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**ZENTRAKLINIK**



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## **Certification**

of the applicability of the clinical diagnostics around

**Venor<sup>®</sup> Mp of the firm Minerva Biolabs GmbH**

For the detection of the respiratory tract pathogen  
*Mycoplasma pneumoniae* in patient samples by means of PCR

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Akkreditiert durch



vertreten im



ZLG-P-826.00.25

*Certificate MB/130202-AR*

The clinical diagnostic kit Venor<sup>®</sup>*Mp* was provided for evaluation by Minerva Biolabs GmbH. Venor<sup>®</sup>*Mp*, registration number DE/CA73/084175, produced in accordance with IVD 98/79/EU for the detection of the respiratory tract pathogen *Mycoplasma pneumoniae* by means of PCR. The comparative investigation was conducted in accordance with the instruction of the product and in conformity with the DIN EN 45001 accredited Quality Management Systems of Dr. A. Roth.

### ***Investigation material and methods***

The investigation was conducted on the basis of 172 frozen DNA extracts. 105 gargled fluids from the throat (GF), 45 bronchoalveolar lavage fluids (BAL), 9 nasopharyngeal aspirates, 4 protected brush specimens, 7 sputa and 2 pleuracentesis had been obtained from 149 in-patients and examined by means of an in-house PCR. From 7 patients with evidence of mycoplasma DNA from materials of the deep respiratory tract (6 BAL, 1 throat swab and 1 sputa), it was additionally possible at the same time to obtain GF samples. Furthermore, from 6 patients with an initial positive PCR result, additional sample material (2 BAL und 5 GF) was obtained after a mean of 12.9 days of treatment and submitted for a second PCR evaluation. A control group was formed from 16 BAL patients with interstitial lung disease of non-infectious etiology.

The in-house PCR, followed by non-radioactive detection of the amplicon by means of dot-blot hybridization, specifies a DNA fragment in the ATPase operon from *M. pneumoniae* (primer slightly modified after Bernet C *et al.*, 1989, Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction. J Clin Microbiol, 27:2492-2496). Five µl and 0.5 µl DNA in a 50 µl PCR reaction mix were analyzed. Only if the reaction with 5 µl DNA was positive was the result depicted as (+).

The Venor<sup>®</sup>*Mp* diagnostic kit consists of 4 color indicated components (the PCR buffer, the primer nucleotide mixture, the positive control as well as the internal control), which together constitutes a PCR reaction mix without *Taq* polymerase for performing a competitive PCR. The polymerase must be procured by the user and is not supplied with the kit. In this investigation the polymerase used was from the company Pharmacia. The latter 3 of the above-mentioned components must be rehydrated with water before use (volumes of either 130 µl or 300 µl). In further proceedings, the method corresponds to a classical PCR, whereby the PCR products are evaluated after 35 amplification cycles in a conventional ethidium bromide stained agarose gel electrophoresis. A smaller volume of 2.5 µl DNA than that indicated in the manual (5 µl) was analyzed in a 25 µl PCR reaction mix.

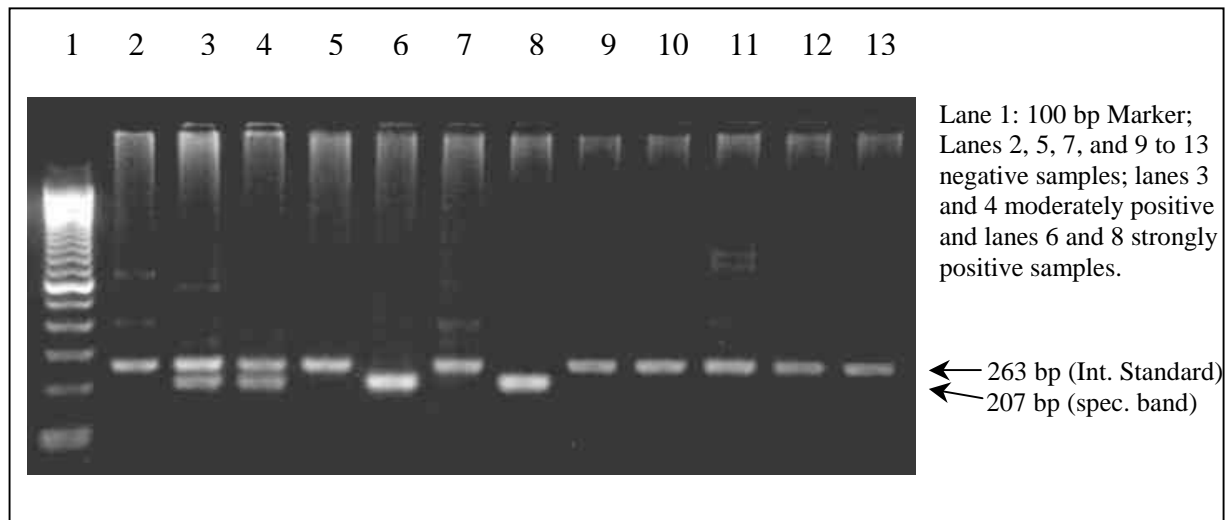
In parallel to the DNA specimen collection, an acute serum was taken from all patients and tested on the basis of a particle immunoassay (PIA) from the company Mast Diagnostica. The time difference between PCR samples and serum was less than 5 days; on average  $\pm 1.2$  days. A follow-up serum was available from 26 patients in the convalescence phase. Titers of 1:160 to 1:320 were considered borderline. Titers of or larger than 1:640 were interpreted as definitely positive, which was the case with 39 patients. Clinical data was collected from patients with a positive PCR, borderline serology (only such with a titer  $> 1:160$ ) or a positive antibody test in order to better understand any discrepancies of the two methods, as well as to get

