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The increased incidence of *Mycoplasma pneumoniae* in France in 2011 was polyclonal, mainly involving *M. pneumoniae* type 1 strains.

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Abstract

An increased incidence of *Mycoplasma pneumoniae* infections was reported in 2011 in two cities in France, Bordeaux and Caen. Two complementary molecular typing methods, PCR-RFLP on adhesin P1 and multilocus variable number tandem repeat analysis (MLVA), were used to determine whether this phenomenon was clonal. In 2011, the percentage of *M. pneumoniae*-positive patients doubled in both cities compared with 2010. Macrolide resistance remained stable at 8.3% of patients. Eighteen MLVA types were identified among 94 *M. pneumoniae*-positive specimens, demonstrating that the phenomenon was multiclonal. Types P, J, U, X and E were the most frequent and 81.6% of the strains were adhesin P1 type 1.

Mycoplasma pneumoniae causes community-acquired respiratory tract infections, particularly in school-aged children and young adults. Epidemics occur periodically, at 3- to 7-year intervals [1], and can last several months. An epidemic of *M. pneumoniae* infection started in 2010 in Denmark [2]. Since this date, a substantial increase in the incidence of *M. pneumoniae* infections has been reported in several European countries [3–5], Israel [6] and Japan [7], making its spread a worldwide phenomenon.

One major question is whether these surges in *M. pneumoniae* infections can be attributed to the spread of unique or distinct clones. Unfortunately, most epidemiological reports have not included genotyping data. Clonal spread has been documented recently in which the clonal spread of a single *M. pneumoniae* MLVA type J strain in children attending the same primary school and in their household contacts was reported [8]. On the other hand, the increased incidence of infections in Jerusalem in 2010 was found to be polyclonal [9],

FIG. 1. Percentage and number of *M. pneumoniae*-positive patients detected in Bordeaux and Caen and the distribution of MLVA types detected in *M. pneumoniae*-positive respiratory tract specimens. (a) Percentage of *M. pneumoniae*-positive patients in Bordeaux between 2000 and 2011 (among a total of 29 805 specimens collected from 24 511 patients) and in Caen between 2010 and 2011 (among a total of 7609 specimens collected from 6431 patients). For each point, 95% CI and the total number of patients tested are presented in parenthesis. Comparison of percentage of *M. pneumoniae*-positive patients was performed using the χ^2 test. (b) Distribution of *M. pneumoniae*-positive patients by month in Bordeaux and Caen between January 2010 and January 2012. (c) Age distribution of *M. pneumoniae*-positive patients in Bordeaux and Caen in 2011. (d) Minimum spanning tree for *M. pneumoniae* MLVA types detected in 2011 from *M. pneumoniae*-positive respiratory tract specimens according to the city. Each circle represents a particular MLVA type. The number in parentheses indicates the number of patients infected with this *M. pneumoniae* MLVA type. The distance between genotypes is expressed as the number of allelic changes. The colour of the circles indicates the city, dark grey for Bordeaux and white for Caen. The numbering system of MLVA types A to X was described by Degrange *et al.* [14]; MLVA type 29, 2-4-6-7-2; MLVA type 35, 2-3-6-7-2; MLVA type 36, 6-4-6-7-2; MLVA type 38, 4-4-5-6-2; MLVA type 41, 2-3-5-7-2. (e) Minimum spanning tree for *M. pneumoniae* MLVA types detected from *M. pneumoniae*-positive respiratory tract specimens in 2011 according to the P1 adhesin type.. The colour of the circles indicates the P1 adhesin type: dark grey for type 1, white for type 2, light grey for cases in which the type was not determined (see d for the minimum spanning tree legend). For a and b, the criteria for *M. pneumoniae* infections were a positive result on *M. pneumoniae*-specific PCR, a positive culture, complement fixation test titres $\geq$ 1:64 and/or the presence of *M. pneumoniae*-specific IgM.

