae* in both children with URTI and CAP.

Keywords Acute respiratory infection · Children · *Chlamydia pneumoniae*

Introduction

Acute respiratory infections (ARIs) including the upper and lower airway respiratory tract infections are the main causes of hospitalizations in developing countries [1]. Community-acquired pneumonia (CAP) is an acute lower respiratory tract infection leading to the deaths of approximately 1 million children under 5 years of age in 2015 alone [2]. This high incidence and mortality related to ARI in children is associated with the presence of risk factors that predispose individuals

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to the development of this disease. The identification and reduction of these factors are necessary to prevent new cases [3]. Another challenge is the early etiological identification of ARI for mortality reduction [4]. In this context, acute respiratory infections are caused by a very variable numbers of bacteria and viruses, and the main pathogens involved are respiratory syncytial virus (RSV), Rhinovirus, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Chlamydia pneumoniae* [5], but the real importance of *C. pneumonia* as causative of ARI hospitalization, pneumonia, and deaths remains unclear. *C. pneumoniae* is an obligate intracellular bacterium and a member of the Chlamydiaceae family. This organism was recognized as a pathogen in 1986 when it was isolated from respiratory tract of patients with acute respiratory disease [6]. It has a biphasic life cycle that includes an infecting phase of elementary bodies and a replicative phase of reticulate bodies [7]. Its infective form exhibits tropism for cells of the respiratory tract and is a common cause of acute respiratory tract infections [8]. However, *C. pneumoniae* is also prevalent in asymptomatic carriage as demonstrated in healthy adults [9, 10] indicating that the detection of this pathogen may not indicate a symptomatic infection.

As an obligate intracellular bacterium, its identification in biological samples is difficult, since it requires non-routine clinical laboratory techniques such as cell culture and DNA-based methods [11]. This may result in an underreporting of respiratory infection cases caused by *C. pneumoniae*. In this context, PCR detection is highly specific and provides fast and sensitive results.

Early diagnosis of *C. pneumoniae* infection is necessary for better clinical management that mainly consists of administration of macrolides or fluoroquinolones [12, 13], because this bacterial species is not susceptible to penicillin [14]. Additionally, persistent infection can be refractory to treatment with macrolide antibiotics [15]. Persistent *C. pneumoniae* infection has been associated with chronic diseases in humans, including asthma, arthritis, and heart disease [16, 17] by likely spreading of the organism via the blood or lymphatic circulation by macrophages [18].

The identification of chlamydial infection in children with respiratory infection and asymptomatic ones may allow for the development of strategies and specific therapies to control ARI in children. Therefore, this study investigated the presence of *C. pneumoniae* in children from São Luís, Brazil, with upper respiratory tract infections (URTIs) and CAP as well as in asymptomatic ones.

Methods

Study population

The study included 416 children of both genders aged 3 months to 10 years (29.7 months of average) during the period from November 1, 2014, to April 30, 2016. Among the participants, 165 were outpatient children with URTI, 150 were hospitalized children with CAP, and 101 asymptomatic children without any respiratory symptoms (control group). Participants with respiratory diseases were recruited from two clinic centers of São Luís, Brazil (Dr. Odorico de Amaral Matos Children's Hospital and Dr. Juvêncio Mattos Children's Hospital). The control group consisted of children without any respiratory symptoms, either currently and in the last 3 months before enrollment, who attended daycare or schools. The case population was selected according to protocol definitions and inclusion criteria. URTI was defined when children presented one of the following respiratory symptoms: low-grade fever, sore throat, runny nose, sneezing, or nasal obstruction, for a period up to 7 days. Pneumonia cases were defined using the Brazilian Society of Pneumology and Tisiology Guideline [19] as follows: (a) presence of consolidation (a dense or fluffy opacity), pulmonary infiltrate (alveolar or interstitial densities), or pleural effusion on chest radiography; (b) two or more symptoms of acute lower respiratory tract illness, cough, fever, difficulty breathing, age-adjusted tachypnea (≥ 50 breaths/ min for children aged 2–11 months; ≥ 40 breaths/min for children aged ≥ 12 months), and/or wheezing. In addition to the inclusion criteria, exclusion criteria were: (a) live attenuated influenza vaccination less than 7 days before enrollment, (b) undergoing otolaryngologic surgery, (c) presence of comprised chronic debilitating disease (anatomic abnormalities of the respiratory tract, cancer, chronic pulmonary illness, asthma, immunological defects, heart disease with clinical repercussion, hemoglobinopathy, liver or kidney disease), and (d) HIV-infected mother.

