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A seroepidemiological study of *Mycoplasma pneumoniae* infections in Denmark over the 50-year period 1946–1995

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Abstract. The epidemiological pattern of *Mycoplasma pneumoniae* infections in Denmark over the 50-year period 1946–1995 is described. The study is based on blood specimens received at the central laboratory at Statens Serum Institut for titration of cold agglutinins (CA), initially for the diagnosis of CA positive primary atypical pneumonia, and during the 1960s of *M. pneumoniae* infection; in addition, specimens from the last 38 years were tested for antibodies specific to *M. pneumoniae*. By retrospective analysis of the test results compiled over the years it was found that intervals of regular periodicity have been interrupted by an era of changes in the pattern. Attention is paid to the significance of CA for this study, and the possible background of the epidemiological pattern is described.

Key words: Epidemics, Denmark, *Mycoplasma pneumoniae*, Primary atypical pneumonia (PAP)

Introduction

In 1943 Peterson et al. described the cold (hem) agglutinin (CA) reaction as the first practical tool for studies of patients with the newly recognized syndrome of Primary Atypical Pneumonia (PAP) [1]. The next year Eaton et al. [2] isolated an agent from patients with CA positive PAP, which in the early 1960s was identified as *M. pneumoniae* [3, 4]. This led to development of practical and specific serological methods; most widely used was the *M. pneumoniae* complement fixation (MpCF) test [5], which in many laboratories replaced the CA test. The aim of the study was to describe the pattern of *M. pneumoniae* epidemics in a well defined population by retrospective analysis of serological data from a period of 50 years. Changes in the picture of this pattern are discussed in the light of a significant change in the social pattern within the population.

Material and methods

Material. The material consisted of patient blood samples collected at hospitals and by general practitioners throughout Denmark from January 1946 till December 1995. In the early years the samples were sent to SSI for the diagnosis of CA positive PAP [1, 6, 7], and from the late 1960s of *M. pneumoniae* infections [2–4]. Since 1958 all CA positive sera have been stored at –30 °C if sufficient material was left for later specific tests for antibodies to an aetiological agent [8, 9]. The random selection of CA positive sera from 1958 to 1972 to be tested for specific anti-*M. pneumoniae* antibodies has been described [8–10]. Since 1972 all sera have been tested routinely by a *M. pneumoniae* complement fixation (MpCF) test in addition to the CA test. Analysis of the data from this retrospective seroepidemiological study is based on the significant association of CA and antibodies to *M. pneumoniae* during a current *M. pneumoniae* infection [8–10], confirmed by recent studies (Table 1) [11]. All sera with a negative CA test were excluded whether they had positive anti-*M. pneumoniae* tests or a rise in titer. This was done for the sake of continuity and comparison in this epidemiological study.

During the period 1946–1957 CA titration was performed on 35,871 blood specimens for the diagnosis of CA positive PAP [12]. The sera were not kept for further studies, but the CA test results were available from the protocols.

From 1 July 1976, to 31 December 1986, a total of 176,045 blood specimens were received for confirmation or otherwise of the clinical diagnosis of *M. pneumoniae* infection. Out of these specimens 12,562 were received from 9,161 patients who had at least one MpCF positive test. This is described in [10] which mentions that there were 2,421 patients with at least two blood specimens taken within a period of 60 days. Among them 496 patients developed significant titer rises in the MpCF test (Table 1).

Except for this 10.5 years period 1976–1986, char-

Table 1. Cold agglutinin (CA) test results in patients' sera with ⩾ 4-fold rises in *M. pneumoniae* complement fixation (MpCF) test by age

Age group (years)	Number of patients	Positive CA test	
		Number	% in group
0–4	20	19	95
5–19	189	180	95
20–39	200	164	82
⩾ 40	87	44	51
All ages	496	407	82

acteristics of the patients, especially their age and clinical diagnosis, have not been recorded regularly over the years, nor was this the case as for the ratio hospital/practitioner for submission of specimens. However, the sample studied these 10.5 years [10] comprised 4,456 (49%) male cases and 4,664 (51%) female cases among 9,158 whose sex was known. Fifty seven per cent had their blood specimens sent from hospitals and 43% from practitioners, who had requested MpCF and CA tests as a diagnostic aid. These figures may be useful representatives in estimates about the total material which constituted a subset of the Danish population.