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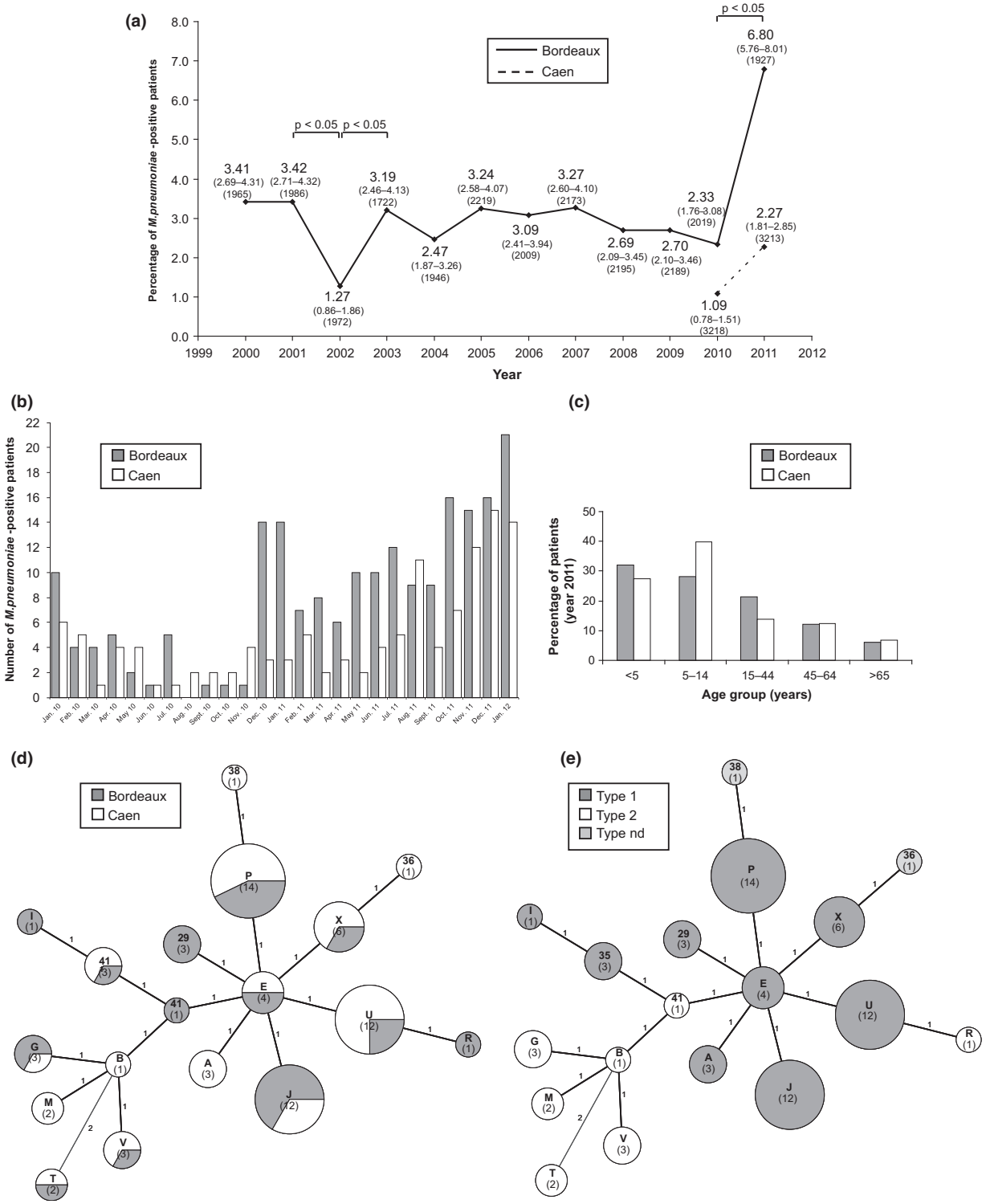
as were cases in England and Wales that occurred during the winters of 2010–2011 and 2011–2012, but fewer than 20 samples were analysed for each period in this study [3].

In this study, we report an increased incidence of *M. pneumoniae* infections in 2011 in two cities in France: Bordeaux, in the southwest of the country, and Caen, in the northwest. We aimed to determine whether this phenomenon was clonal or multiclonal using two complementary *M. pneumoniae* molecular typing methods, PCR-RFLP of the adhesin P1 gene and multilocus variable number tandem



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repeat analysis (MLVA). The incidence of macrolide resistance was also determined.