Clinical and epidemiological data were obtained from the medical records of the children and from a questionnaire filled out by the parents or guardians during the period of hospitalization or attendance at daycare or schools.

The recorded data were the following: age, gender, malnutrition based on the WHO criterion of a Z-score cutoff point of ≤ 2 standard deviations, type of pregnancy, low birth weight based on the WHO criterion of less than 2.5 kg regardless of gestational age, exclusive breastfeeding for 6 months, breastfeeding for < 12 months, immunization history according to the Brazilian vaccine calendar (incomplete immunization was defined when the child did not receive at least one vaccine), length of maternal education (a low maternal education level was defined as < 9 years of schooling), type of delivery, family income (a low family income was defined as less than 2 times the minimum wage), numbers of smokers living in the house, and clinical data (signs, symptoms, and history of pneumonia).

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of Ceuma University under the Certificate of Presentation for Ethical Consideration (CAAE #467.131) and was in compliance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from parents or caregivers prior to enrollment.

Biological samples and DNA extraction

Nasopharyngeal samples were collected with a sterile swab (Plast Labor, Rio de Janeiro, Brazil). The swab was introduced into one nostril until resistance and then rotated 180°. Collected samples were stored in 2 mL of 0.9% NaCl and transported under refrigeration (4–8 °C) until processing (maximum of 2 h later). Samples were centrifuged at 10,000*g* for 10 min. To the pellet, 180 μL of 200 mg/mL lysozyme was added and samples were incubated at 37 °C for 30 min. Then, DNA was isolated using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Construction of *C. pneumoniae* DNA standard curve

C. pneumoniae strain AR-39 DNA (kindly provided by the School of Pharmaceutical Sciences, University of Sao Paulo, São Paulo, SP, Brazil) was used as positive controls. The *C. pneumoniae* 16S rRNA gene was amplified by PCR using protocols and primers previously described by Lima-Neto et al. [20]. The expected PCR product size of 125 bp was verified by agarose gel electrophoresis and the PCR products were purified and cloned using the pGEM-T System kit (Promega Corporation) following the manufacturer's instructions as previously described [21].

Copy numbers of pGEM-T *C. pneumoniae*–recombinant DNA were determined as previously described [21] and the DNA concentration was adjusted to 10^7 copies/mL, serially diluted (1:10) to obtain DNA standard solutions with copy numbers of 10^3 to 10^6, and used for construction of standard curves and as positive controls in quantitative real-time PCR (qPCR) assays.

Quantification of *C. pneumoniae* DNA in biological samples

DNA extracted from the swabs and standard DNA (from *C. pneumoniae* strain AR-39) were amplified by qPCR. The quantification of the copy numbers of *C. pneumoniae* DNA/mL was realized in an optimized qPCR assay contained primers and probe at 200 nM, 1× GoTaq® Probe qPCR Master Mix (Promega Corporation), and 30-ng DNA in a final volume of 25 μL as previously optimized [21]. The qPCR assays were carried out in the QuantStudio™ 6 Flex (Thermo Fisher Scientific, Waltham, MA, USA) using the following program: one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, and 60 °C for 1 min.

To determine the copy numbers of *C. pneumoniae* DNA/mL (also described as DNA density) of each nasopharyngeal sample, the cycle threshold (Ct) result was compared with that of the standard curve, and then the value obtained was normalized by the number of 16S gene copies in the *C. pneumoniae* genome [21].

qPCR specificity, sensitivity, and precision

All primers and probe were checked for possible cross-hybridization with bacterial or human genes using a database similarity search program, BLAST (Basic Local Alignment Search Tool). Additionally, the specificity of the *C. pneumoniae* 16S rRNA gene primers and probe were also tested in a qPCR assay performed using DNA extracted from pure cultures of bacterial clinical isolates from normal throat flora or common respiratory tract agents including *Streptococcus pyogenes* (ATCC 19615), *Streptococcus pneumoniae* (ATCC 55143), *Staphylococcus aureus* (ATCC 25923), *Haemophilus influenzae* (ATCC 53876), *Pseudomonas aeruginosa* (ATCC 27283), and *Klebsiella pneumoniae* (ATCC 10031) and clinical isolates of *Neisseria* spp., *Mycoplasma pneumonia* and *Chlamydia trachomatis*.