Prospective clinical studies comprising 276 patients with acute lower respiratory tract infection were included to evaluate the predictive values of a positive (PV+) and a negative (PV–) CA test. Blood specimens were received for MpCF and CA testing, because the patients were suspected of having *M. pneumoniae* disease. Sixty patients showed a ⩾ 4-fold rise in MpCF titre during illness, and 216 were MpCF negative (titre < 64) in all consecutive samples. In Table 2 the patients are grouped according to CA positivity and stratified by age.

In addition, CA reactions were measured in paired sera from 132 patients with respiratory tract infections in whom at least 4-fold rises of antibodies against viral or chlamydial antigens were demonstrated (Table 3). These sera were all MpCF negative.

Serological methods. Standard methods for CA and MpCF tests were used throughout the study as follows briefly:

CA test. Two-fold dilutions of sera were prepared in veronal buffered saline pH 7.6 (VBS), from 1:16 to 1:2,048. To 0.4 ml amounts were added 25 µl of a 2.5% suspension in VBS of washed human group 0I erythrocytes from a CA negative healthy donor. After overnight incubation at 4 °C, the rack was placed in an ice bath and the tubes were shaken lightly and examined against an illuminated green frosted glass screen for clearly visible agglutination. The highest dilution with definite agglutination was taken as the endpoint, and the reciprocal of the dilution value was recorded as the titer. For CA tests of specimens received in the years 1946–57 a slightly different two-fold titration method was used which included three volumetric dilution steps [13]. The two methods were in agreement with respect to titer levels, and in both a titre of ⩾ 64 was considered a positive result.

MpCF test. A slight modification of the method of Kenny and Grayston [5] was used, as described previously [Appendix in 8]. Lipid antigen was extracted from glass or plastic-adherent organisms with chloroform and methanol. After evaporation, the extract was dissolved in 96% ethanol and stabilized with 5% bovine serum albumin in VBS. Sera diluted 1:8 were heat inactivated at 56 °C for 30 min, and diluted two-fold through 1:4,096 in microtiter plates followed by addition of 1.5 U antigen and 2.0 U guinea pig complement. After an incubation for

Table 2. Cold agglutinin reaction in 276 patients with or without acute *M. pneumoniae* infection[a] stratified by age group

Patients	Age group	Total number	CA negative number	CA positive number	%
With *M. pneumoniae* infection	0–4	0	0	0	
	5–19	11	1	10	90.9
	20–39	36	8	28	77.8
	⩾ 40	13	4	9	69.2
	Total	60	13	47	78.3
Without *M. pneumoniae* infection	0–4	2	1	1	50.0
	5–19	24	22	2	9.1
	20–39	51	45	6	11.8
	⩾ 40	139	131	8	5.8
	Total	216	199	17	7.9
Both groups		276	212	64	

[a] For definition see text.

Table 3. Cold agglutinin reaction in 132 paired sera with ⩾ 4-fold rise in titre of antibodies against viral and chlamydial antigens indicated. All sera were negative for antibodies to *M. pneumoniae*

Antigen	Number of paired sera	Cold aggl.-test positive (%)
Influenza A virus	52	8 (15.4)
Influenza B virus	13	1 (7.7)
Influenza C virus	5	2 (40)
Adenovirus	34	1 (2.9)
Respiratory syncytial virus	9	0 (–)
Cytomegalovirus	5	0 (–)
Chlamydia antigen	14	0 (–)
Total	132	12 (9.1)

1 hour at 37 °C, sensitized (with rabbit anti-sheep erythrocyte serum) sheep erythrocytes were added, and a second incubation performed. The antibody titre was defined by the highest serum dilution producing 60% inhibition of haemolysis.

