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ORIGINAL ARTICLE

Mycoplasma pneumoniae multilocus variable-number tandem-repeat analysis genotypes are associated with inflammatory biomarker levels in children with lower respiratory tract infections

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Abstract

The multilocus variable-number tandem-repeat analysis (MLVA) typing method is commonly used in *Mycoplasma pneumoniae* (*M. pneumoniae*) epidemiology. It remains unknown if clinical manifestations of lower respiratory tract infections (LRTI) in children differ between different MLVA genotypes. We aimed to determine if specific *M. pneumoniae* MLVA genotypes indicate the severity of LRTI in children. We performed a retrospective study of children younger than 18 years with signs of acute *M. pneumoniae* LRTI from January 1, 2009, to December 31, 2014. All patients who were PCR-positive for *M. pneumoniae* from pharyngeal swabs and had MLVA genotype successfully defined were included in the study. We compared the epidemiological and clinical data of children infected with different MLVA genotypes. In total, 429 patients (mean age 7.4 years, SD 3.4 years; 54% boys) met the study inclusion criteria. We compared the data of patients infected with the three most common MLVA types: MLVA-3,5,6,2 (86/429), MLVA-3,6,6,2 (71/429) and MLVA-4,5,7,2 (256/429). MLVA-3,5,6,2-infected patients over 5 years of age presented with a significantly higher median C-reactive protein level (34 vs 23 vs 19 mg/L, $p = .008$) and a higher median white blood cell count (9.4 vs 7.9 vs 8.5×10^9/L, $p = .040$) compared to MLVA-3,6,6,2- and MLVA-4,5,7,2-infected patients. No such difference was observed in the group of younger than 5 years. The results from our large cohort indicate that different MLVA genotypes may have different pathogenic potential and that children with MLVA-3,5,6,2 LRTI may present with higher inflammatory marker levels in comparison with other MLVA types.

Keywords *Mycoplasma pneumoniae* · Genotype · Respiratory tract infection · Children

Introduction

Mycoplasma pneumoniae (*M. pneumoniae*) is a common cause of community-acquired lower respiratory tract infections (LRTI) in children, being responsible for up to 40% of community-acquired pneumonia in children over 5 years of age [1–3]. *M. pneumoniae* LRTI are often mild and self-limiting; however, patients can develop severe disease [4, 5]. Factors determining the severity of *M. pneumoniae* LRTI are only partly understood [1].

M. pneumoniae strains can be classified into different genetic groups by using several typing methods, most frequently P1 typing, multilocus variable-number tandem-repeat analysis (MLVA) and multi-locus sequence typing (MLST) [6, 7]. P1 typing based on P1 gene sequencing was the most commonly used genotyping method, until newer methods, such as MLVA, were developed [6–11]. In comparison with P1 typing, which separates isolates into two major subtypes, P1 type 1 and P1 type 2, MLVA offers a more discriminative categorisation of isolates based on the variable copy numbers of tandem repeat sequence at specific loci [6, 11].

We recently described an association between P1 genotype and severity of LRTI in children [12]. However, it remains largely unknown if the clinical severity of acute

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M. pneumoniae LRTI in children differs between different MLVA genotypes as well. A large number of *M. pneumoniae* LRTI in children during two recent *M. pneumoniae* epidemics in Slovenia [13] provided us with a unique opportunity to study the association between *M. pneumoniae* MLVA genotype and clinical characteristics of acute *M. pneumoniae* LRTI in children.

Materials and methods

Study subjects

Children younger than 18 years, referred to the University Children's Hospital Ljubljana and Department of Infectious Diseases Ljubljana with clinical signs of acute mycoplasmal LRTI from January 1, 2009, to December 31, 2014, were tested for *M. pneumoniae*. The diagnosis of LRTI was made based on clinical and/or radiographic appearance.

All patients who were PCR-positive for *M. pneumoniae* from pharyngeal swabs and had MLVA genotype successfully defined were identified from a laboratory database and included in the study. We excluded cases when the underlying condition could affect the severity of the disease (i.e. immunocompromised children and children with chronic pulmonary disease) and where additional testing provided evidence that other infectious agents were the more likely cause of the disease. The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (No 0120–8/2018/4).