Results from all specimens submitted for *M. pneumoniae* diagnosis at the Department of Bacteriology of the University Hospital of Bordeaux between 1 January 2000 and 31 January 2012 and at the Department of Virology of the University Hospital of Caen during 2010 and 2011 were collected retrospectively. Both departments received samples from adult and paediatric wards. In Bordeaux, the diagnosis of *M. pneumoniae* infection was based on real-time PCR amplification [10], culture in modified Hayflick medium [11] and serology, including complement fixation test and detection of *M. pneumoniae* IgM using *M. pneumoniae* IgM-ELISA (Medac Hamburg, Germany). In Caen, diagnosis was made using a commercial ELISA to detect specific IgG and IgM (Immuno-Well, BMD, Croissy Beaubourg, France) and commercial multiplex PCR tests (RespiFinder® SMART 22, PathoFinder).

A total of 29 805 specimens from 24 511 patients were submitted for *M. pneumoniae* diagnosis at the Bordeaux hospital between 2000 and 2012, and 785 patients were diagnosed with a *M. pneumoniae* infection (3.20%; 95% CI, 2.99–3.43) according to a positive PCR, a positive culture, a complement fixation test titre $\geq$ 1/64 and/or the presence of *M. pneumoniae*-specific IgM. The percentage of *M. pneumoniae*-positive patients ranged between 1.27 and 3.42% before 2010 (Fig. 1a); it was 2.33% in 2010 (2478 specimens tested from 2019 patients, 2119 by serology and 359 by both PCR and culture) and it significantly increased in 2011 (p 1.3 10^{-11}, χ^2 test) to reach a rate of 6.80% (2282 specimens tested from 1927 patients, 1848 by serology and 434 by both PCR and culture). A significant increased incidence of *M. pneumoniae* infections was also observed in Caen (Fig. 1a), with the percentage of infected patients doubling from 1.1% in 2010 (3797 specimens tested from 3218 patients, 485 by serology and 3312 by PCR) to 2.3% in 2011 (3812 specimens tested from 3213 patients, 399 by serology and 3413 by PCR) (p 0.0002, χ^2 test). The distribution of the number of cases of *M. pneumoniae* infections by month revealed that the epidemic in Bordeaux started in December 2010 (Fig. 1b). In Caen, the epidemic started later, in autumn 2011, despite a peak in August 2011 (Fig. 1b).

In 2011, in Bordeaux, the median age of *M. pneumoniae*-positive patients was 8.5 years (interquartile range (IQR) 4.1–36.8 years), ranging from 1 week old to 93 years old. In Caen, the median age was 8.3 years (IQR 4.6–24.2 years), and patients ranged from 2 months to 82 years old. The majority of patients, 59.6% in Bordeaux and 67.1% in Caen, were aged 14 years or under, while 21.4% of patients in Bordeaux and 13.7% of patients in Caen were 15–44 years old (Fig. 1c). In both cities, the age profiles of *M. pneumoniae*-positive patients were similar to those in 2010 prior to the surge of *M. pneumoniae* infections. This age distribution was also similar to the one reported in Scotland between 2008 and 2011, where a majority of patients (53%) were aged 14 and under [12].

All respiratory tract specimens from patients detected positive for *M. pneumoniae* by PCR in 2011 (44 specimens from 39 patients in Bordeaux and 50 specimens from 50 patients in Caen) were typed using a PCR-RFLP method targeting the P1 adhesin gene [13] and using the *M. pneumoniae* MLVA typing method [9, 14]. Macrolide resistance-associated mutations in the 23S rRNA gene were searched using a real-time PCR and melting curve analysis method [15].