Interpretation of test results. The patient was considered to have a current or recent *M. pneumoniae* infection if the MpCF test demonstrated a ⩾ 4-fold rise in titre to ⩾ 64 in at least two consecutive sera. This criterion was used in studies of the significance of CA in *M. pneumoniae* infection in the 496 patients with MpCF titre rises (Table 1). It was also the criterion in the clinical studies when the predictive values of a positive (PV+) and a negative CA test (PV–) were evaluated (Table 2).

However, in the major part of the material the epidemiological studies were based on results from testing single sera for anti-*M. pneumoniae* and CA and on knowledge of the close association between the two types of antibodies during a *M. pneumoniae* infection.

Results

In the 15 years from 1958 to 1973 the disease occurred in a regular pattern of epidemics culminating every 4,5 years (Figure 1). Then followed two 'premature' epidemics in 1975 and 1977/78 culminating at intervals of 3 and 2.5 years, respectively, succeeded by a 9-year hyperendemic to hypoendemic period, which ended in a big epidemic in the winter 1987/88. Then, after 4 years of low prevalence, the next epidemic followed in the winter of 1991/92. The subsequent 4 years of low endemic prevalence ended in an increase of cases greater than usually seen between epidemics, however, still not characteristic of an epidemic.

The quarterly numbers of CA positive specimens received during the period 1946-57 are shown on Figure 1. A significant high prevalence (arrow) indicates that another epidemic of *M. pneumoniae* infection had ocurred in 1949/50, nine years before the 1958/59 epidemic.

Among the 496 patients selected from the 10.5-year period 1976–1986 with a current *M. pneumoniae* infection according to our criterion, the average prevalence of a positive CA test for all ages was 82% (Table 1). In the age group below 20 years, the CA test was positive in 95% of cases. The prevalence declined in the older age groups to a mean value of 51% among those 40 years or older. A similar declining prevalence of CA positivity in the older age group of patients with acute *M. pneumoniae* infection was seen in the clinical studies (Table 2).

Based on the data in Table 2 the sensitivity of the CA test was 78% (47/60) and the specificity of the CA test was 92% (199/216). If according to our experience we assume a prevalence of 10% for *M. pneumoniae* infection during, and 2% outside epidemics (data not shown), then the PV+ for the CA test if performed alone would be 52% and PV– 97% during epidemics; in endemic periods the figures would be 17% and 99.5%, respectively [14].

Discussion

This epidemiological study was based on a retrospective analysis of results from testing sera by a CA test and *M. pneumoniae* antibody tests during the period 1958–1995. Usually a serological test for an infectious disease is diagnostic only when a significant rise in titre can be demonstrated in blood specimens taken during the course of an illness. In the available serological material a follow-up of titres in a particular patient had not been possible for all patients, and in many cases only a single specimen had been taken. However, with the known significant association between formation of CA and specific antibodies to *M. pneumoniae* in a patient, a positive result in both tests of a single serum will yield a reasonably safe diagnosis. As demonstrated this holds true especially for patients in the younger age groups, who are at the highest risk of getting the infection (Table 1). It may be added that while an anti-*M. pneumoniae* test can remain at a positive level for several months, the CA test will usually turn negative much earlier [9, 15].

CA are antibodies of the immunoglobulin (Ig)M class. As demonstrated and discussed by Uldum et al. [16], there is a direct association between the occurrence of *M. pneumoniae* antibodies of the IgM class measured by an enzyme immunoassay and CA positivity in the younger age groups. In accordance with the present study it was found that some, mainly older, patients neither produced IgM antibodies to *M. pneumoniae* nor CA at all, but did produce IgG antibodies, probably as a result of reinfection.

