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Comparison of detectability of *Mycoplasma pneumoniae* infections in children, using PCR-test and serological methods: indirect immunofluorescence and immunoenzymatic assay

B. Maczyńska, K. Matusiewicz, J. Chiciak, M. Stankiewicz, B. Sozańska, A. Boznański

Wrocław, PL

Objectives:

Mycoplasma pneumoniae is an important cause of community acquired infections of upper and lower respiratory tracts in children, mainly atypical pneumonia. The aim of our study was the comparison of the serological procedures used in routine diagnostic (enzymatic- and fluorescence assays) with the polymerase chain reaction (PCR) which is currently the most reliable and sensitive method applied in *M. pneumoniae* diagnostic.

Materials and methods:

The materials from 100 children hospitalized in Pediatric and Alergology Department of Medical University of Wrocław were analyzed. The PCR assay using *M. pneumoniae* Diagnostic Kit Venor Mp (Minerva, Germany) was performed to amplify the gene encoding the P1 adherent protein. PCR was used to examine the presence of *M. pneumoniae* in throat swab specimens. The level of IgM and IgG antibodies were evaluated in sera samples using two immunoenzymatic tests: with membrane P1 protein (ELISA DiaSorin, USA) and with whole cell mycoplasma antigen (ELISA Euroimmun, Germany) and indirect immunofluorescence test with Biochip technique-fibroblastic cells infected by *M. pneumoniae* (Euroimmun, Germany). Swabs and sera were taken from children in acute infection.

Results:

Fifty-two percent throat swabs from children investigated by PCR were positive. Depending on serological test used, different percentages of positive results (IgM or both IgM and IgG antibodies) were detected in sera samples-30% using ELISA from DiaSorin, 27% in fluorescence test and 10% in ELISA from Euroimmun. Additional in fluorescence test 10% of samples were considered as the positive on the basis of the high titer (>200) of IgG antibodies. In 15% of cases, positive results in PCR were negative in all serological methods.

Conclusions:

Detectability of *M. pneumoniae* was highest with PCR test applied. In serological test used in routine diagnostic, the false negative results could be obtained. The indirect fluorescence test was considered as the most reliable and sensitive in comparison with PCR assay.

Comparison of detectability of *Mycoplasma pneumoniae* infections in children, using PCR-test and serological methods: indirect immunofluorescence and immunoenzymatic assay.

B. Mączyńska*, K. Matusiewicz, J. Chiciak,
M. Stankiewicz,¹ B. Sozańska, A. Boznański².

¹Department of Microbiology , ²Department of Pediatric and Alergology, Wrocław Medical University, Poland.

*beciam@mbio.am.wroc.pl

INTRODUCTION AND PURPOSE

Mycoplasma pneumoniae is an important etiological agent of community acquired infections of upper and lower respiratory tracts in children and young adults, mainly atypical pneumonia. *M. pneumoniae* has been reported as the cause of 25-30 % of all pneumonia cases. This pathogen has also been associated with non respiratory diseases as meningitis, encephalitis, pancreatitis or arthritis. Diagnosis of infection by this organism is difficult because culture is specific but slow (may require 3 week for results) and insensitive. For this reason the diagnostics is usually based on serological methods. Serological procedures require demonstration of a rise in antibody titer or detection of several different immunoglobulins, mainly IgG and IgM antibodies. Unfortunately these serological methods often give a false positive or a false negative results. Sometimes the obtained results are difficult to interpret and do not allow for the rapid application of an effective treatment. Generally in *M. pneumoniae* infection is difficult to

set up criteria for the “gold standard” to detect acute or remote infection. The aim of our study was the comparison of the serological procedures used in routine diagnostics (enzymatic- and fluorescence assays) with the polymerase chain reaction (PCR) which is currently the most reliable and sensitive method applied in *M. pneumoniae* diagnostic.

MATERIALS AND METHODS

In our investigations the materials from 100 children hospitalized in Pediatric and Alergology Department of Medical University of Wroclaw due to atypical pneumonia were analysed. The serum samples for antibody testing and the throat swabs for polymerase chain reaction (PCR) were obtained from children in acute infection. The PCR assay using *Mycoplasma pneumoniae* Diagnostic Kit Venor[®]Mp VenorMp Minerva Biolabs (Germany) was performed. The level of IgM and IgG antibodies were evaluated in sera samples using two different immunoenzymatic tests ELISA DiaSorin (USA) and ELISA Euroimmun (Germany) and indirect immunofluorescence test with Biochip technique - Euroimmun (Germany).