MLVA typing was performed from archived anonymised swabs or DNA extracts from swabs [13], and these data from the laboratory database were included in our study. The protocol of this study was approved by the National Medical Ethics Committee (No 0120–244/2021/3).

Study design

The main objective of our observational retrospective study was to assess if *M. pneumoniae* MLVA genotype is associated with the severity of LRTI in children.

Data on age, gender, MLVA type, P1 type, macrolide susceptibility, interval between disease onset and antibiotic therapy initiation, laboratory markers of inflammation, presence of extrapulmonary manifestations, hospital admission, duration of hospital stay and data related to treatment and complications were collected in all patients.

We compared the epidemiological and clinical data of patients with the most frequent *M. pneumoniae* MLVA genotypes: MLVA-3,5,6,2, MLVA-3,6,6,2 and MLVA-4,5,7,2. The clinical impact was assessed by comparing several markers of disease severity: inflammatory markers, hospital admission, duration of hospital stay, oxygen treatment and admission to the critical care unit. The patients were further divided into two age groups (< 5 years and 5–18 years), and their epidemiologic and clinical characteristics were compared.

Methods

Pharyngeal swabs were subjected to DNA isolation using MagNA Pure Compact (Roche, Germany) and later tested by *M. pneumoniae* real-time PCR (Argene, France). The remainder of each PCR-positive sample was cultivated as described previously [13]. MLVA typing was performed using a standardised MLVA protocol by amplification of four variable-number tandem repeat (VNTR) loci (Mpn13, Mpn14, Mpn15 and Mpn16) [11]. P1 typing was performed using pyrosequencing that targets the *M. pneumoniae* MPN141, MPN528a genes, and macrolide resistance was recognised by pyrosequencing two parts of domain V in the 23S rRNA gene [13].

Multiplex PCR was performed on the nasopharyngeal aspirate specimens to assess viral co-detection, including respiratory syncytial virus, influenza virus, parainfluenza virus, adenovirus, human bocavirus, metapneumovirus, rhinovirus, enterovirus and coronavirus at the time of LRTI diagnosis.

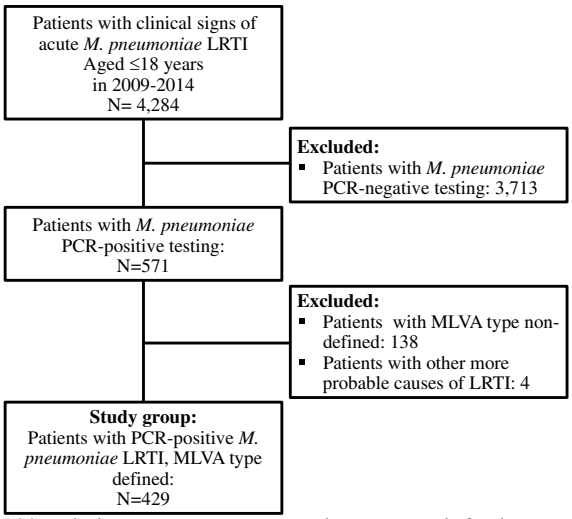
Analysis

Continuous variables were presented as mean (SD) or median (IQR) where appropriate. Categorical variables were described with counts and percentages.

Continuous variables in multiple independent groups were compared using one-way ANOVA or the Kruskal–Wallis test, where appropriate, whereas continuous variables in two independent groups were compared using independent samples *T*-test or Mann–Whitney *U*-test, where appropriate. Categorical variables were compared by using the Pearson chi-square test. The differences were considered statistically significant when the *p* value was less than 0.05.

Results

During the study period, *M. pneumoniae* was detected in a pharyngeal swab by PCR in 571 of 4284 children with LRTI. After applying the inclusion and exclusion study criteria, we evaluated data from 429 patients (mean age 7.4 years, SD 3.4 years; 54.1% boys) with acute *M. pneumoniae* LRTI and MLVA genotype defined (Fig. 1). Twenty-six percent of patients (113/429) were younger than 5 years at presentation. MLVA typing revealed eight distinct MLVA types: MLVA-3,5,6,2 (20.0%, 86/429), MLVA-3,5,6,3 (0.5%, 2/429), MLVA-3,6,6,2 (16.6%, 71/429), MLVA-4,4,7,2 (0.2%,



Abbreviations: LRTI, Lower respiratory tract infection; MLVA, Multilocus variable-number tandem-repeat analysis.