Positive and negative DNA controls were also used in every qPCR assay done on the clinical specimens to avoid false-negative and false-positive results. All samples were performed in duplicate.

qPCR assays were performed using a serial dilution of *C. pneumoniae* DNA (10 to 10^6 copies/mL) to determinate the limit of detection (LOD) and the limit of quantification (LOQ). The LOD was defined as the lowest amount of *C. pneumoniae* DNA detectable in a single reaction. The LOQ was the lowest amount of *C. pneumoniae* DNA that can be quantified. In the same assay, the qPCR efficiency was evaluated through the determination of the slopes of the standard curves using an equation previously described [22].

For precision analysis, repeated analysis was performed for 25% of the DNA samples, which were randomly selected.

Radiographic findings

Patients with clinical suspicion of CAP were submitted to chest radiograph (CRX) and two independent pediatric radiologists analyzed the CXR and registered in accordance with x-ray standardized interpretation [23]. All chest radiographs were recorded daily and were evaluated separately by the two pediatric radiologists who read each of all the CRX, independently. Further evaluation was done by a third pediatric radiologist who unaware of the clinical data and of the

pediatricians' evaluation. The presence of the consolidation, infiltration, non-obstructive pulmonary atelectasis, and pleural effusion were registered.

Statistical analysis

Data were analyzed using the GraphPad Prism version 6, Epi-Info version 6.0, and IBM SPSS Statistics 20.0 software. Categorical variables were compared by chi-square test or Fisher's exact test. Continuous variables were compared by *t* test (normal distribution) and Mann-Whitney test (without normal distribution).

In order to evaluate the risk factors associated with ARI, univariate logistic regression was applied and odds ratios (ORs) were assessed including the 95% confidence interval (CI). Multivariate logistic regression was applied after adjusting the model for the covariates that presented $p < 0.02$. Statistical significance was considered $p < 0.05$.

Results

Table 1 shows the clinical data for the children with URTI and with CAP. The majority of the patients with URTI or CAP presented with dyspnea (62.8% and 84.0%, respectively), fever (59.1% and 83.1%, respectively), and wheezing (50.5% and 72.6%, respectively). Additionally, 77.4% of children with CAP had less than 10 days of symptoms, 52.0% had cough, 50.0% had tachypnea, and 42.1% had history of pneumonia.

The epidemiological data of children with URTI or CAP are shown in Table 2. Among these children, incomplete immunization, low maternal education level, and > 1 people per child bedroom were associated with both the URTI and CAP groups. In addition, cigarette smoker living in the house was only associated with the URTI group and malnourishment, preterm birth, low birth weight, and lack of prenatal treatment were only significantly associated with the CAP group.

Univariate and multivariate logistic regression of risk factors for URTI are shown in supplementary Tables 1 and 2, respectively. According to the univariate logistic regression analysis,> 1 person per child's bedroom had increased risk for URTI. After adjusting the model with the factors that presented $p < 0.02$, multivariate logistic regression showed that > 1 people per child bedroom still was associated with increased risk for URTI. Furthermore, malnourished children, incomplete immunization, and low maternal education level had increased risk for CAP (supplementary Table 3). After adjusting the model with the factors that presented $p < 0.02$, multivariate logistic regression showed that incomplete immunization and low maternal education level still were associated with increased risk for CAP (supplementary Table 4).

As shown in Fig. 1, *C. pneumoniae* DNA was detected more frequently in swab samples from the respiratory tracts of both URTI (38.2%) and CAP children (18.0%) than in those of the control group (7.9%, $p < 0.001$). Additionally, the median density of *C. pneumoniae* DNA among 165 cases with URTI (3.4 $\log_{10}$ copies/mL) exceeded that of control cases (2.9 $\log_{10}$ copies/mL, $p < 0.05$) as shown in Fig. 2. On the other hand, there was no significant difference in the density of *C. pneumoniae* DNA for the CAP group (median density of 3.0 $\log_{10}$ copies/mL) when compared with the controls ($p > 0.05$).