PCR-test (VenorMp Diagnostic kit Minerva Biolabs – Germany)

According to the producer the PCR test is reliable with as little material as 1-5 *Mycoplasma pneumoniae* particle per sample. The primers used in the Venor[®]Mp kit were selected to exclusively amplify *Mycoplasma pneumoniae* I and II DNA encoding the P1 adherent protein. The reliability of the test is guaranteed by content of internal

control (PCR quality check) – sequence of the HTLV-I-tax gene a size of 263 bp. For verification of the results there is a positive control included (DNA fragment of *M. pneumoniae* prepared by PCR). Templates for PCR analysis were prepared by DNA extraction from material obtained by washing away throat swabs using manual method with guanidide isothocianiate (de Bruijn at al.) We got similar effects using the simplest thermal method in which material obtained from swabs is heated to 94°C for 10 minutes (according to Reznikov at al.). Preparation of PCR samples, dNTP's, buffer, polymerase, internal and positive control concentration was specified by a producer. The temperature profile is also given. Amplified product were separated and visualised by standard agarose electrophoresis in 2% gel stained with ethidium bromide (TAE buffer , 80 V). In samples containing *M.p* DNA (as well as the positive control a 207 bp band was detected. Successfully performed reaction was indicated by a 263 bp internal control product, although this product might be not seen in positives samples with high concentration of the template competition between templates in the PCR.

Indirect immunofluorescence test (Euroimmun – Germany)

M. pneumoniae infected and non-infected cells covering the reaction areas of a BIOCHIP Slide were incubated with a diluted serum samples. The important step before specific IgM antibodies detection was removing of IgG class immunoglobulins from patient's serum by immunoabsorption (EUROSORB reagent). This was to prevent false IgM positive test result cause by possibly existing rheumafactors of class IgM reacting with specifically bound

IgG. False IgM negative test results brought about by specific IgG competing with the IgM for the antigenic binding sites were also avoided. Furthermore, the immunoadsorption of the IgG leads to an elimination of rheumafactors from the sample. If the sample was positive, specific antibodies of IgM and IgG classes attach to the bacterial antigens. In a second step the attached antibodies were stained with fluorescein-labelled anti-human antibodies and made visible with the fluorescence microscope. Antibodies against *M.p* caused a granular to coarse drop-like fluorescence in infected cells, mainly in the area of the cytoplasm. If the serum sample contained anti-*M.p*, essentially the same pattern was observed as for the positive control serum. For comparison purposes, the second BIOCHIP of each test field was coated with non-infected cells. The important is that in this assay the antibodies titer may be detect. The serum samples showed the presence IgM or both IgM and IgG antibodies (Table 2) and samples with the high titer (>200) of IgG antibodies (Table 3) were considered as positive (current, acute infection).

Immunoenzymatic test – ELISA ETI-MP IgG, IgM (Dia Sorin, USA)

The antigen used in the ETI-MP test-the purified fraction of *M. pneumoniae* P1 membrane protein was coated on microtiter plates. Cover plates with diluted 1/105 sera samples, negative control and three calibrators (P10, P50, P100) were incubated 1h at 37oC. In next step anti-human conjugate with horseradish peroxidase (HRP) were added and the plate were incubated 1h at 37oC. The next step is the incubation with the substrate. After stopping the reaction, the ODs of

samples were transformed to arbitrary units determined from the calibration curve (10, 50 and 100 BU/ml). Antibodies titer between 10 and 50 BU/ml was considered as significant and a value > 100 BU/ml was considered a high titer. The serum samples showed the presence IgM or both IgM and IgG antibodies in significant titer were recognised a positive.

Immunoenzymatic test – ELISA IgG, IgM – Euroimmun (Germany)

Diluted 1/101 patient's samples were incubated in the wells of plates coated with whole cell extract of *M. pneumoniae* strain 'FN', for 30 minutes at a room temperature. Afterwards an enzyme conjugate was added. After the next incubation the chromogen/substrate was used. The reaction was stopped by means of stop solution. Before a patient's serum was tested for specific antibodies of the IgM class, antibodies of IgG had been removed by immunoabsorption. The extinction value were interpreted by calculating a ratio according to following formula: extinction of controls /extinction of calibrator (cut-off). The ratios>1.0 are to be considered as positive, ratios<1.0 as negative.