Fig. 1 Study flowchart. LRTI, lower respiratory tract infection; MLVA, multilocus variable-number tandem-repeat analysis

1/429), MLVA-4,5,6,2 (0.2%, 1/429), MLVA-4,5,7,2 (59.7%, 256/429), MLVA-4,5,7,3 (2.3%, 10/429) and MLVA-4,6,7,2 (0.5%, 2/429). The three predominant MLVA types were MLVA-4,5,7,2, MLVA-3,5,6,2 and MLVA-3,6,6,2, which in total accounted for 96.3% of total isolates. Almost exclusively, specific MLVA types belonged to either P1 type 1 or type 2, with only a few exceptions. In the MLVA-3,5,6,2 and MLVA-3,6,6,2 type, 84/86 and 71/71 isolates were of P1 type 2, respectively, while in MLVA-4,5,7,2 type, 256/256 isolates were of P1 type 1. The majority of isolates (425/429) were macrolide susceptible, and 4 (0.9%) were found to contain an A2063G mutation in the *M. pneumoniae* 23S rRNA domain V that infers macrolide resistance. Three of the resistant isolates belonged to MLVA-3,5,6,2 and one to MLVA-4,5,7,2, with no difference between MLVA types ($p = .358$).

We detected a viral co-infection in 26.8% (90/336) of the cases. Co-detected respiratory viruses included respiratory syncytial virus, influenza virus, parainfluenza virus, adenovirus, human bocavirus, metapneumovirus, rhinovirus, enterovirus and coronavirus. Rhinovirus (50.0%, 45/90), human bocavirus (16.7%, 15/90) and parainfluenza virus (7.8%, 7/90) were the predominant co-detected viruses. There was no statistically significant association between a specific viral co-detection and MLVA genotype.

The three main MLVA types cocirculated in the study period (Fig. 2). The characteristics of patients with *M. pneumoniae* LRTI infected with either MLVA-3,5,6,2, MLVA-3,6,6,2 or MLVA-4,5,7,2 strains are summarised in Table 1. Data of 86 patients infected with MLVA-3,5,6,2 were compared with 71 patients infected with MLVA-3,6,6,2 and 256 patients infected with MLVA-4,5,7,2. Despite no difference in X-ray observations, incidence of extrapulmonary manifestations and rate of viral co-detection, patients infected with MLVA-3,5,6,2 had a higher median baseline CRP level and a higher median WBC compared to MLVA-3,6,6,2 and MLVA-4,5,7,2 (Table 1). Further analysis showed the MLVA-3,5,6,2-infected patients had a higher median CRP level compared to MLVA-4,5,7,2 (28 vs 18 mg/L, $p = .024$) and a higher median WBC compared to MLVA-3,6,6,2 (10.2 vs 8.1×10^9/L, $p = .007$) and MLVA-4,5,7,2 (10.2 vs 8.8×10^9/L, $p = .038$).

Out of 429 patients, 31.9% (137/429) required hospital treatment. The characteristics of hospitalised patients infected with the three most frequent MLVA types are summarised in Table 2. There was no difference in the rate of

