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Research article

IMMUNE RESPONSE STATUS AND DEVELOPMENT OF *STREPTOCOCCUS PNEUMONIAE* CARRIAGE AS HEALTH RISK FACTORS FOR WORKERS ENGAGED IN COKE PRODUCTION AND BASIC OXYGEN STEELMAKING

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Occupational hazards affect immunological reactivity and increase the risk of infection with respiratory pathogens.

The purpose of our work was to study the mechanisms and causes of developing carriage of streptococci possessing genetic determinants of resistance to antibiotics as health risk factors for workers engaged in coke production and basic oxygen steelmaking.

We examined 136 workers of a ferrous metals industry and compared the prevalence of the carriage of Streptococcus pneumoniae, Streptococcus spp. *and macrolide resistance genes (Mef, ErmB) in those engaged in coke production and basic oxygen steelmaking. We also collated the results of immunological examination of the carriers and non-carriers of* S. pneumoniae. *The control cohort was formed of engineering and technical personnel.*

We established that, compared with the controls, Streptococcus pneumoniae *was a more frequent finding in the workers engaged in coke production and basic oxygen steelmaking ($p < 0.05$). The cycle threshold value of* Streptococcus spp. *in them was also statistically higher. The Mef gene was more abundant in the steelmakers, including those carrying* Streptococcus pneumoniae *($p < 0.05$), and a change in immunological reactivity was detected in the cases carrying* Streptococcus pneumoniae. *In those engaged in basic oxygen steelmaking, significant changes were detected at the cellular level expressed by a reduced absolute number of mature T-lymphocytes (CD3+), T-helpers (CD4+), and T-suppressor/cytotoxic cells (CD8+) ($p \leq 0.05$). At the humoral level, both the relative and absolute number of B-lymphocytes (CD19+) and the level of IgM were reduced, while that of IgG was, on the opposite, increased ($p \leq 0.05$). In the coke plant workers carrying* Streptococcus pneumoniae, *the level of IgG was significantly higher than the control and reference values.*

We conclude that Streptococcus pneumoniae *carriage in the steelmakers as a health risk factor was accompanied by a change in immunological reactivity and higher abundance of the Mef gene compared to the controls while in the coke production workers the related differences were detected only in the IgG level.*

Keywords: *immunity, ferrous metallurgy, industrial aerosols,* Streptococcus pneumoniae, *pneumococcal disease, antibiotic resistance genes, immune status, bactericidal activity.*

For several decades, much attention has been paid to studying the mechanisms of the development of diseases of the immune system in relation to occupational exposures. Alterations in the immune system can manifest as allergies, immunosuppression, or autoimmunity

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[1–3]. Through Toll-like receptors, airborne pollutants, just like microorganisms, activate innate cells, which produce cytokines, including chemotactic factors. Macrophages, dendritic cells, and neutrophils migrate to lymph nodes and tissues, where adaptive immune cells are activated with the following development of immunological response to chemical and biological antigens [4, 5]. *Streptococcus spp*. localize on the mucous membrane of respiratory organs, oral cavity, and intestines. Workers with the compromised immune system found to be asymptomatic carriers can spread antibiotic-resistant microorganisms. Multi-drug resistance to antibiotics is a global healthcare challenge. Anthropogenic "…chemical stresses, such as metals, hydrocarbons, organic compounds, etc., are the source of such resistance." [6] Many microorganisms are capable of modifying antibiotics and target sites and reducing intracellular accumulation, thus altering membrane permeability and increasing efflux activity [6–9]. At present, information about anthropogenic risk factors for the spread of antibiotic resistance is being accumulated, making it relevant to study effects of different industrial aerosols on the mechanisms and causes of developing carriage of streptococci that possess genetic determinants of antimicrobial resistance.

Our aim was to study the mechanisms and causes of developing carriage of streptococci that have genetic determinants of antibiotic resistance as a health risk factor for coke production workers and steelmakers.

Materials and methods. We conducted immunological testing of 136 ferrous industry workers, including 44 steelmakers working in the basic oxygen furnace shop and 40 coke production employees working in the coke shop and the coke tar shop. The control group consisted of 52 white-collar workers from the sales support, procurement and material support department, analytics and external logistics department. All the subjects were men matched by age and length of employment. The mean age of the steelmakers was 42.1 ± 1.06 years and their mean length of employment was 17.6 ± 1.1 years. The mean age of the coke production workers was 45.2 ± 1.13 years and their mean work experience was 18.7 ± 1.45 years. In the control group, these means were 43.6 ± 1.29 years and 18.1 ± 1.45 years, respectively.