The highest *C. pneumoniae* DNA copy number observed in the control group 3.98 $\log_{10}$ copies/mL was used as the cutoff value and it was observed that 8.5% (14/165) of the children with URTI and 3.3% (5/150) with CAP presented high copy numbers of *C. pneumoniae* DNA. Of the five children with CAP who also had high *C. pneumoniae* DNA copy numbers, it was observed that all children presented less than 10 days of symptoms, had antibiotic changed during treatment, and presented infiltrate on chest radiograph (Table 3).

Table 1 Clinical data of children with URTI and CAP

Symptoms		URTI (165)	CAP (150)	*p**
Dyspnea		62.8% (103)	84.0% (126)	< 0.0001
Fever (≥ 38.0 °C)		59.1% (97)	83.1% (123)	< 0.0001
Wheezing		50.6% (83)	72.6% (109)	0.0018
Duration of symptoms				
	Less than 10 days	–	77.4% (113)	
	10 to 20 days	–	11.6% (17)	
	More than 20 days	–	11.0% (16)	
Cough		–	52.0% (78)	
Tachypnea		–	50.0% (75)	
History of pneumonia		25.0% (41)	42.1% (62)	0.0026

The values are presented as percentage and number of subjects in parenthesis. *URTI*, upper respiratory tract infections; *CAP*, community-acquired pneumoniae

Table 2 Epidemiological data and *C. pneumoniae* DNA detection in children with upper respiratory tract infections, with community-acquired pneumonia and asymptomatic controls

Variables	URTI (165)	CAP (150)	Control (101)	*p* value[1]	*p* value[2]
Male gender	54.5% (90)	56.6% (85)	43.5% (44)	0.100	0.053
Age < 5 years old	79.4% (131)	89.3% (134)	80.2% (81)	1.000	0.065
Exclusive breastfeeding for 6 months	46.7% (77)	50.0% (75)	48.5% (49)	0.801	0.791
Breastfeeding for < 12 months	55.3% (83)	56.6% (85)	57.5% (48)	0.705	0.159
Cesarean delivery	34.1% (56)	41.6% (62)	44.5% (45)	0.092	0.696
Malnourished children	7.9% (13)	20.0% (30)	5.9% (6)	0.632	*0.001*
Preterm birth	10.4% (17)	12.7% (19)	4.0% (4)	0.098	*0.024*
Low birth weight (< 2.5 kg)	10.1% (16)	15.6% (22)	4.9% (5)	0.241	*0.021*
Incomplete immunization	22.7% (39)	25.3% (38)	11.8% (12)	*0.024*	*0.009*
Lack of prenatal treatment	7.9% (13)	14.7% (22)	3.0% (3)	0.118	*0.002*
Maternal education level < 9 years	77.6% (128)	54.6% (82)	3.9% (4)	*< 0.001*	*< 0.001*
Maternal occupation of housewife	50.3% (83)	62.4% (93)	50.5% (51)	0.540	0.090
Low family income	59.4% (98)	72.9% (108)	70.3% (71)	0.088	0.777
People per child's bedroom (> 1)	89.7% (148)	80.5% (120)	43.6% (44)	*< 0.001*	*< 0.001*
Cigarette smoker living in house	25.5% (42)	18.0% (27)	11.0% (11)	*0.004*	0.152

Categorical variables were compared by chi-square test or Fisher's exact test. *URTI*, upper respiratory tract infections; *CAP*, community-acquired pneumonia; *p value*[1] , compared between URTI versus control group; *p value*[2] , compared between CAP versus control group

Additionally, none of the children had leukocytosis and high CRP levels.

It should be noted that negative controls (non-template reaction) were used in all qPCR assays done and no amplification was observed. Additionally, other common respiratory pathogens and human DNA samples were tested in qPCR assays for specificity and showed no amplification by either primer pairs or probe. The LODs of the qPCR assays were 100 DNA copies/mL for *C. pneumoniae*.

Discussion

Some well-conducted studies have associated classic risk factors with ARI [24] and it is important to understand risk factors that contribute to the development of ARI whereas they may offer clues to prevention of the disease. In this study, it was observed that low birth weight, preterm birth, malnutrition, incomplete immunization, and high numbers of people per child's bedroom are risk factors associated with CAP but other classical risk factors were not related; this disagreement suggests that new studies with higher numbers of subjects are necessary to resolve the relevance of these risk factors in the development of CAP.