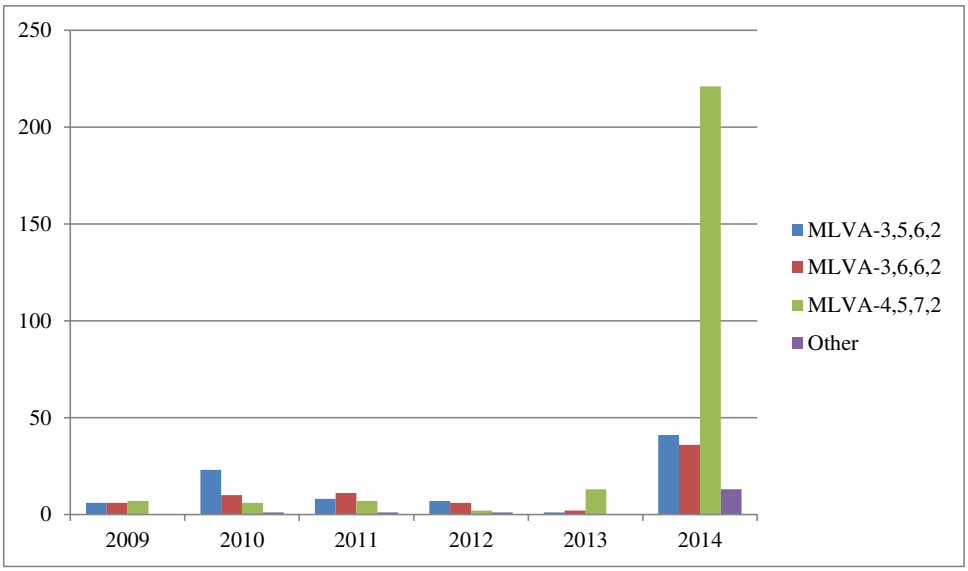


Fig. 2 Distribution of MLVA genotypes from 2009 to 2014 in patients with *M. pneumoniae* lower respiratory tract infection. Bars indicate the number of patients

Table 1 Characteristics of patients infected with either *M. pneumoniae* MLVA-3,5,6,2, MLVA-3,6,6,2 or MLVA-4,5,7,2 strains. Data are presented as median (IQR) or as percentage (proportion of subjects). Significant differences ($p < .05$) are highlighted in bold

	MLVA-3,5,6,2	MLVA-3,6,6,2	MLVA-4,5,7,2	*p* value
Subjects, *N*	86	71	256	
Boys/girls (%)	53.5%/46.5%	67.6%/32.4%	50.0%/50.0%	**.036**
Age (years)	7.5 (IQR 4.2–10.2)	7.9 (IQR 5.8–9.8)	6.9 (IQR 4.6–9.8)	.600
< 5 years	29.1% (25/86)	22.5% (16/71)	27.0% (69/256)	.642
5–18 years	70.9% (61/86)	77.5% (55/71)	73.0% (187/256)	
Interval between disease onset and antibiotic therapy initiation (days)	7 (IQR 5–10)	6 (IQR 4–9)	7 (IQR 5–10)	.070
CRP (mg/L)	28 (IQR 9–48)	23 (IQR 8–47)	18 (IQR 8–37)	.050
WBC ($\times 10^9$/L)	10.2 (IQR 7.7–12.8)	8.1 (IQR 6.3–11.6)	8.8 (6.9–11.9)	**.020**
X-ray effusion	23.2% (16/69)	28.1% (16/57)	26.3% (55/209)	.810
Extrapulmonary manifestations	11.9% (10/84)	14.9% (10/67)	12.1% (31/256)	.810
Viral co-detection	26.2% (11/42)	14.3% (7/49)	27.2% (41/151)	.182

Continuous variables were compared using the Kruskal–Wallis test, whereas categorical variables were compared by using the Pearson chi-square test

Abbreviations: *CRP*, C-reactive protein; *IQR*, interquartile range; *WBC*, white blood cell count

Table 2 Characteristics of hospitalised patients infected with either *M. pneumoniae* MLVA-3,5,6,2, MLVA-3,6,6,2 or MLVA-4,5,7,2 strains. Data are presented as median (IQR) or as percentage (proportion of subjects)

	MLVA-3,5,6,2	MLVA-3,6,6,2	MLVA-4,5,7,2	*p* value
Subjects, *N*	29	30	78	
Age (years)	6.2 (IQR 3.8–9.1)	5.9 (IQR 3.0–8.2)	6.2 (IQR 3.3–8.3)	.846
< 5 years	44.8% (13/29)	40.0% (12/30)	42.3% (33/78)	.932
5–18 years	55.2% (16/29)	60.0% (18/30)	57.7% (45/78)	
Admission rate	33.7% (29/86)	42.3% (30/71)	30.5% (78/256)	.173
Hospital stay (days)	3 (IQR 1–4)	2 (IQR 1–4)	3 (IQR 2–5)	.204
Oxygen therapy	37.9% (11/29)	46.7% (14/30)	46.2% (36/78)	.765
Viral co-detection	21.1% (4/19)	10.0% (2/20)	30.2% (16/53)	.186

