Methodological Approaches to Deciphering the Etiological Structure of Non-Nosocomial Pneumonia

L. V. Artemova*,, V. A. Beskhlebnaya*, L. G. Ezhova*, M. Yu. Kirillov**, E. G. Kravtsov*, and M. V. Dalin***

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 136, No. 10, pp. 475-478, October, 2003
Original article submitted June 21, 2003

Comparative analysis of the efficiency of PCR and microbiological methods for deciphering of the etiological structure of non-nosocomial pneumonias demonstrated the diagnostic significance of detecting *Streptococcus pneumoniae* DNA in the blood, but not sputum. Mechanisms of penetration of *S. pneumoniae* into the blood are discussed.

Key Words: *Streptococcus pneumoniae; non-nosocomial pneumonia; polymerase chain reaction*

Pneumonia remains one of the most prevalent diseases in the XXI century. Mortality from non-nosocomial pneumonia reaches 5% and as high as 20% among patients whose condition required hospitalization. According to bacteriological studies, *Streptococcus pneumoniae* is responsible for up to 30% of non-nosocomial (outpatient) pneumonias (pneumonias other than nosocomial) [1]; *Mycoplasma pneumoniae* are detected in 20-30% cases, *Haemophilus sp.* in 10-15%, and *Chlamydia pneumoniae* in 8% cases [2]. In 20-30% cases the etiology of pneumonia is undetectable and drug therapy is prescribed empirically.

The main method used for identification of the agent of pneumonia was microbiological analysis of the sputum. The threshold sensitivity of this method is no higher than 10^3 CFU/ml. For microorganisms highly sensitive to conditions of culturing (*S. pneumoniae*, *H. influenzae*, *etc.*) negative results of bacteriological study are not always true. Sputum inoculations in pneumonia caused by *H. influenzae* can be false negative in 34-47% cases [8].

Highly sensitive specific molecular genetic methods for identification of pneumonia etiology have been developed recently; this prompted us to evaluate the efficiencies of the new methods for identification of etiological agents of non-nosocomial pneumonias.

MATERIALS AND METHODS

Forty-five patients (30 men and 15 women) hospitalized with non-nosocomial pneumonia in Clinical hospital No. 64 (Moscow) were observed. The patients' ages varied from 16 to 86 years. The criteria for selection of patients were emergence of the disease anywhere but not in hospital and the presence of clinical and X-ray signs of pneumonia. Sputum and blood specimens for microbiological analysis and PCR were collected on admission to hospital. Microbiological analysis of the sputum was carried out in 60% patients, its molecular biological testing in PCR in 93%. If *S. pneumoniae* DNA was detected in the sputum, its presence in the blood was tested.

Control group consisted of 20 men and 32 women aged 22-83 years (healthy workers of the same hospital and patients hospitalized for cardiovascular diseases without respiratory symptoms). Nasopharyngeal washings and blood samples were collected from all controls.

DNA was isolated from whole blood by modified method described by J. K. Kulski and T. Price [6], from the sputum and nasopharyngeal washings by modified method presented by R. Boom *et al.* [3], but in

*Russian University of Peoples' Friendship, Moscow; **LAGIS Firm, Moscow

0007-4888/03/1364-0423$25.00

the former case the biological substrate was pretreated with N-acetyl-L-cysteine. The resultant DNA preparations were stored at -20°C before PCR.

Four test-systems were used for PCR. *S. pneumoniae* DNA was detected by Spn test (with insertion element (IS 1381) serving as the target for primer pair), *H. influenzae* DNA in Hibn test (with hpd gene coding for immunoglobulin d-binding protein as target for primer pair), *M. pneumoniae* DNA in Mpn test (with p1 gene coding for adhesin as the primer pair target), and *M. tuberculosis* DNA was detected using Mtu test system (with insertion element serving as the target for primer pair). All these test systems were developed and tested at Laboratory for Gene Engineering Systems (LAGIS). The choice of targets for primer pairs was based on published sequences of *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, and *M. tuberculosis* [11,12].

The reaction mixture for PCR (final volume 25 μl) contained 10 mM Tris HCl (pH 8.7), 50 mM KCl, 2 mM $MgCl_2$, 0.25 mM each dNTP, 10 pmol of each primer, 1 U Taq polymerase, and 5 μl DNA preparation. Amplification was carried out in a Tertsik amplifier (DNK Tekhnologiya Firm) using traditional 3-staged protocol (denaturing, annealing, synthesis). An aliquot of the resultant reaction mixture was analyzed in 1.5% agarose gel stained with ethidium bromide. Amplified products were photographed in UV light.