the time interval between the beginning of the illness and the point of the investigation. The charts of patients with negative PCR and negative serology were not clinically analysed, so that it cannot be excluded, due to a potential false negative PCR in one or another case, that an acute mycoplasma infection could have been present before antibody formation. Therefore, the clinical sensitivity of the PCR cannot be calculated with respect to the entire collective of 149 patients.

## Results and Discussion

In view of the provided ready for use reagents and the manufacturer's quality-assurance, the execution of the Venor<sup>®</sup>Mp diagnostic kit was substantially easier than the in-house method. Detection by means of electrophoresis even involves less manual input than with a dotblot hybridization, and it offers the large advantage of rapidity (40 min.). The PCR and electrophoresis can be executed in 2 hours (hands-on time for 24 samples: 15 minutes for the PCR and 20 minutes for electrophoresis). Extraction of the DNA from 24 samples can be performed in 2 to three hours depending upon the method. Electrophoresis is also considerably less expensive than any other method, particularly than hybridization. An example of an electrophoresis produced in this study is shown in Figure 1: (i) the specific band after PCR amplification of *M. pneumoniae* DNA (207 bp) can be detected very clearly, (ii) it can be very well distinguished from the amplification band of the internal standards (inhibitor control) and (iii) a semi-quantitative estimate of the DNA in the sample, which can be further verified, is possible based on the relation of two bands strength to each other (competitive setup, see lanes 3 and 4 with moderately positive samples and lanes 6 and 8 with highly positive samples).

**Figure 1.** Gel electrophoresis from 12 patient samples by PCR amplification by means of Venor<sup>®</sup>Mp diagnostic kit



The results determined with 172 samples are represented in **Tab. 1**, including the patient samples indicating the course of the mycoplasma infection after treatment. 43 samples were determined to be positive with both PCR methods while 123 corresponding samples were negative. All 16 samples of the control group were likewise negative with both PCR methods (97% agreement for the two PCR procedures with a total of 188 samples).

**Tab. 1:** Agreement of the results of the Venor<sup>®</sup>*Mp* PCR procedure with comparison analysis (in-house PCR) in relation to serology.

<i>Number of Samples</i>	Serology of the patient and titer	Both PCR methods corresponding positively <sup>1</sup>	Only in-house PCR positive <sup>2</sup>
Negative			
79	<1:40	1	-
9	1:40	2	-
5	1:80	0	-
Indeterminable			
12	1:160	1	-
15	1:320	7	-
Positive			
11	1:640	7	1
12	1:1280	6	2
2	1:2560	1	-
11	1:5120	5	1
6	1:10240	6	-
3	1:20480	3	-
7	1:40960	4	2
<b>172</b>		<b>43</b>	<b>6<sup>3</sup></b>

<sup>1</sup>: 123 of the examined samples were in negative agreement through both PCR methods.

<sup>2</sup>: With none of the examined samples was only the Venor<sup>®</sup>*Mp* positive and the internal PCR negative.

<sup>3</sup>: 1 BAL and 5 GF, 2 of these 6 samples were obtained after 10 days treatment (see Table 3.)

With 35 patients (38 samples) corresponding to a titer greater than 1:320 and with both PCR procedures positive for the pathogen, one deduced that a mycoplasma infection was present. The clinical and laboratory diagnostic findings of these patients were compatible with mycoplasma pneumonia. The samples for the PCR were obtained from these patients not later than 2 weeks after the beginning of the illness, or not later than 5 days after beginning of treatment. From Tab. 1, one can infer that in some cases with borderline or positive serology, the pathogen could not be detected by means of PCR (see comments Tab. 2, Groups 2, 3 and 4). Three samples were positive in the PCR while negative in the serology. The results of 26 samples (24 patients), with which discrepancies occurred between one of the three methods, are more precisely represented in Tab. 2 concerning the clinical data and the point in time of the sample production in relation to the beginning of the illness

(process samples obtained directly after treatment were excluded; see below explanations to Table 3): the samples are divided in four groups and the evaluation of the results are described in the right column.