Continuous variables were compared using the Kruskal–Wallis test, whereas categorical variables were compared by using the Pearson chi-square test

Abbreviations: *IQR*, interquartile range

hospital admission, requirement for oxygen treatment or the average duration of hospital stay between hospitalised patients. No intensive care treatment was required in either group.

To better assess the possible differences observed between MLVA genotypes related to age, the study group was divided into 110 patients younger than 5 years and 303 patients older than 5 years (Table 3). Patients infected with MLVA-3,5,6,2 strains had a higher median baseline CRP level and a higher median WBC compared to MLVA-3,6,6,2 and MLVA-4,5,7,2 in the group of 5- to 18-year-olds with no difference in the rate of viral co-detection between MLVA genotypes. No such difference was observed in the group of younger than 5 years. To point out any differences related to age, additional characteristics of patients younger than 5 years and older than 5 years were compared (Table 4). Younger patients had a significantly higher rate of viral co-detection (44.1% vs 15.9%, $p < .001$). Further on, when assessing patients with no viral co-detection, younger patients had a higher median WBC and a higher rate of hospital admission with no difference in the requirement for oxygen treatment.

Discussion

Typing of *M. pneumoniae* strains is mainly used to define *M. pneumoniae* epidemics and is not routinely used in clinical practice to predict the presentation and severity of *M. pneumoniae* LRTI [14]. This study investigated the association between *M. pneumoniae* MLVA genotype and clinical characteristics of acute *M. pneumoniae* LRTI in children.

We collected a large cohort group of 429 patients with *M. pneumoniae* LRTI and MLVA genotype defined, due to two *M. pneumoniae* epidemics in Slovenia, one from summer 2009 to winter 2010/2011, and another from winter 2013/2014 to winter 2014/2015 [13]. The three predominant MLVA types, MLVA-3,5,6,2, MLVA-3,6,6,2 and MLVA-4,5,7,2, accounted for 96.3% (413/429) of total isolates.

Table 3 Comparison of markers of disease severity of patients infected with either *M. pneumoniae* MLVA-3,5,6,2, MLVA-3,6,6,2 or MLVA-4,5,7,2 strains divided into two age groups. Data are presented as median (IQR) or as percentage (proportion of subjects). Significant differences ($p < .05$) are highlighted in bold

	MLVA-3,5,6,2	MLVA-3,6,6,2	MLVA-4,5,7,2	*p* value
< 5 years				
Subjects, *N*	25	16	69	
CRP (mg/L)	21 (IQR 5–30)	22 (IQR 7–58)	18 (IQR 5–38)	.816
WBC ($\times 10^9$/L)	11.8 (IQR 9.6–14.8)	10.5 (IQR 7.9–14.9)	10.5 (IQR 7.8–15.0)	.623
X-ray effusion	22.2% (4/18)	30.0% (3/10)	34.5% (19/55)	.617
Admission rate	52.0% (13/25)	75.0% (12/16)	47.8% (33/69)	.146
Hospital stay (days)	2 (IQR 2–4)	2 (IQR 2–4)	4 (IQR 2–5)	.122
Oxygen therapy	46.2% (6/13)	50.0% (6/12)	57.6% (19/33)	.782
Viral co-detection	47.1% (8/17)	21.4% (3/14)	49.1% (26/53)	.173
5–18 years				
Subjects, *N*	61	55	187	
CRP (mg/L)	34 (IQR 16–49)	23 (IQR 9–42)	19 (IQR 8–38)	**.008**
WBC ($\times 10^9$/L)	9.4 (IQR 7.4–12.7)	7.9 (IQR 6.0–10.5)	8.5 (IQR 6.4–11.1)	**.040**
X-ray effusion	23.5% (12/51)	27.7% (13/47)	23.4% (36/154)	.829
Admission rate	26.2% (16/61)	32.7% (18/55)	24.1% (45/187)	.436
Hospital stay (days)	3 (IQR 1–5)	2 (IQR 1–4)	3 (IQR 2–5)	.735
Oxygen therapy	31.3% (5/16)	44.4% (8/18)	37.8% (17/45)	.799
Viral co-detection	12.0% (3/25)	11.4% (4/35)	15.3% (15/98)	.813

