MOLECULAR EPIDEMIOLOGICAL MONITORING OF *STREPTOCOCCUS PNEUMONIAE* STRAINS ISOLATED IN ELDERLY PATIENTS WITH COMMON-ACQUIRED PNEUMONIAES

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The problem of microbiological monitoring and epidemiological surveillance over *S. pneumoniae* strains as one of ubiquitous agents causing common-acquired pneumoniaes and other respiratory infections of different severity is still urgent.

Multilocus sequence typing is a promising technique of molecular epidemiological monitoring enabling to identify epidemically dangerous clones of ubiquitous agents.

The aim of the investigation was to assess the spread of epidemically significant *Streptococcus pneumoniae* strains by multi-locus sequence typing in elderly patients with common-acquired pneumoniae, bronchites, and agents, and identify a dominant genotype.

Materials and Methods. We studied 14 strains isolated in patients with common-acquired pneumoniaes (among them 7 — multi-resistant), 8 strains — in patients with chronic obstructive pulmonary disease, 4 strains — in the carriers. Multilocus sequence typing was carried out in accordance with M.C. Enright and B.G. Spratt technique (1998).

Results. All strains isolated in three populations are relative isolates of *Streptococcus pneumoniae*, and the majority of them (18 of 26) have a unique genotype determining the presence of one sequence type for each strain. From 14 strains isolated in elderly patients with common-acquired pneumoniae, 6 belonged to *Taiwan 19F-14*. Among the strains isolated in the carriers, the strain identical to *R6* strain prevailed. No genotype was found to prevail among the strains isolated in patients with chronic obstructive pulmonary disease.

Conclusion. Multi-locus sequence typing enables to identify new genotypes of *S. pneumoniae* and prognosticate the appearance of epidemically dangerous strains with new properties.

Key words: multi-locus sequence typing; pneumococcal pneumoniaes; community-acquired pneumoniaes; *S. pneumoniae*.

Community-acquired pneumonia continues to be an urgent problem of the modern medicine among the aged population [1–4]. Prevalence of the disease in Moscow amounts to 17.4‰, and in the USA — to 20–40‰ [4]. Morbidity of community-acquired pneumonia in elderly people is 2 times higher than in young individuals, the rate of hospitalization increasing 10-fold with age [5, 6]. Lethality in pneumonia among the patients over 60 is 10 times higher than in other age groups, reaching 10–15% in pneumococcal pneumonias [4].

Taking into consideration the fact, that *S. pneumoniae* is one of the main causative agents of community-acquired pneumonias in different age groups, and carriage of this agent is extremely common [4–7], conducting epidemiological studies of pneumococcal pneumonias in elderly people and assessing their significance is of great interest.

The most important problem in the study of pneumococcal pneumonias in elderly is organization of control over the spread of antibiotic-resistant isolates [7–9], as well as molecular epidemiological monitoring of the identified isolates. At present, this kind of monitoring is carried on by means of genotyping method, which allows obtaining standard information on the spread of this or that isolate. This information about the character of the revealed *S. pneumoniae* strains makes it possible to plan prophylactic measures in relation to pneumococcal infection.

One of the methods designed for molecular epidemiological monitoring is multilocus sequence typing

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(MLST), based on the study of fragment variability, enabling creation of individual allelic profile of each strain and description of its epidemic significance [10–13].

Application of MLST for investigation of pneumococcus, possessing high capability to natural transformation, enables determination of separate clonal groups and revealing their epidemic significance [9, 12]. It should be taken into consideration that sequence types, differentiated by MLST, may have various serotypes (phenotypic variability), caused by microevolution processes going on in microbiocenosis, *S. pneumoniae* being a part of it.

The aim of the investigation is to evaluate the character of spreading epidemically significant *Streptococcus pneumoniae* strains in elderly patients with community-acquired pneumonia, bronchitis and in carriers, and identify a dominant genotype using multilocus sequence typing technique.

Materials and Methods. Since one of the most important epidemic characteristics is resistance to antibacterial chemopreparations, including multiresistance, equal number of multiresistant pneumococcus strains and strains sensitive to antibiotics were taken for investigations: group 1 (n=14) — strains isolated from the elderly patients with community-acquired pneumonia (7 — multiresistant and 7 — sensitive to antibiotics); group 2 (n=8) — strains isolated from patients with chronic obstructive pulmonary disease (COPD) (4 multiresistant and 4 sensitive strains); group 3 (n=4) — strains from aged carriers. Multiresistance meant resistance of pneumococcal strains to penicillin and erythromycin.