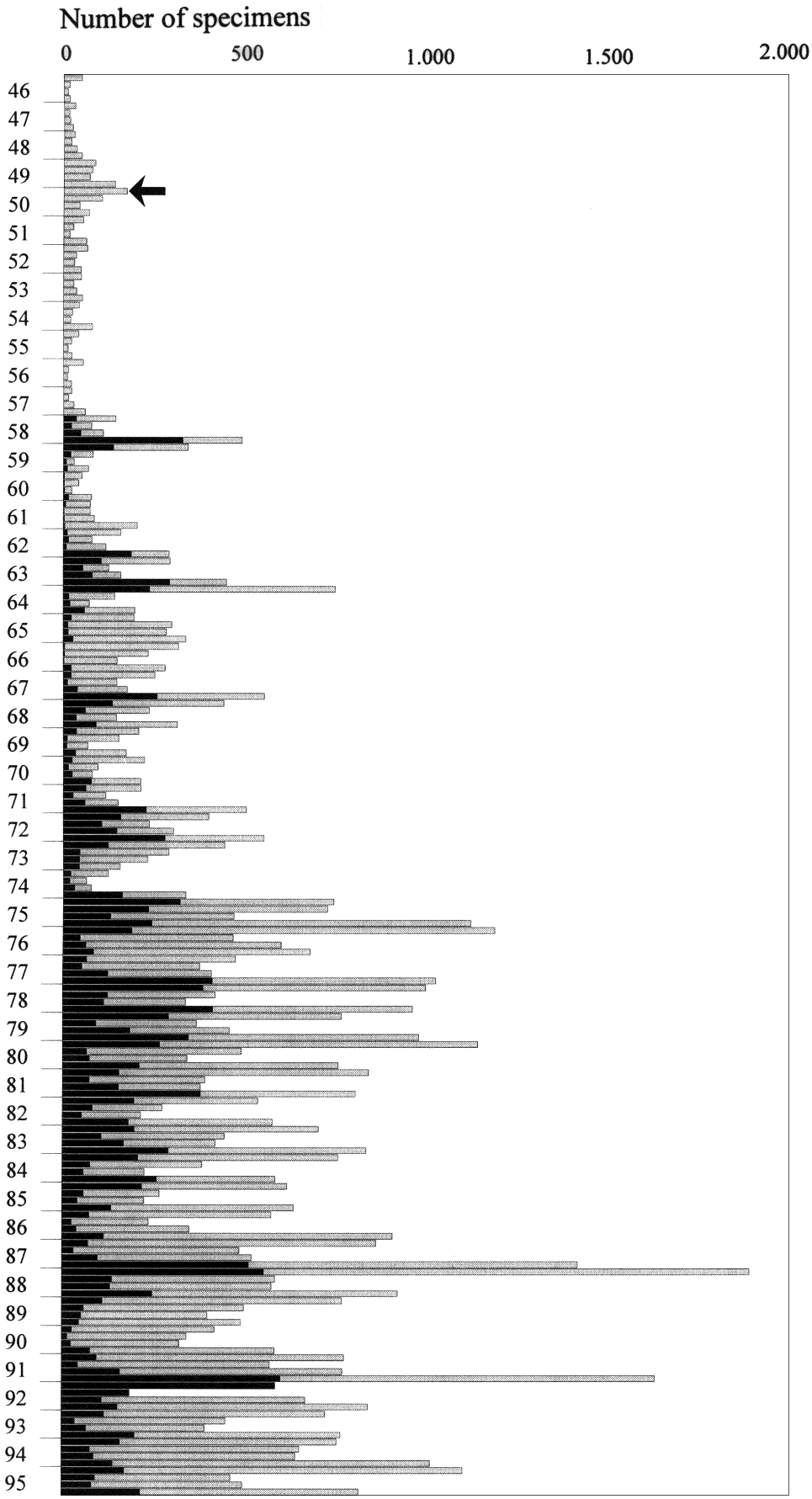

Figure 1. Quarterly numbers of cold agglutinin (CA) positive specimens (grey columns) and anti-*Mycoplasma pneumoniae* positive *plus* CA positive specimens (black columns) received at Statens Serum Institut during 50 years. Arrow indicates the first *M. pneumoniae* epidemic. In the first two quarters of 1992 CA tests were not performed.