In the basic oxygen furnace shop, industrial aerosols mainly contain inorganic compounds (iron, vanadium (V) oxide, and manganese), while organic compounds (aromatic hydrocarbon and aldehydes) prevail in those generated in coke production. Measured levels of the above contaminants were found to be 2.5–3 times higher than the maximum permissible ones. The working conditions of the subjects were classified as hazardous and corresponding to Class 3, subclasses 3.1 and 3.2. The response of the immune system to industrial metal-containing aerosols can be characterized by sensitization, immunosuppression or autoimmunization, usually associated with the risk of developing increased susceptibility to infectious antigens. And although the mechanism of damage to the immune system caused by organic pollutants is poorly studied, numerous experiments have demonstrated T cell mediated immunosuppression. Another potential risk factor for developing bacterial carriage is the damage to epithelium and inflammation of the mucous membrane of the upper respiratory tract induced by oxidative stress. The controls were not exposed to industrial aerosols.

DNA of *Streptococcus pneumoniae*, *Streptococcus spp*., and streptococcal antimicrobial resistance genes were detected by real-time polymerase chain reaction using the Rotor-Gene® Q cycler by QIAGEN, Germany, and the test system by Litekh LLC, Russia. We established the presence of the *Mef* gene, which is a determinant of resistance to macrolides (erythromycin, azithromycin, clarithromycin, and roxithromycin), and the *ErmB* gene, the emergence of which accounts for phenotypic resistance to clarithromycin, azithromycin, clindamycin, and erythromycin. For this purpose, pharyngeal swabs were taken using sterile swab sticks and then kept at -20 °C in transport medium until DNA extraction. The latter was carried out using the NA-sorbent kit by Litekh LLC, Russia, in accordance with the instructions for use. The cycle threshold (Ct) value showing the number of cycles required for the fluorescent signal to cross the threshold value was applied to compare the bacterial load. It allowed us to conclude on the relative amount of the gene in the sample: the lower the Ct value, the higher the amount of DNA of the gene under study.

Immunological testing included determination of the cellular component of the immune system by flow cytometry, immunoglobulins A, M, and G by enzyme immunoassay, and bactericidal activity of neutrophils in the nitroblue tetrazolium (NBT) test.

Statistical analysis of the data was performed using StatSoft Statistica, version 10.0. We applied methods of descriptive statistics, the Mann – Whitney test for intergroup comparison, and the Kruskal – Wallis one-way analysis of variance. Differences were considered significant at $p \leq 0.05$.

All the workers gave written informed consent for study participation and personal data processing.

The study has certain limitations related to the small size of the sample tested for *S. pneumoniae* carriage. Despite the epidemiological characteristics of the microorganism carried by 5–10 % of the adult population, studying its prevalence in the working population is of certain interest since it is a stage in the development of invasive and non-invasive pneumococcal diseases.

Results and discussion. Of 136 workers examined, *Streptococcus pneumoniae* was detected in 10 % (14), of which 29 % (4) are steelmakers, 64 % (9) are coke oven workers, and 7 % (1) are the controls. Compared with the controls, pneumococcus was more often detected in the subjects engaged in coke production and basic oxygen steelmaking, $p < 0.05$. The number of *Streptococcus spp.* carriers in the groups was comparable (Table 1).

We established that the PCR Ct value for *Streptococcus spp.* was lower in the steelmakers (16.5 ± 1.7) and coke oven workers (17.2 ± 2.5) than in the controls (23.9 ± 3.9), $p < 0.05$, which indicates a higher bacterial load. The Ct value for *Streptococcus pneumoniae* in the steelmakers is lower than in the controls (20.9 ± 4.03 against 24.43 ± 6.86, $p \leq 0.05$), which also reflects a higher bacterial load. The increase in the rate of respiratory infections related to exposure to aromatic hydrocarbons has been demonstrated by Låg et al. [10]. The pathogenesis is associated with a whole number of reactions triggered by aromatic hydrocarbons: firstly, the metabolism in epithelial cells changes through activation of the aryl hydrocarbon receptor, and, secondly, the epithelial barrier is directly damaged due to disruption of tight intercellular and gap junctions [11]. Metals (zinc, copper, cobalt, manganese, and arsenic), both in the ionic form and as nanoparticles, and polycyclic hydrocarbons activate adaptation mechanisms of streptococci, which are associated with the ability to pump glutathione from the human body, to protect from oxidative stress and, as a consequence, increase the expression of virulence factors [12]. Besides, through mutation or horizontal transfer, microorganisms acquire genetic resistance to chemical pollutants, which is an alarming precursor of the spread of bacteria with double resistance, both to metals and antibiotics. It has been established that environmental pollution with metals can play an important role in the emergence and maintenance of antibiotic resistance genes in bacteria [12]. We studied the prevalence of two types