MLST was performed according M.C. Enright and B.G. Spratt method [11]. Primers used in the study may be presented in the following way:

ddl-up, 5′ TGCC/TCAAGTTCCTTATGTGG and *ddl*-dn, 5′ CACTGGGTG/ AAAACCA/ TGGCAT;

gdh-up, 5′ ATGGACAAACCAGCNAGC/ TTT and *gdh*-dn, 5′ GCTTGAGGTCCCATG/ACTNCC;

gki-up, 5′ GGCATTGGAATGGGATCACC and *gki*-dn, 5′ TCTCCCGCAGCTGACAC;

recP-up, 5′ GCCAACTCAGGTCATCCAGG and *rec*P-dn, 5′ TGCAACCGTAGCATTGTAAC;

spi-up, 5′ TTATTCCTCCTGATTCTGTC and a*spi*-dn, 5′ GTGATTGGCCAGAAGCGGAA;

xpt-up, 5′ TTATTAGAAGAGCGCATCCT and *xpt*-dn, 5′ AGATCTGCCTCCTTAAATAC.

We used this method in order to compare control data with those obtained in the course of MLST, since serotyping technique is commonly accepted in the world practice of epidemiological surveillance of pneumococcal infection. All strains were serotyped according to standard technique [12] using Pneumotest panel (Statens Seruminstitut, Copenhagen, Denmark), serotypes not included in the panel were identified in the Institute" Statens Seruminstitut" (Denmark) [12].

In the process of serotyping single colonies of 24-h culture were taken: a drop (25 μl) was placed in the corresponding section of the panel. A saline solution in the equal volume was used as a control. Then, a small quantity of the culture obtained was put in the drop of antiserum by means of a toothpick or microbiologic loop, mixed carefully, distributing it uniformly throughout the whole volume. Further, the panel in which reaction was made was swung slightly till the appearance of agglutination, which occurred in case of reaction of bacterial antigens with specific antiserum during 1 min at the room temperature, and was studied by the phase contrast microscopy technique according to the manufacturer's recommendations (Statens Serum Institute, Denmark).

The following assay reagents were used for serotyping: omni-serum identifying pneumococcus capsule; group-specific antiserum (groups A and I, P and T); type serum for identification of separate serotypes; group serum reacting with all serotypes of pneumococci within a group; a serum for intrageneric typing [12].

Results and Discussion. To study the possibility of forming hospital multiresistant strain of *S. pneumoniae*, originating from a single clone, we conducted MLST of the pneumococcus strain population isolated from various biotypes and possessing various antibacterial resistance. Population of strains isolated from patients with community-acquired pneumonia (n=14 strains: 7 strains resistant to penicillin and erythromycin, and 7 strains sensitive to antibacterial drugs), strains isolated from patients with COPD (n=8 strains: 4 of them resistant to penicillin and erythromycin, and 4 — sensitive to antibacterial drugs) and strains isolated from the carriers (2 strains sensitive to antibacterial drugs and 2 strains not sensitive to antibacterial drugs) were compared. A total of 26 strains were involved in the study.

The analysis of the populations studied by MLST method showed that all strains isolated from different groups of patients and carriers are related isolates of *S. pneumoniae* species, the majority of which (18 of 26) possess a unique genotype, determining the presence of one sequence type for each strain. Besides, 6 cluster groups may be formed from the variety of the examined strains. Those groups contained the strains isolated from various patients, had different molecular epidemiological characteristics (serotype, resistance to antibacterial preparations), and were genetically closely related.

The results of MLST for the strains separated from the patients with community-acquired pneumonia (Fig. 1) led to the conclusion that multiresistant strains were formed by horizontal transfer of the genetic material in community-acquired pneumonias: it was confirmed by the presence of two clonal complexes, in one of which 1 multiresistant strain was present (clonal complex, containing ST-1.1, ST-4, ST-13), and 2 complexes in the other (ST-2, ST-8.1, ST-11.1). All this shows the possibility of forming multiresistance in pneumococcal strains in patients with community-acquired pneumonia.

The analysis of spreading genetically closely related strains shows that of the four multiresistant pneumococcal isolates separated by us from the carriers two are representatives of a single clonal complex. It suggests, that processes of intragenome strain reorganization going on in carriers, may also lead to the formation of hospital isolate, and, consequently, require prophylactic measures.

Having compared the findings of MLST for all multiresistant strains (Fig. 2) we came to the conclusion, that there was a great probability of horizontal transfer in multiresistant strains separated in aged individuals with COPD, as well as in the strains separated from the patients with community-acquired pneumonia (See the Table).