RESULTS

Table 1. Percentage of positive samples in all investigated method

N=100 Using method	Percentage of samples detected: as a positive as a negative	
PCR VenorMp – Minerva Biolabs	52	48
Indirect Immunofluorescence - Euroimmun	36	64
ELISA ETI-MP – DiaSorin	32	68
ELISA - Euroimmun	14	86

Table 2. Detection of *M. pneumoniae* IgM and IgG antibodies in two ELISA tests and immunofluorescence test.

N=100 (100 %)	Percentage of sera samples detected by test:		
	ELISA DiaSorin	ELISA Euroimmun	Immunofluorescence Euroimmun
Absence of antibodies in both classes	57	73	51
Presence of IgM and IgG antibodies	14	14	23
Presence of IgM antibodies only	18	0	4
Presence of IgG antibodies only	11	13	22

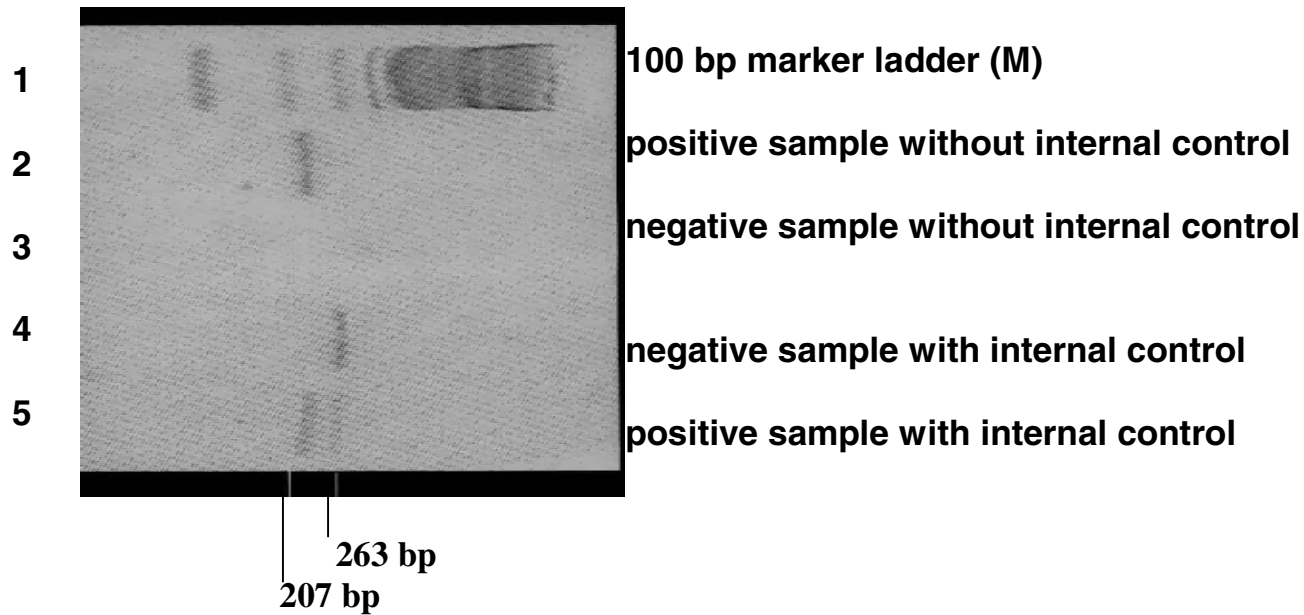
Table 3. Analyse of IgG antibodies detected by immunofluorescence test

N=22 (100 %)	Number (percentage) of sera samples detected by immunofluorescence test with IgG antibodies only)
IgG antibodies with titer > 200	9 (40 %)
IgG antibodies with titer > 100	5 (24 %)
IgG antibodies with titer > 10	8 (36 %)

Table 4. Sensitivity and specificity serological method using PCR as a reference test

	ELISA DiaSorin	ELISA Euroimmun	Immunofluorescence
Sensitivity	77 %	59 %	87 %
Specificity	81 %	76 %	82 %

Visualisation of electrophoretic products of PCR in agarose gel.



Amplified PCR products are visualised by standard gel electrophoresis. A sample containing *Mycoplasma pneumoniae* DNA will produce a distinct 207-bp band as seen with the positive control reaction. This band is absent in reaction performed with negative patient samples and in the negative control. The internal control DNA should be present in every reaction and will produce a 263 bp band.