Macrolide resistance-associated mutations were found in 8.8% and in 7.9% of patients out of 34 and 38 patients for whom amplification was obtained in Bordeaux and Caen, respectively (Table 1). Thus the overall resistance rate was 8.3% of patients (95% CI, 3.9–17.0). This rate of macrolide resistance remained stable in Bordeaux compared with 2005–2007 [15]. Four A2058G substitutions, one A2059G substitution and one A2062G substitution (*E. coli* numbering) were identified. The A2062G mutation was previously reported in *in vitro* selection of resistant mutants [16] but to our knowledge, this is the first instance in which this mutation has been identified in a *M. pneumoniae*-positive clinical specimen. In Bordeaux and Caen, 13 and 14 distinct MLVA types were identified, respectively (Table 1, Fig. 1d). The MLVA types in Bordeaux were similar to those in Caen with only a few exceptions (Fig. 1d). In both cities, MLVA types P, U, J, E and X predominated, MLVA types P, U and J being the three most frequent. In Bordeaux, the proportion of type 1 strains was 81.2% (26/32) in 2011 (Table 1). Type 1 strains also predominated in Caen, at a proportion of 82.1% (32/39) (Table 1). Thus, the overall proportion of type 1 strains in both cities was 81.6% (95% CI, 71.1–89.0) (Fig. 1e). Interestingly, a shift toward type 1 strains has been noted in Bordeaux since 2009–2010. We have previously reported that both PCR-RFLP types were present in the same proportion in Bordeaux from 2000 to 2006 [17]. It was the same in 2007 and 2008, with 38.1% (8/21) and 50% (5/10) of type 1 strain, respectively, then type 1 strains became more prevalent in 2009 and 2010, with 66.6% of type 1 strain (4/6 and 2/3, respectively), to finally reach the rate of 81.2% of *M. pneumoniae*-positive specimens in 2011. The alternation of the two types over time has been described previously [1].

Our data demonstrate that the surge of *M. pneumoniae* infections in 2011 in France was a multiclonal phenomenon, as was the case in Israel, England and Wales [3, 9, 18], but that the great majority of cases involved *M. pneumoniae* type 1 strains. The predominance of type 1 strains is consistent with results from China, where 93% of strains from clinical isolates collected in 2008 and 2009 were reported to be *M. pneumo-*

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TABLE 1. Characteristics of 94 *M. pneumoniae*-positive respiratory tract specimens collected at the Bordeaux and Caen University Hospitals, France, in 2011