According to Liu et al. [25], *C. pneumoniae* infections have been studied widely in developed countries, but in developing countries, they are not well studied. Our analysis of respiratory samples of children from São Luís, Brazil, with URTI or CAP for the presence of *C. pneumoniae* DNA using a highly specific and sensitive qPCR assay revealed a high prevalence of *C. pneumoniae* infection. These results underline the need for considering *C. pneumonia* whenever diagnosing and treating respiratory infections in children.

Recent reports from other developing countries have shown a relatively low frequency of *C. pneumoniae* in respiratory samples of child or adult patients with acute

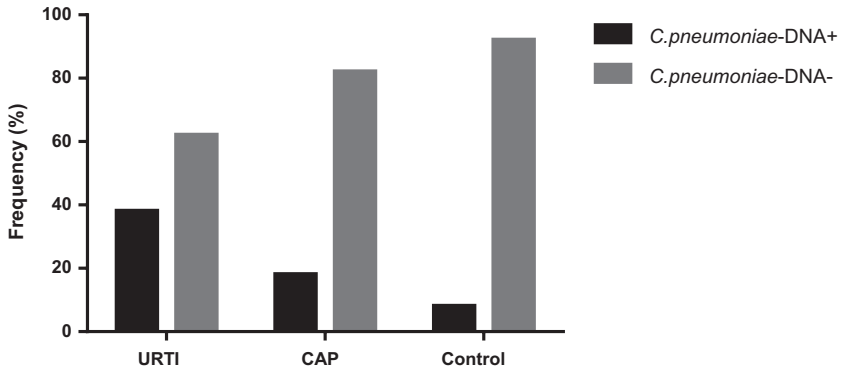

Fig. 1 Positivity for *C. pneumoniae* DNA in nasopharyngeal swab samples of children with URTI, CAP, and control groups. URTI, upper respiratory tract infections; CAP, community-acquired pneumonia. Values are presented as percentage of *C. pneumoniae* DNA positivity and compared by Fisher's exact test

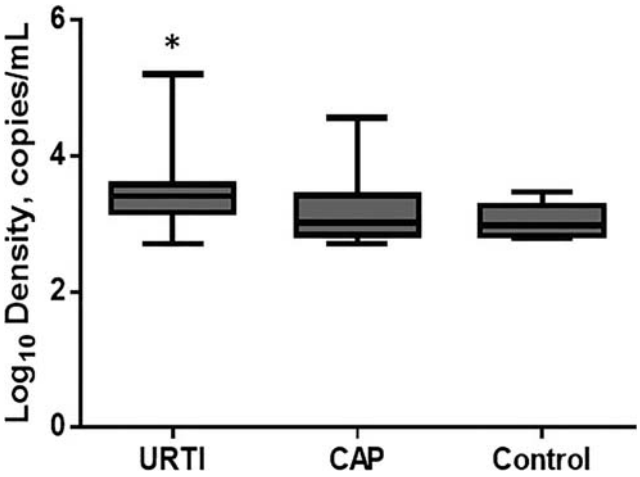

Fig. 2 *C. pneumoniae* DNA density by URTI, CAP, and control groups. Horizontal lines through boxes, group medians; Boxes extend to the 25th and 75th percentiles and whiskers to minimum and maximum values. URTI, upper respiratory tract infections; CAP, community-acquired pneumonia. Significances were calculated by Kruskal-Wallis test or unpaired *t* test followed by the Dunn's multiple comparisons test analysis. $*p < 0.05$ differs from control group

respiratory infection, including CAP, which varies between 0.08 and 7.9% [26–30]. However, a recent study involving 675 children with ARI from Peru identified, using conventional PCR, *C. pneumoniae* DNA in respiratory samples of 10.5% of the children [31]. Our findings showed a high percentage of *C. pneumoniae* than those found in other studies with respiratory infections. These discrepant results in the *C. pneumoniae* positivity are suggestive that they depend on the sample size, patient age, severity of the respiratory disease, seasonality, outbreaks, used method, gene target, and primers or probe design. In addition, more than one type of samples should be collected when possible from patients with ARI, such as sputum and tracheal aspirates. These samples are more representative of lower acute respiratory infections than nasopharyngeal swab but the tracheal aspirates are hard to be collected and sputum sampling is easier but limited, since a *C. pneumoniae* infection does not always generate sputum production [33].