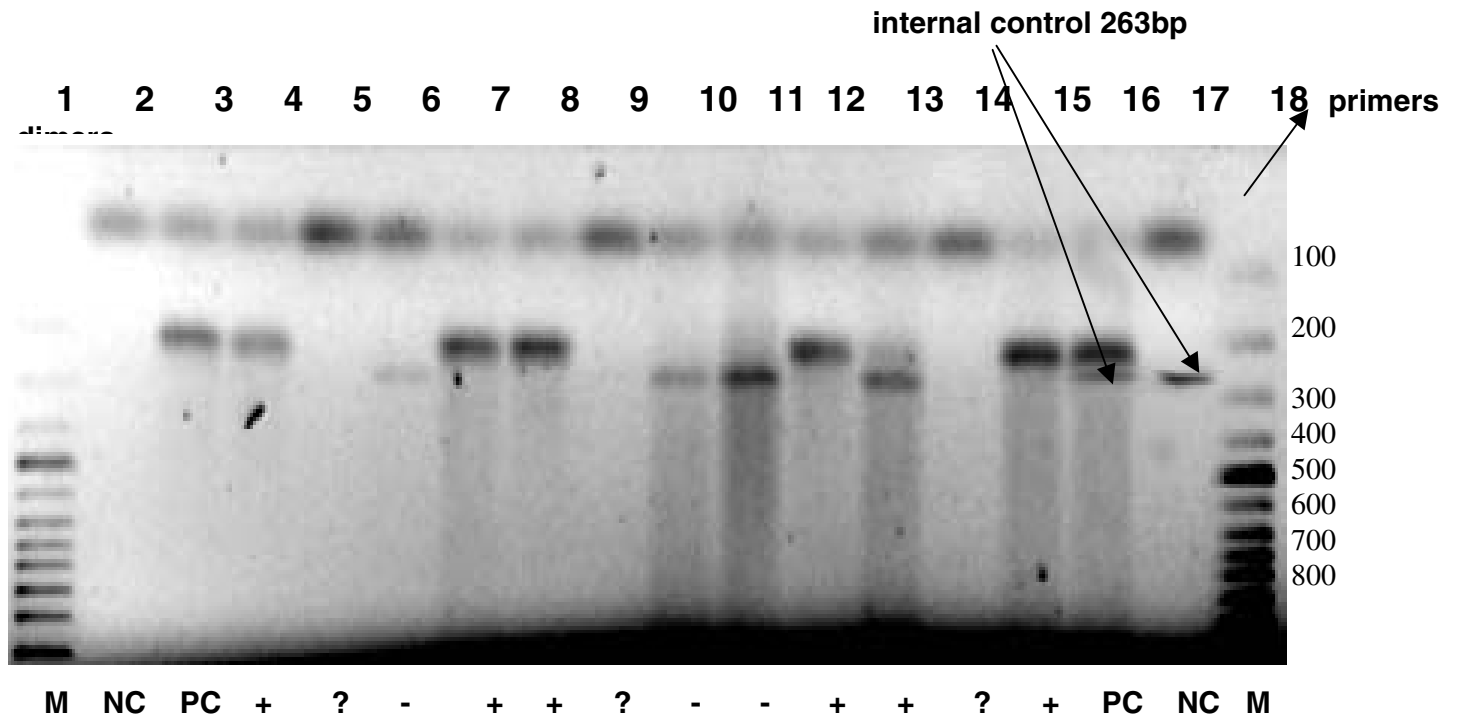
The example of detection *M. pneumoniae* DNA in throat swabs.

M- molecular weight marker

NC (line 2)- negative control without internal control

PC (line 3)- positive control without internal control

+ positive samples
 - negative samples
 ? uncertain samples



CONCLUSIONS

- **52 % throat swabs from children investigated by PCR were positive.**
- **Venor^R Mp diagnostic kit for conventional PCR in throat swabs is suitable for rapid, accurate and early diagnosis of the *M. pneumoniae* infection in children.**
- **Depending on the serological test used, different percentages of positive results were detected in sera samples.**
- **The indirect fluorescence was recognised as a best reliable from investigated serological methods.**
- **In 16 % of cases, positive results in PCR were negative in all serological methods.**
- **In comparison with the PCR test the serological methods showed low sensitivity and specificity.**

- **The combination of PCR and IgM serology should thus allow to obtain the highest number of diagnoses at a very early phase of *M. pneumoniae* infection in children.**