City[a]	Specimen designation	Source[b]	Patient's age (years unless specified)	Date of collection	MLVA type[c]	PCR-RFLP type	Macrolide resistance genotype[d]
C	C12-51	Nose	7	Feb.-11	U	1	wt
C	C12-53	Nose	4 months	Mar.-11	Nd[e]	nd	no amp
C	C12-54	NPA	6 months	Apr.-11	E	1	no amp
C	C12-55	Throat	12	May-11	U	1	wt
C	C12-1	Nose	2	May-11	P	1	wt
C	C12-2	Trach. asp.	21	Jun.-11	P	1	wt
C	C12-3	Nose	6	Jun.-11	P	1	wt
C	C12-4	Nose	10	Jul.-11	X	1	wt
C	C12-5	Nose	30	Jul.-11	nd	nd	wt
C	C12-6	NPA	2	Jul.-11	P	1	wt
C	C12-7	Nose	67	Aug.-11	U	1	wt
C	C12-8	Nose	43	Aug.-11	A	1	no amp
C	C12-9	Nose	78	Aug.-11	35	1	wt
C	C12-10	Nose	1	Aug.-11	35	1	wt
C	C12-11	Nose	59	Aug.-11	nd	nd	no amp
C	C12-12	NPA	9 months	Aug.-11	X	1	wt
C	C12-13	Nose	2 months	Aug.-11	nd	nd	no amp
C	C12-14	Nose	14	Aug.-11	P	1	wt
C	C12-15	Nose	2	Aug.-11	nd	nd	no amp
C	C12-16	Throat	18	Sep.-11	A	1	wt
C	C12-17	ANP	10	Sep.-11	P	1	wt
C	C12-18	Nose	3	Oct.-11	36	nd	A2058G
C	C12-19	Nose	4	Oct.-11	A	1	wt
C	C12-20	Nose	4	Oct.-11	38	nd	no amp
C	C12-21	Nose	6	Oct.-11	V	2	wt
C	C12-22	Throat	10 months	Oct.-11	X	1	A2058G
C	C12-23	Nose	1	Oct.-11	X	1	wt
C	C12-24	Nose	3	Oct.-11	J	1	wt
C	C12-25	Nose	9	Nov.-11	J	1	wt
C	C12-26	Nose	4	Nov.-11	J	1	no amp
C	C12-27	Nose	8	Nov.-11	G	2	wt
C	C12-28	Nose	58	Nov.-11	nd	nd	no amp
C	C12-29	Nose	3	Nov.-11	U	1	no amp
C	C12-30	Nose	6	Nov.-11	U	1	wt
C	C12-31	Nose	7	Nov.-11	P	nd	wt
C	C12-32	Nose	25	Nov.-11	U	1	wt
C	C12-33	NPA	7	Nov.-11	U	1	wt
C	C12-34	Nose	12	Nov.-11	nd	nd	wt
C	C12-35	Nose	4	Dec.-11	nd	nd	wt
C	C12-36	Nose	8 months	Dec.-11	P	1	wt
C	C12-37	NPA	5	Dec.-11	U	1	no amp
C	C12-38	Nose	29	Dec.-11	E	1	wt
C	C12-39	Nose	5	Dec.-11	V	1	wt
C	C12-40	NPA	1	Dec.-11	J	1	wt
C	C12-41	Nose	73	Dec.-11	M	2	wt
C	C12-42	NPA	16	Dec.-11	U	1	wt
C	C12-43	BAL	62	Dec.-11	T	2	wt
C	C12-44	Nose	60	Dec.-11	nd	2	A2058G
C	C12-45	Nose	9 months	Dec.-11	B	2	wt
C	C12-46	Nose	82	Dec.-11	M	2	wt
B	Mpn-4840	Throat	17	Jan.-11	nd	nd	no amp
B	Mpn-4872	Throat	40	Jan.-11	J	1	wt
B	*Mpn-4838*[f]	Throat	5	Jan.-11	J	1	wt
B	*Mpn-4864*[f]	Throat	5	Jan.-11	J	1	wt
B	Mpn-4837	Throat	9	Jan.-11	J	1	wt
B	Mpn-4873	Throat	8	Jan.-11	J	1	wt
B	Mpn-4883	Throat	4	Jan.-11	nd	nd	no amp
B	Mpn-4885	Throat	1	Jan.-11	nd	nd	no amp
B	*Mpn-4845*[f]	Throat	4	Jan.-11	J	1	wt
B	*Mpn-4861*[f]	Throat	4	Jan.-11	J	1	wt
B	Mpn-4889	Throat	38	Feb.-11	nd	nd	no amp
B	Mpn-4940	Throat	54	Mar.-11	R	2	wt
B	Mpn-4941	BAL	8	Mar.-11	U	1	wt
B	Mpn-4945	NPA	4	Mar.-11	J	1	wt
B	Mpn-4999	NPA	36	Apr.-11	X	1	wt
B	Mpn-4977	Throat	39	Apr.-11	X	1	wt
B	Mpn-4994	Trach. asp.	4	Apr.-11	J	1	wt
B	*Mpn-Lim1*[f]	NPA	10	May-11	I	1	wt
B	*Mpn-5063*[f]	Sputum	10	Jun.-11	I	1	A2059G
B	Mpn-5079	Throat	2	Aug.-11	nd	nd	no amp
B	Mpn-5087	Throat	7	Aug.-11	35	1	wt
B	Mpn-5096	BAL	2	Aug.-11	41	2	A2062G
B	Mpn-5104	Bronch. asp.	5	Aug.-11	nd	nd	wt
B	Mpn-5094	NPA	3	Aug.-11	J	1	wt
B	Mpn-5213	NPA	34	Sep.-11	P	1	wt
B	Mpn-5160	BAL	5	Oct.-11	29	1	wt
B	Mpn-5156	NPA	1	Oct.-11	29	1	wt
B	Mpn-5149	NPA	3	Oct.-11	P	1	A2058G
B	Mpn-5163	Throat	5	Oct.-11	E	1	wt
B	Mpn-5144	Throat	1	Oct.-11	G	2	wt
B	Mpn-5209	NPA	1 week	Nov.-11	nd	nd	wt