**Tab. 2:** Discrepancy of results with the comparison of the in-house PCR, the Venor<sup>®</sup>Mp PCR procedure and serology.

Patient		PCR			Serology (PIA)		Group and Comment <sup>2</sup>
No.	Age	Material	In-house <sup>1</sup>	Venor <sup>®</sup> Mp	Acute serum	Follow-up	
<b>12</b>	28	BAL	+	+	<b>1:40</b>	1:12800	<b>1.</b> Negative serology or positive PCR after titer increase, clinically acute <i>Mycoplasma pneumoniae</i> . The PCR is accurately positive in the acute phase of the infection.
<b>65</b>	33	Sp	+	++	<b>1:160</b>	1:1280	
<b>139</b>	36	GF	+	+	<b>1:40</b>	1:40	
<b>151</b>	36	GF	+	+	<b>Neg</b>	-	
<b>25</b>	31	GF	Neg	Neg	1:1280	1:640	<b>2.</b> Negative PCR and increased titers. The samples for the PCR were obtained later than 2 weeks after the beginning of the illness or after more than 5 days following commencement of treatment. With 7 patients no pneumonia was present (bronchitis) and no antibiotic was administered. The PCR is accurately negative to a high degree of probability when specimens are obtained late or after treatment.
<b>35</b>	88	GF	Neg	Neg	1:1280	-	
<b>60</b>	1	GF	Neg	Neg	1:5120	-	
<b>60</b>	1	GF	Neg	Neg	1:5120	-	
<b>69</b>	22	GF	Neg	Neg	1:320	1:80	
<b>101</b>	26	GF	Neg	Neg	1:640	-	
<b>102</b>	15	GF	Neg	Neg	1:320	-	
<b>108</b>	12	GF	Neg	Neg	1:320	-	
<b>110</b>	41	GF	Neg	Neg	1:640	1:320	
<b>116</b>	19	GF	Neg	Neg	1:320	-	
<b>117</b>	46	GF	Neg	Neg	1:2560	1:640	
<b>118</b>	55	GF	Neg	Neg	1:320	1:320	
<b>131</b>	24	GF	Neg	Neg	1:320	-	
<b>31</b>	15	<b>GF</b>	(+)	<b>Neg</b>	1:1280	-	
<b>36</b>	15	<b>GF</b>	(+)	<b>Neg</b>	1:640	1:320	
<b>81</b> <sup>3</sup>	35	<b>BAL</b>	(+)	<b>Neg</b>	1:1280	-	
<b>122</b>	20	<b>GF</b>	(+)	<b>Neg</b>	1:5120	-	
<b>81</b> <sup>3</sup>	35	<b>GF</b>	<b>Neg</b>	<b>Neg</b>	1:1280	-	<b>4.</b> Both PCR procedures are negative, increased titers. The PCR samples were obtained in the acute phase of a clinically determined mycoplasma infection and before commencement of treatment. Both PCR procedures are false negative.
<b>98</b> <sup>3</sup>	46	<b>GF</b>	<b>Neg</b>	<b>Neg</b>	1:5120	-	
<b>132</b>	42	<b>GF</b>	<b>Neg</b>	<b>Neg</b>	1:320	-	
<b>134</b>	36	<b>GF</b>	<b>Neg</b>	<b>Neg</b>	1:320	-	
<b>136</b>	53	<b>GF</b>	<b>Neg</b>	<b>Neg</b>	1:640	-	

<sup>1</sup>: (+): only the PCR with 5 µl DNA addition was positive. In contrast, the PCR with 0.5 µl DNA was negative.<sup>2</sup>: False negative results of the PCR or the serology are indicated in bold print.<sup>3</sup>: With this patient the sputum (No. 81) and the BAL (No. 98) were positive with both PCR procedures.

Together with the above-mentioned 35 patients and the 10 patients from Tab. 2 (Groups 1, 3 and 4 excluding Patients No. 81 and 98, which were already accounted for with the 35 patients) 43 patients had an active mycoplasma infection, which was not older than 2 weeks at the point in time of the sample collection and treated for not longer than 5 days (51 samples). Five of these 51 samples were not detected as positive both by the in-house and the Venor<sup>®</sup>*Mp* PCR (see Tab. 2, Group 4). Furthermore, 4 samples with very weak positive results from the in-house PCR could not be detected with the Venor<sup>®</sup>*Mp* procedure. Thus 46 of the 51 samples (90%) were positive by means of in-house PCR and 42 samples (82%) were positive by means of the Venor<sup>®</sup>*Mp* PCR. The in-house PCR was positive with at least one of the 40 samples from the 43 patients (93%), and the Venor<sup>®</sup>*Mp* PCR was positive with 37 patients (86%). It should be noted that in the comparison of a hybridization detection method and an electrophoretic method, a higher sensitivity within the hybridization should be expected. Yet, the Venor<sup>®</sup>*Mp* PCR displays similar levels of sensitivity as the in-house method. This may be due to the fact that Venor<sup>®</sup>*Mp* uses a repetitive gene for amplification, and that the amplification efficiency was highly optimized. Tab. 2 further shows that from the investigation of acute serum, the serology produced a false negative result in 3 cases at the point in time of the diagnostic efforts, and gave an ambiguous result in one patient sample. Additionally, we can state that none of the positive PCR results were false positive (specificity 100%) because all PCR positive patients had – with high probability on grounds of serological and clinical signs –