Table 1

Results of studying pneumococcal carriage and antibiotic resistance genes in the workers

Indicator	Steelmakers $n = 44$	Coke oven workers $n = 40$	Control group $n = 52$
Streptococcus pneumoniae DNA	9 % (4)	23 % (9)	2 % (1)
Streptococcus spp. DNA	100 % (44)	100 % (40)	100 % (52)
Mef gene	100 % (44)	100 % (40)	94 % (49)
ErmB gene	80 % (35)	87,5 % (35)	63 % (33)

Table 2

DNA replication factors of the determinants of antibiotic resistance in the workers depending on *S. pneumoniae* carriage

Cycle threshold (Ct)	Steelmakers $M \pm m$		Coke oven workers $M \pm m$		Control group $n = 52$ $M \pm m$
	Streptococcus pneumoniae				
	Carriers $n = 4$	Non-carriers $n = 40$	Carriers $n = 9$	Non-carriers $n = 31$	
Mef gene	19.2 ± 1.7*	21.1 ± 4.5	20 ± 2.7	21.2 ± 2.6	25.9 ± 3.2
ErmB gene	25.5 ± 3.3	28.6 ± 3.5	27 ± 2.7	29 ± 2.8	30.3 ± 3.4

Note: * Statistically different from the control group ($p \leq 0.05$).

of genes: the Mef gene, which encodes the ability to quick clearance of antibiotics and metals from the body, and the ErmB gene encoding the ribosomal methylase that dimethylates a single adenine in 23S rRNA and is involved in target modification [13]. In our study the Mef gene was found in 97 % (133) of the examined: 33 % of them (44) are steelmakers, 30 % (40) are coke production workers, and 37 % (49) are the controls. The ErmB gene was found in 76 % (103) of the examined: 34 % of them (35) are steelmakers, 34 % (35) are coke production workers, and 32 % (33) are the controls. The emergence of such a gene in streptococci leads to the development of the highest level of resistance to macrolides, lincosamides, and streptogramin B (the MSLB resistance phenotype) [14, 15].

The lower Ct value of the Mef gene obtained in the steelmakers carrying *Streptococcus pneumoniae* indicates a higher genetic burden (Table 2). As shown above, through the genetic apparatus of pneumococci, metal-containing aerosols are likely to increase the efficiency of the efflux pump as a mechanism of adaptation to toxicants, which ultimately affects the prevalence of antimicrobial resistant bacteria.

The risk of developing streptococci-related diseases and primarily *S. pneumoniae* increases with the impairment of nonspecific and/or specific defense mechanisms. It has been shown that occupational risk factors for employees at ferrous metal processing, such as manganese and its compounds, iron, vanadium(V) oxide, and cadmium, adversely affect the immune system [9, 16]. In this study we revealed statistical differences in indicators of immune status between *S. pneumoniae* carriers and non-carriers (Table 3).

The response of the immune system to metal-containing aerosol exposures includes both cellular and humoral immune responses. Previous experimental and clinical studies have shown that damage to organs and cells of the immune system mainly occurs due to oxidative stress [17]. Compared to the controls, the steelmakers demonstrated a decreased total lymphocyte count attributed to lower counts of mature T lymphocytes (CD3+) in the populations of T helpers (CD4+) and cytotoxic T lymphocytes (CD8+).

As shown in the literature, immunosuppression of the T-cell component and a decrease in blood lymphocytes disrupts the mechanism of replenishing the tissue lymphocyte pool, thus reducing the effectiveness of defense against respiratory infections and provoking long-term persistence of streptococci on the mucous membrane of airways [18]. In their experiment Zhang et al. [19] proved the possibility of *Streptococcus pneumoniae* recolonization of the mucosa of the upper respiratory tract in laboratory mice deficient in CD4+ lymphocytes [19]. Changes in humoral immunity were found only among the *S. pneumoniae* carriers. Compared to the controls, they had a decreased count and rate of B lymphocytes (CD19+), decreased IgM, increased IgG, and decreased bactericidal activity of neutrophils. The observed response is possible due to the depletion of the pool of B