Continuous variables were compared using the Kruskal–Wallis test, whereas categorical variables were compared by using the Pearson chi-square test

Abbreviations: *CRP*, C-reactive protein; *IQR*, interquartile range; *WBC*, white blood cell count

Table 4 Characteristics of patients with *M. pneumoniae* lower respiratory tract infection with no viral co-detection divided into two age groups. Data are presented as median (IQR) or as percentage (proportion of subjects). Significant differences ($p < .05$) are highlighted in bold

	< 5 years	5–18 years	***p*** value
Subjects, *N*	72	174	
Boys/girls (%)	62.5%/37.5%	47.1%/52.9%	**.028**
Interval between disease onset and antibiotic therapy initiation (days)	7 (IQR 5–8)	7 (IQR 5–10)	.282
CRP (mg/L)	23 (IQR 6–37)	21 (IQR 9–41)	. 572
WBC ($\times 10^9$/L)	10.6 (IQR 7.8–13.7)	8.7 (IQR 6.5–11.3)	**.001**
Extrapulmonary manifestations	11.4% (8/70)	10.0% (17/170)	.742
X-ray effusion	25.0% (14/56)	26.5% (39/147)	.842
Admission rate	61.1% (44/72)	31.2% (54/173)	**< .001**
Hospital stay (days)	3 (IQR 1–4)	3 (IQR 1–4)	.899
Oxygen therapy	53.5% (23/43)	38.9% (21/54)	.151

Continuous variables were compared using the Mann–Whitney *U*-test, whereas categorical variables were compared by using the Pearson chi-square test

Abbreviations: *CRP*, C-reactive protein; *IQR*, interquartile range; *WBC*, white blood cell count

MLVA types cocirculated in the study period. During the first epidemic, majority of isolates belonged to MLVA-3,5,6,2 and MLVA-3,6,6,2 whereas in the second epidemic, MLVA-4,5,7,2 prevailed [13].

We compared the epidemiological and clinical data of 86 MLVA-3,5,6,2-, 71 MLVA-3,6,6,2- and 256 MLVA-4,5,7,2-infected patients with signs of acute LRTI. Patients infected with MLVA-3,5,6,2 strains older than 5 years presented with a higher median baseline CRP level and a higher median WBC, compared to the other two MLVA types. Plasma levels of inflammatory markers, especially CRP, can rapidly and dramatically increase in response to infection, inflammation and tissue injury [15]. Moreover, it was shown that the magnitude of inflammatory marker increase usually correlates with disease severity, including LRTI [16–18]. The results from our large cohort study therefore suggest that different MLVA types may have different pathogenic potential and that LRTI caused by MLVA-3,5,6,2 strains may have a more severe disease course than those with other MLVA strains in children, which is indicated by

higher inflammatory marker levels. However, we observed no difference in other markers of disease severity, such as the requirement for oxygen therapy, duration of hospital stay or need of intensive care treatment.

Interestingly, when dividing our patients into two age groups, we found no similar impact of MLVA genotype on inflammatory levels in the age group of < 5-year-olds. Previous studies have shown that a high prevalence of co-detected respiratory viruses in younger children suggests that viruses could play a role in making pneumonia clinically apparent in this age group [19]. Moreover, clinical manifestations of *M. pneumoniae* LRTI in young children are supposed to be milder than those in older children [1, 19]. Indeed, younger children had a significantly higher rate of viral co-detection in our study. On the contrary, however, when assessing the differences between the two age groups with no viral co-detection, we observed that younger patients had a higher median WBC and a higher rate of hospital admission, suggesting a severe disease course also in this age group. Apparently, the factors determining *M. pneumoniae* LRTI severity in < 5-year-olds are only partly understood. This could explain why we found a relationship between MLVA genotype and severity of LRTI in older children, but not in younger ones where probably other factors prevail over MLVA genotype in influencing LRTI severity, such as for example a reduced innate immune response in comparison with older children [20, 21].