mycoplasma pneumonia, as discussed above (with 2 patients, No. 139 and 151, serology was negative and a titer increase could be verified).

A GF could be obtained at the same time from 7 patients with confirmed mycoplasma by means of PCR using sample materials from the deep respiratory system (6 BAL, 1 throat swab and 1 sputum). 5 of these patients were also positive in the GF, and 2 patients were negative in the obtained GF (Tab. 2, No. 81 and 98).

**Tab. 3** lists the samples depicting the course of the illness following treatment (average of 12.9 days) from 6 patients with an infection confirmed by both positive PCR and positive serology. The pathogen concentration noticeably drops very rapidly under the PCR detection limit after the commencement of treatment. Although only a few patients here examined, the results of Tab. 3 indicate that the PCR can be regarded as a specific method for the early diagnostics of an acute infection. All BAL of the control group was likewise negative. The concern that the specificity of the PCR may be influenced by false

**Tab. 3:** Influence of the treatment on the PCR results (process samples)

Patient No.	Age	PCR			PIA	
		Days after treatment began	Material	In-house		Venor <sup>®</sup> <i>Mp</i>
14	46		BAL	+	+	1:10240
		20	BAL	+	(+)	1:20480
26	31		BAL	+	+	1:10240
		20	GF	Neg	Neg	1:5120
65	33		Sp	+	++	1:160
		7	GF	Neg	Neg	1:1280
98	46		BAL	+	++	1:5120
		10	GF	Neg	Neg	1:5120
105	33		BAL	+	+	1:40960
		10	GF	(+)	Neg	1:40960
128	64		GF	+	++	1:5120
		10	GF	(+)	Neg	1:40960
		13	BAL	Neg	Neg	1:40960

positive results with healthy carriers is clearly weakened by the results presented here.

Patients with pneumonia usually display symptoms during a longer duration until hospitalized or receive treatment as out-patients. This is consistent with the fact that in the predominant number of samples specified in Tab. 2 (Group 2), no DNA of the pathogen was detectable despite high antibody titers of the patients. However, it cannot be definitively answered whether this was due to a late point in time of the sample collection, or by the absence of the pathogen in the upper respiratory tract, or by too small a sensitivity of the PCR. It is remarkable that GF was almost exclusively affected by discrepant results, while BAL, which constituted almost a third of the investigated specimens, revealed a false negative result in no case with the in-house PCR and in one case with Venor<sup>®</sup>Mp. This points into the direction that a higher sensitivity can be achieved with materials from the deep respiratory tract, at least with respect to pneumonia, and that the pathogen density is not always high enough in GF for definitive detection by PCR. Likewise it is very important to obtain the investigative sample as early as possible after the beginning of the disease. One can conclude the PCR could have a substantially higher sensitivity than serology with out-patients.

### ***Evaluation***

This investigation shows that the diagnostic kit Venor<sup>®</sup>Mp is a sensitive and very specific procedure for detection of acute mycoplasma infections. The method offers in particular the advantage of speed for patients still lacking antibody formation or those with low antibody titers at the beginning of the illness. Contrary to indirect methods for pathogen detection, Venor<sup>®</sup>Mp can be used effectively to confirm suspected infection through the high specificity of the PCR method. The illness however cannot be fully excluded by a given negative result. The internal control of the kit offers, in addition to the advantage of the internal control of each individual amplification, the advantage of a semi-quantitative determination of the pathogen. If necessary, this could also be used for therapy control.

This latter fact and the information which resulted from this investigation concerning the sample preparation, should be further specified in the Instruction Manual of this diagnostic kit. It should also be noted that the method lacks a sequence analysis procedure (e.g. hybridizing with a probe), and particular attention must therefore be given to the estimation of the correct size of the 207 bp amplicon (direct proximity to the amplicon of the internal standard).

The product Venor<sup>®</sup>Mp can be certified for very good suitability and practicability for the direct detection of *Mycoplasma pneumoniae* in materials of the upper and lower respiratory tract.

Berlin, March 11, 2002

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