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Table 1 (*Continued*)

City[a]	Specimen designation	Source[b]	Patient's age (years unless specified)	Date of collection	MLVA type[c]	PCR-RFLP type	Macrolide resistance genotype[d]
B	Mpn-5188	Trach. sec.	1	Nov.-11	29	1	wt
B	Mpn-5171	Sputum	1	Nov.-11	E	1	wt
B	Mpn-5206	BAL	1	Nov.-11	V	2	wt
B	Mpn-5220	NPA	11	Nov.-11	P	1	wt
B	Mpn-5173	NPA	1	Nov.-11	G	2	wt
B	*Mpn-5200*[g]	Trach. asp.	32	Nov.-11	T	2	wt
B	*Mpn-Cla1*[g]	Throat	32	Nov.-11	T	2	wt
B	Mpn-Cho	BAL	25	Nov.-11	U	1	wt
B	Mpn-5227	Sputum	19	Dec.-11	P	1	wt
B	*Mpn-5263*[g]	BAL	6	Dec.-11	P	1	wt
B	*Mpn-5264*[g]	Pleural liq.	6	Dec.-11	P	1	wt
B	Mpn-5249	Sputum	55	Dec.-11	P	1	wt
B	Mpn-5221	Bronch. asp.	6	Dec.-11	U	1	wt

[a]C, Caen; B, Bordeaux.
[b]NPA, nasopharyngeal aspirate; Trach. asp., tracheal aspirate; BAL, bronchoalveolar lavage; Bronch. asp., bronchial aspirate; Pleural liq., pleural liquid.
[c]The numbering system of MLVA types A to X was described by Degrange *et al.* [14]; MLVA type 29, 2-4-6-7-2; MLVA type 35, 2-3-6-7-2; MLVA type 36, 6-4-6-7-2; MLVA type 38, 4-4-5-6-2; MLVA type 41, 2-3-5-7-2.
[d]wt, wild type; no amp, no amplification with the real-time PCR assay used to detect 23S rRNA mutations associated with macrolide resistance according to Peuchant *et al.* [15]. The lack of amplification concerned 17% (16/94) of the studied specimens. Substitutions A2058G, A2059G and A2062G (*E. coli* numbering) correspond to substitutions A2063G, A2064G and A2067G, respectively, using *M. pneumoniae* numbering.
[e]nd, not determined. The absence of type determination concerned 17% (16/94) and 19% (18/94) of the specimens using MLVA and PCR-RFLP typing, respectively.
[f]Specimens from the same patients are italicized. Three distinct patients had two sequential specimens. They were collected 8 days apart for specimens Mpn-4838 and Mpn-4864, and specimens Mpn-4845 and Mpn-4861, and 48 days apart for specimens Mpn-5063 and Mpn-Lim1.
[g]Specimens from the same patients are italicized. Two patients had two concurrent specimens collected on the same day (Mpn-5200 and Mpn-Cla1 for one patient and Mpn-5263 and Mpn5264 for the second one).

niae type 1 [19], and results from an Italian outbreak in 2010, where 82% of the strains belonged to type 1 [20].

In conclusion, we confirm that the recent increased incidence of *M. pneumoniae* infection in France was not a clonal spread but rather a multiclonal phenomenon. This is certainly the result of the simultaneous spread of several clones in the community, each of which was able to spread clonally in a closed or semi-closed community, such as a family or school. However, despite several distinct MLVA types, most of these clones were characterized as adhesin P1 type 1.

Authorship/Contribution

Conceived and designed the experiment: SP, JPL, AV and CB. Performed the experiment: SP, AT, JPL and AC. Analysed the data: SP, AT, JPL, AC and CB. Contributed reagents/materials/analysis tools: SP, JPL, AV and CB. Wrote the paper: SP, JPL, AV and CB.

Transparency Declaration

Nothing to declare.

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