Isolation and identification of the agents from the sputum were carried out by standard microbiological methods on an ATB-expression device (Bio-Merie).

RESULTS

X-ray findings confirmed pneumonia in 41 patients, pulmonary tuberculosis was detected in 3 patients, and in one patient pneumonia was not confirmed by X-ray findings. The right lower lobe was involved in 33 patients (80%), the left lower lobe in 5 (11%), two lower lobes in 1 (2%), right upper and lower lobes in 1 (2%), and the middle and lower lobes in 1 (2%) patient. Bacteriological analysis of the sputum was carried out in 27 patients. *S. pneumoniae* as monoinfection was detected in 8 cases and in combination with other agents in 5 cases. Other microorganisms were isolated from the sputum of 20 (74%) patients.

Sputum was analyzed by PCR in 41 patients. *Streptococcus pneumoniae* DNA was detected in 33 samples, in 23 of these (70%) pneumococcal DNA was detected in associations with other agents, such as *H. influenzae* (20 cases), *M. pneumoniae* (4 cases), and *M. tuberculosis* (3 cases). DNA of other microorganisms was detected in 28 patients.

Blood was tested for *S. pneumoniae* by PCR in 38 patients (84% of the total sampling). DNA of *S. pneumoniae* was detected as a monoinfection in 10 (26%) patients, in 9 of these patients positive signal for *S. pneumoniae* DNA was found in both sputum and blood.

We hypothesized that the appearance of *S. pneumoniae* DNA in the blood is due to high level of contamination of the sputum. In order to verify this hypothesis, we investigated all cases with isolation of *S. pneumoniae* culture from the sputum in a titer of 10^5 CFU/ml, confirmed by a positive PCR signal in the sputum, except one case in which no PCR analysis of the sputum was carried out. Seven blood samples from these 8 patients were tested for *S. pneumoniae* DNA (Table 1). The signal was positive in only 1 sample; hence, there is no speaking about correlation between the detection of *S. pneumoniae* by microbiological methods in the sputum and PCR identification of the agent in the blood. Therefore, the level of pneumococcal contamination of the sputum $>10^3$ CFU/ml determined by microbiological methods does not guarantee the appearance of the agent in the blood.

We tried to detect factors essential for the development of invasive process in pneumonia. We considered that the presence of DNA in the blood indicated an invasive process. Groups of patients with invasive process confirmed by PCR and without this process were compared by the following factors: sex, age, harmful habits, chronic diseases, and presence of DNA of other agents in the sputum. None of these

TABLE 1. Detection of *S. pneumoniae* by Microbiological Methods and Identification of the Agent in the Blood by PCR

Material and method of investigation	Patient No.							
	2	21	22	31	32	33	35	45
Sputum (microbiological method)	+	+	+	+	+	+	+	+
Sputum (PCR)	+	+	+	+	+	+	+	Not detected
Blood (PCR)	+	—	—	Not detected	—	—	—	—

Note. «+»: presence of *S. pneumoniae* DNA (microbiological inoculation); «—» absence of *S. pneumoniae* DNA.

TABLE 2. Effects of Various Factors on the Development of Invasive Process Caused by *S. pneumoniae*

Parameter	Presence of *S. pneumoniae* DNA in blood	No *S. pneumoniae* DNA in blood
Cases, total	10	28
Sex (m/f)	8/2	21/7
Mean age	46.3 (22-72)	42.8 (19-74)
Tobacco smoking	6	12
Alcoholism	3	2
Presence of DNA of other agents in sputum**	7 (70%)	20 (71%)
M. tuberculosis	2	1
H. influenzae	6	15
M. pneumoniae	1	4
Chronic respiratory diseases	6	8
Subjects healthy before disease	2 (20%)*	13 (46%)*

Note. *Data are significant ($p<0.05$). **DNA of two agents could be present in the sputum simultaneously.

factors (except health status before disease) was essential for the incidence of invasive process (Table 2). The rate of detection of *S. pneumoniae* DNA was markedly lower only in the patients without chronic diseases ($p<0.05$).

We hypothesized that other agents present in the sputum can modulate the development of invasive process in pneumonia caused by *S. pneumoniae* and found that neither *M. pneumoniae,* nor *H. influenzae* stimulated the development of invasive process (Table 3). *S. pneumoniae* DNA was more often present in the blood of patients with tuberculosis, but these differences were statistically insignificant, which can be explained by small size of the sampling.

S. pneumoniae DNA was detected in 39 (75%) samples (nasopharyngeal washings) from controls, mixed infection pneumococcus+*H. influenzae* was detected in 28 (54%) cases and that with *H. influenzae* and other agents in 38 (73%) cases.