Thus, as the result of the analysis of genetic diversity of *S. pneumoniae* strains among the studied 26 strains sensitive and not sensitive (i.e. moderately resistant and resistant) to penicillin and erythromycin, 22 various sequence types and 4 clonal complexes were distinguished, 2 complexes being presented by more than one isolate.

Notably, that of 14 strains isolated from elderly individuals with community-acquired pneumonia, 6 were referred to *Taiwan 19F-14* profile, of them 4 possessed similar profile of resistance: were resistant/moderately resistant to penicillin, erythromycin and clindamycin. Genotypic characteristics of the analyzed strains also demonstrated a high degree of sample uniformity: all isolates had resistance determinants to macrolide antibiotics — genes *mefE* and *ermB* (which is in line with phenotypic data). The main characteristics of *S. pneumoniae* strain, genotype *Taiwan 19F-14*, is its multiresistance primarily in relation to beta-lactates and macrolides, that is independent of the geographic area of isolation of these microorganisms. This sequence type prevails mainly in South Korea, Vietnam and Hong Kong.

Among the strains caused COPD in elderly MLST method helped identify 2 different sequence types, each of which was presented by 3 isolates (See Fig. 2).

Though the sample was not large, the strains separated from the carriers turned out to be phenotypically not uniform: 2 srains were moderately resistant to

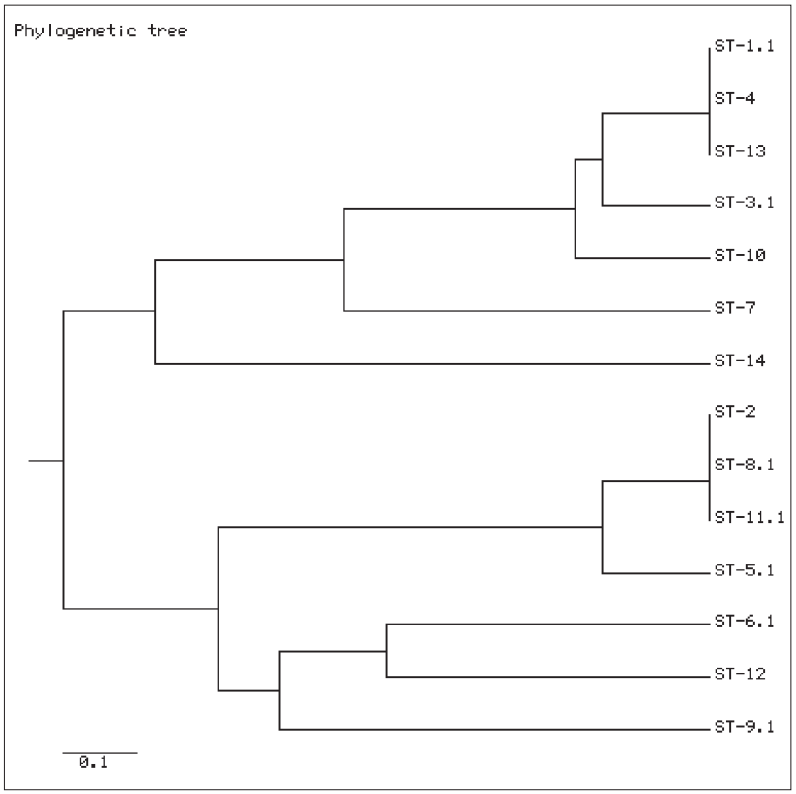

Fig. 1. Findings of multilocus sequence typing of *S. pneumoniae* strains isolated from patients with community-acquired pneumonia (strains with expansion 1 are multiresistant, without expansion — sensitive to antibiotics), n=14 strains