Few clinical studies to date have investigated the influence of *M. pneumonia* MLVA genotype on the severity of *M. pneumoniae* LRTI. Similar to our study, a recent research showed that infections with MLVA-3,5,6,2 have a higher risk of progressing to severe pneumonia [22]. Although the substantial difference in the rate of macrolide-resistant *M. pneumoniae* (MRMP) between the two studies, we still found a similar correlation between MLVA typing and clinical severity. In comparison with their study, in which they included only inpatients, we included outpatients as well as hospitalised patients in our study in order to better assess the difference in disease severity between different MLVA genotypes. In another study, patients infected with MLVA-4,5,7,2 had a higher pneumonia severity index [23]. However, no children were enrolled in their study, and their results could be partly influenced by the high rate of MRMP, which was present in less than 1% of specimens in our study. In addition, we found no association between MLVA genotype and MRMP that was suggested in previous papers [24–26].

One of the limitations of our study is definitely its retrospective design. A prospective design would allow for assessing other measures of inflammation and disease severity, including host factors, to better understand the pathogenic role of the *M. pneumoniae* genotype. Regardless, our sample size of 429 patients is one of the largest study samples addressing this question either in children or adults. All our patients were recruited from university hospitals, which may have led to a disproportionate number of cases with more severe *M. pneumoniae* LRTI. In addition, one could think that previous infections would offer protection against previous circulating types and influence the severity of the resulting illness as observed in previous studies [27]. There was no information about prior infection with *M. pneumoniae* in any of the patients included in our study. However, it is interesting that MLVA-3,5,6,2 still resulted presumably more pathogenic even though we could speculate that, according to our data, the population might have been more naive to MLVA-4,5,7,2.

M. pneumoniae LRTI represent a significant healthcare burden, as *M. pneumoniae* is the most commonly detected bacteria in school-aged children hospitalised with CAP [2]. To date, typing *M. pneumoniae* strains has been mainly used to define *M. pneumoniae* epidemics and is not routinely performed when assessing an individual patient with *M. pneumoniae* LRTI. Our results suggest that MLVA typing could be utilised not only to quickly predict the severity of the disease course and individual selection of treatment plans, but also to prepare treatment regimens in view of future *M. pneumoniae* epidemics with possible more pathogenic *M. pneumoniae* types. Prospective collection of respiratory samples for *M. pneumoniae* culture and typing could further elucidate the impact of *M. pneumoniae* genotype on disease presentation and severity of LRTI.

Conclusions

The results from our large cohort suggest that different MLVA subtypes may have different pathogenic potential and that MLVA-3,5,6,2 LRTI may present with higher inflammatory marker levels in comparison with other frequent MLVA types in children. This was only observed in children older than 5 years. MLVA typing is not routinely performed but could be used to predict the presentation and severity of the disease course which could lay the groundwork for not only individualised treatment plans but also as a preparation for future epidemics with possibly more pathogenic *M. pneumoniae* strains.

Previous studies on the clinical presentation of *M. pneumonia* LRTI have generally not considered the role of MLVA genotype. Therefore, it is essential that future studies of other patient populations or other epidemics examine the relationship of *M. pneumoniae* genotypes to the severity of LRTI.

Author contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Jasna Rodman Berlot, Tatjana Mrvič and Darja Keše.

The first draft of the manuscript was written by Jasna Rodman Berlot, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during the current study are not publicly available but are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval The study was approved by the National Medical Ethics Committee (20 February 2018, No 0120–8/2018/4).

Consent to participate This is a retrospective observational study. No consent was obtained from the participants or their parents as the information is anonymised, and the submission does not include images or other data that may identify the person. The study design was approved by the National Medical Ethics Committee (20 February 2018, No 0120–8/2018/4).

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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