It is well known that CA production is a non-specific response to a variety of diseases such as infection by Influenza, Morbilli, Epstein-Barr viruses and Adenovirus [7, 17–22], as well as rheumatic diseases and those caused by proliferative changes in lymphoid tissues [23, 24]. Many of these conditions can be differentiated from *M. pneumoniae* infection on clinical grounds. Some non-*M. pneumoniae* respiratory pathogens may, however, cause a CA production, but this is significantly less common than in *M. pneumoniae* infection, as for instance shown in Table 3 and seen by comparison of Tables 1 and 3.

The CA test has been performed at SSI during all 50 years, and the uniformity of this test over the years is a reliable background for using the results in this retrospective study, in which the prevalence of CA positive results accurately mirrors *M. pneumoniae* epidemics [11].

This knowledge was applied in an analysis of data from 1946 to 1957 where only CA test results were available because sera had not been kept for later tests of specific antibodies. Prevalences of CA positivity showed a significant peak in the winter of 1949/50 (Figure 1), just 9 years before the first recorded *M. pneumoniae* epidemic in 1958. A peak in the winter of 1954 is not significantly higher than peaks of the adjacent winters. Still, the 1949/50 peak would fit into the regular pattern of the following period 1958–1972 with *M. pneumoniae* epidemics culminating every 4.5 years if we ignore the insignificant peak in 1954.

The increase of cases at the end of 1995 was above the average seen at this season outside epidemics; time will show if it announces the next epidemic spanning two winters like those of 1962/63–1963/64 and 1971/72–1972/73 as described previously [8].

It is likely that the reported culminations of epidemics may be delayed for a few weeks. This is due partly to the time of antibody development after onset of illness, partly to the fact that usually some time will pass before doctors' attention is drawn to the development of an epidemic. This delay, however, will hardly influence the time intervals between tops of the epidemic curves, which are most relevant for this study.

Long-term studies of the infection in other populations have indicated a periodicity of 3–6 years [11, 25–29]. Niitu et al. [30] conducted a study over 22 years among pupils of the primary, middle and high schools in Sendai, Japan, which showed a strictly 4-year periodicity. A 50-year long-term study of *M. pneumoniae* infection in a population of the size investigated here has not been described before. In this period the Danish population increased from 4.1 to 5.1 million inhabitants.

We do not know what governs the periodicity of these epidemics. The duration of protective immunity of an individual has been estimated from a few published cases to last about 4 years with a range of 2 to 10 years [31–33]. In a previous publication [9] we have discussed the possible significance of herd immunity in connection with a more than six-fold increase in the number of children in day-care institutions and day-care in private homes in Denmark in the period 1961–86. This increase was most steep in the years of change from an epidemic to an endemic situation (see Figure 1). In 1986 almost one quarter of the 0–10 years old children was in day-care [9]. This well-known risk factor for transmission of *M. pneumoniae* diseases among children may have resulted in an increasing protective immunity in a significant proportion of the population. With the known limited duration of protective immunity the balance between protected and susceptible individuals may have tipped towards the end of the hypoendemic period thus paving the way for the following big epidemic in 1987/88. Investigations in protective immunity in a population are still needed as a basis for strategies in prevention and treatment of the disease.

The conclusion is that although MpCF, and especially CA tests, have been available for serodiagnosis for many years, their performance have not been compared when applied to all suspected cases in a large population as was the case at SSI where patients were tested over a period of 50 years. This provided a large data base for comparison of these two classical serological tests for *M. pneumoniae* infection. The prevalence of these reactions traced parallel epidemic patterns reflecting the epidemiology of M.pneumoniae disease in the population. Not reported previously is the age-related tendency for CA formation, which parallelled the age-related occurrence of *M. pneumoniae* disease. As discussed before [16], this means that patients with *M. pneumoniae* infection, especially those above 40 years of age, are underreported if the MpCF plus CA tests are employed on single sera alone. If in all such patients' paired sera could have been collected the prevalence of *M. pneumoniae* infections may be higher than reported, however, this would hardly have influenced the description of the epidemiological pattern, which is the main subject of this study.

CA were not found in some groups of patients with respiratory tract infections of diverse aetiological diagnosis (Table 3), suggesting that the CA non-specificity may not be as important as commonly believed.

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