Testing of 52 blood samples from controls by PCR showed a positive signal for *S. pneumoniae* DNA in only 1 sample (2.0±1.8%). This patient (female, aged 57 years) was hospitalized for suspected coronary disease and cardiac fibrillation. The diagnosis of myocardial infarction was made clinically, no symptoms of inflammatory processes in the lungs were detected.

TABLE 3. Rate of PCR Detection of *S. pneumoniae* DNA in the Blood in Mixed Infection (%)

Microorganisms whose DNA was detected in sputum	Signal for *S. pneumoniae* DNA in blood	
	positive	negative
M. pneumoniae	10	15
H. influenzae	60	46
M. pneumoniae, *H. influenzae*	20	14
M. tuberculosis	20	3

Our study included evaluation of the place of PCR in the algorithm of examination of patients with non-nosocomial pneumonia. The results indicate that the use of PCR notably extends the potentiality of detecting *S. pneumoniae* in biological substrates (sputum) in comparison with the standard microbiological methods (80 and 50%, respectively, $p<0.01$). However, the detection of *S. pneumoniae* DNA in the sputum cannot be regarded as a reliable diagnostic criterion [9], as, like *H. influenzae*, *S. pneumoniae* is a commensal bacteria in humans and their presence in the sputum can attests to carriership or disease development with equal certainty [4,10]. This can be well illustrated by comparison of the rates of *S. pneumoniae* detection by PCR in the sputum of patients with non-nosocomial pneumonias and in nasopharyngeal washings from normal subjects (80 and 75%, respectively). Coincidence of these detection rates indicates high sensitivity of PCR method, which in this situation is a shortcoming, not allowing differentiation between disease and carriership. Therefore testing of normally sterile media of the organism can be more perspective [7]. G. L. Mandell *et al.* [8] indicate that the efficiency of the agent isolation from the blood in non-nosocomial pneumonia can vary from 1 to 16% [8]. In our study use of PCR led to detection of *S. pneumoniae* DNA in 26% of blood samples from patients with non-nosocomial pneumonia. *S. pneumoniae* DNA was detected in only one patient (2%) from the control group. This confirms the data of S. H. Gillespie *et al.* who noted that *S. pneumoniae* can sometimes appear in the blood of patients without any clinical symptoms [5].

Hence, the probability of etiological significance of *S. pneumoniae* in disease development in case the species marker is detected in the blood is not absolute and amounts to 98.0±1.8%.

The mechanisms of appearance of *S. pneumoniae* in the blood remain unclear. The factors we studied seem to be of no key importance. It was found that only premorbid state of the organism could to a certain measure influence *S. pneumoniae* invasion. Tuberculous infection is an important factor: if pneumococcal infection developed in the presence of tuberculosis, the probability of bacteremia was higher. Other studies evaluating the status of *S. pneumoniae* population and the effect of various pathogenicity factors on generalization of the infectionare required.

REFERENCES

1. *Antibacterial Therapy. Practical Handbook*, Eds. L. S. Strachunskii *et al.* [in Russian], Moscow (2000).
2. S. V. Sidorenko, A. I. Sinopal'nikov, and S. V. Yakovlev, *Antibacterial Therapy of Pneumonia in Adults (Recommendations for Practitioners)* [in Russian], Moscow (2000).
3. R. Boom, C. J. Sol, M. M. Salimans, C. L. Jansen, *et al.*, *J. Clin. Microbiol.*, **28**, No. 3, 495-503 (1990).
4. J. R. Catterall, *Thorax*, **54**, No. 10, 929-937 (1999).
5. S. H. Gillespie and I. Balakrisnan, *J. Med. Microbiol.*, **49**, 1057-1067 (2000).
6. J. K. Kulski and T. Price, *J. Clin. Microbiol.*, **34**, No. 8, 1985-1991 (1996).
7. M. L. Lorente, M. Falguera, A. Nogues, *et al.*, *Thorax*, **55**, No. 2, 133-137 (2000).
8. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*, Eds. G. L. Mandell, *et al.*, 5th ed., Philadelphia (2000).
9. D. R. Murdoch, T. P. Anderson, Beynon K. A., *et al.*, *J. Clin. Microbiol.*, **41**, No. 1, 63-66 (2003).
10. S. Obaro and R. Adegbola, *J. Med. Microbiol.*, **51**, No. 2, 98-104 (2002).
11. A. R. Sanchez-Beato, E. Garcia, R. Lopez, and J. L. Garcia, *J. Bacteriol.*, **79**, No. 7, 2459-2463 (1997).
12. H. Tettelin, K. E. Nelson, and I. T. Paulsen, *Science*, **293**, No. 5529, 498-505 (2001).