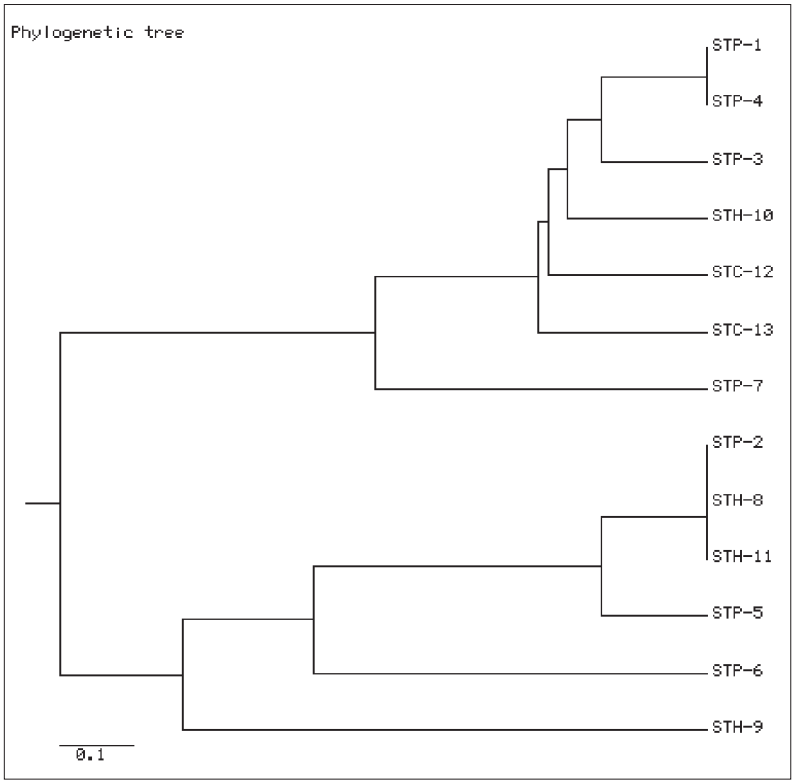

Fig. 2. Findings of multilocus sequence typing of multiresistant pneumococcal strains isolated in all groups of patients (n=13 strains)

Molecular epidemiological characteristics of *S. pneumoniae* strains multiresistant to antibiotics according to MLST data

Serotipe	Housekeeping genes								Resistance			Minimally inhibiting concentration, mg/ml
	aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*	MLST-type	to penicillin	to erythromycin	to clindamycin	
Strains isolated in group 1 (community-acquired pneumonia, n=14)												
19F	7	11	10	1	6	8	14	162	S	R	R	**1.4**
9V	7	11	10	1	6	8	14	158	S	R	R	0.2
19F	1	5	4	12	5	3	8	423	S	R	R	0.5
6B	20	28	1	1	15	14	14	315	S	MR	R	0.2
19F	4	4	2	4	4	1	1	81	S	R	R	0.2
23F	7	5	1	1	13	31	14	440	S	R	MR	**0.2**
14	1	5	4	5	5	1	8	9	S	MR	R	0.3
N/t	1	5	4	5	5	1	8	9	R	MR	R	0.5
14	7	5	1	8	14	11	14	124	R	MR	R	0.2
18C	7	2	1	1	10	1	21	113	R	MR	R	0.2
6A	2	7	4	10	10	1	27	176	R	MR	R	1
15B	8	13	14	4	17	4	14	95	MR	MR	R	0.5
15C	8	13	14	4	17	4	14	201	R	MR	R	0.15
N/t	8	13	14	4	17	4	14	199	R	R	R	1
Strains isolated in group 2 (COPD, n=8)												
9V	7	11	10	1	6	8	14	162	MR	MR	S	0.2
3	7	15	2	10	6	1	22	180	MR	MR	R	0.2
19F	1	8	9	1	6	4	6	311	MR	R	R	0.8
6A	2	7	4	10	10	1	27	65	R	R	R	1
19F	1	8	4	1	1	4	6	36	R	MR	R	2
23F	1	8	9	1	6	4	6	311	MR	MR	R	2
18C	7	2	1	1	10	1	21	113	R	R	R	1
23F	7	13	8	1	10	6	37	272	MR	MR	S	0.5
Strains from carriers (n=4)												
19F	1	8	10	4	9	1	3	341	S	MR	R	1
19A	12	14	11	2	6	17	22	785	MR	MR	R	1
6B	8	37	9	29	2	12	53	344	MR	MR	R	0.02
6A	2	13	9	1	6	19	14	490	MR	MR	S	0.04

H e r e : N/t — nontyped; R — resistant; MR — moderately resistant; S — sensitive.

penicillin and 2 strains — sensitive to it. At the same time, all 4 strains were not sensitive to erythromycin. And 2 strains belonged to sequence type identical to *R6* strain, which is avirulent and may be considered as opportunistic microflora.

Conclusion. Method of multilocus sequence typing must be considered to be the most accurate technique for differentiating streptococci and identifying *S. pneumoniae*, and the most important tool of micrbiological monitoring of the strains of this microorganism, which makes it possible to perform typing and predict appearance of strains with new properties, which are likely to become hospital isolates and demand absolutely new activities in relation to epidemiological surveillance.

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