In general, signs and symptoms of respiratory infections are of little value in the diagnosis of *C. pneumoniae* infections considering that they cause asymptomatic infections even in healthy individuals [10, 32]. In this context, the presence of nasopharyngeal *C. pneumoniae* DNA was also evaluated in asymptomatic children and we found eight cases of asymptomatic infection. Hyman et al. [9] proposed that healthy persons with asymptomatic upper airway infection and carriage with *C. pneumoniae* may represent a reservoir for this pathogen in the community.

To our knowledge, only a single study performed by Kuoppa et al. [33] used qPCR to quantify *C. pneumoniae* DNA in 105 different respiratory samples (nasopharyngeal, sputum, and throat specimens) from 35 patients, obtaining a positivity rate of 21.9% among samples, with a range of 600 to 50,000 *C. pneumoniae* DNA copies/μL in the nasopharynx samples. In our study, we found a range of 511 to 131,971 *C. pneumoniae* DNA copies/μL (2.7 to 5.2 $\log_{10}$ copies/mL) in the nasopharynx samples of children with UTRI or CAP. In addition, we evaluated whether *C. pneumoniae* DNA density was associated with symptomatic respiratory infections (URTI or CAP) and we found an association between high DNA density and URTI. Whether there is a threshold of DNA copy number that correlates with active (replicating) *C. pneumoniae* infection is unknown. In this context, we considered the highest *C. pneumoniae* DNA copy number observed in the control group representing a cutoff of active infections and we observed main characteristics of atypical CAP in the five children with CAP and with high *C. pneumoniae* DNA copy numbers. Despite a hallmark of chlamydial infections having the ability to establish persistent infection, in which the organism cannot be cultured, presumably due to an arrest of the developmental cycle, further studies comparing DNA density, with the gold standards of culture and serology on the same patient along with clinical

Table 3 Main characteristics of the five children with CAP and with high *C. pneumoniae* DNA copy numbers

Patient	Age/ gender	Fever	Duration of symptoms	Antibiotic changed during treatment	DNA copy numbers	Chest radiograph	Leukocytosis	High CRP levels	Outcome
1	36/F	No	Less than 10 days	Yes. Ceftriaxone to Azithromycin	4.97	Interstitial infiltrate	No	No	Recovered
2	108/M	No	Less than 10 days	Yes. Ceftriaxone to Azithromycin	4.31	Interstitial infiltrate	No	No	Recovered
3	60/M	No	Less than 10 days	Yes. Ceftriaxone to Azithromycin	4.30	Perihilar infiltrate	No	No	Recovered
4	5/M	No	Less than 10 days	Yes. Ceftriaxone to Azithromycin	4.56	Interstitial infiltrate	No	No	Died
5	14/M	Yes	Less than 10 days	Yes. Ceftriaxone to Azithromycin	4.42	Perihilar infiltrate	No	No	Recovered

Age was presented in months; Fever was considered when ≥ 38.0 °C; High CRP levels were considered when > 60 mg/L; Leukocytosis was considered when $> 15 \times 10^9$ cells per L. *CAP*, community-acquired pneumonia; *F*, female; *M*, male

symptoms, are needed to determine whether there is any clinical significance of higher copy number.

Conclusion

A high frequency of *C. pneumoniae* in nasopharyngeal samples was found in both children from São Luís, Brazil, with URTI and CAP. However, additional studies are necessary to determine what high density of *C. pneumoniae* DNA clinically means in children with URTI.

Author contribution Each author has contributed significantly to this work. EMS, AGA, LAC, ICPAS, MER, MHH, and LGLN contributed to the conception and the design of the work. MSA, MSC, HLSF, ESAS, KKPF, EMS, AGA, GMB, and LGLN contributed to data acquisition and in performing the experiments. SGM, MHH, RDCH, and LGLN analyzed the data. LAC, MER, RDCH, GMB, and LGLN wrote the manuscript. AGA, LAC, MER, RDCH, GMB, and LGLN revised it critically for important intellectual content and final approval of the version to be published.

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Compliance with ethical standards

This study was approved by the Research Ethics Committee of Ceuma University under the Certificate of Presentation for Ethical Consideration (CAAE #467.131) and was in compliance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from parents or caregivers prior to enrollment.

Conflict of interest The authors declare that they have no competing interests.

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