Table 3

Indicators of immune status in *S. pneumoniae* carriers and non-carriers

Indicators of immune status	Steelmakers $M \pm m$		Coke oven workers $M \pm m$		Control group $n = 52$ $M \pm m$	Reference values
	Streptococcus pneumoniae					
	Carriers $n = 4$	Non-carriers $n = 40$	Carriers $n = 9$	Non-carriers $n = 31$		
Leukocytes, 10^9/L	5.4 ± 0.28	6.5 ± 0.28	7.9 ± 0.51	6.7 ± 1.96	6.7 ± 0.14	4.0–9.0
Lymphocytes, %	29.9 ± 1.27*	29.5 ± 1.25*	27.9 ± 0.28*	31.4 ± 1.02	32.6 ± 0.68	19–37
Lymphocytes, 10^9/L	1.6 ± 0.07*	1.8 ± 0.08*	2.1 ± 0.11*	2.06 ± 0.08	1.96 ± 0.49	1.33–2.59
IgA, g/L	3.1 ± 0.51	2.8 ± 0.16	3.53 ± 0.39	2.9 ± 0.06	2.8 ± 0.08	0.9–4.5
IgM, g/L	0.88 ± 0.46*	1.37 ± 0.23	1.57 ± 0.27	1.2 ± 0.96	1.4 ± 0.09	0.6–2.8
IgG, g/L	18.2 ± 2.83*	16.8 ± 0.99	24.9 ± 1.74*°@	17.2 ± 0.97*	15.9 ± 0.51	8.6–19
IgA secr., mg/L	233.9 ± 106.3	204.5 ± 24.1	219.4 ± 59.1	230.2 ± 21.7	220.6 ± 11.7	115.3–299.7
CD3+, %	70.7 ± 4.31	70.2 ± 1.23	72.7 ± 1.87	73.01 ± 1.36	70.6 ± 0.72	55.0–83.0
CD3+, mm^3	1121 ± 111.5*	1287.8 ± 53*	1527.8 ± 77	1519.3 ± 75.1	1515.7 ± 42	700–2100
CD4+, %	45.9 ± 4.6	40.2 ± 1.2	44.7 ± 2.7	42.6 ± 1.4	43.9 ± 3.71	28.0–57.0
CD4+, mm^3	729 ± 101.3*	737.8 ± 35.1*	959 ± 59.4	881.9 ± 48.3	863.5 ± 25.3	300–1400
CD8+, %	19.6 ± 1.02*	24.8 ± 1.28	23.4 ± 1.71	24.6 ± 1.32	24.5 ± 0.66	10.0–39.0
CD8+, mm^3	309.5 ± 25.1*	451.1 ± 24.8*	498.8 ± 45.1	439.9 ± 36.8	528.6 ± 21.6	200–900
CD19+, %	8.6 ± 1.11*	10.2 ± 0.64	10.7 ± 1.25	10.9 ± 0.66	10.4 ± 0.34	6.0–19.0
CD19+, m^3	146.8 ± 34.3*	187.5 ± 13.5*	230.1 ± 33.8	223.8 ± 14.8	223.6 ± 9.34	100–500
NBT, %	4.01 ± 3.5*	7.6 ± 8.7	6.2 ± 3.8	3.9 ± 3.3*	14.5 ± 8.9	6.0–12.0

Note: * $p \leq 0.05$ for the difference between the exposure groups and the controls; ° $p \leq 0.05$ for the difference between the group positive for *Streptococcus pneumoniae* and the controls; @ $p \leq 0.05$ for the difference between the group positive for *Streptococcus pneumoniae* and the reference value.

lymphocytes in the organs of the immune system resulting from the combined exposure to two factors: pneumococci and industrial toxicants. The IgM level decreased in the steelmakers carrying *Streptococcus pneumoniae*, possibly owing to the switch in the synthesis of IgM to IgG, as confirmed by the increase in the latter compared with the controls. Immunoglobulin G retains memory of antigens for a long time and ensures a stronger immune response [20]. Results of the nitroblue tetrazolium (NBT) test showed a decrease in the neutrophil bactericidal activity in the *S. pneumoniae* carriers working in the basic oxygen furnace shop compared to the controls.

In the workers engaged in coke production, we established higher levels of IgG compared with the controls, regardless of *S. pneumoniae* carriage. When compared with the reference values, IgG levels in the carriers were statistically higher. Stimulation of B lymphocytes can be associated with activation of the aryl hydrocarbon receptor in immunocompetent cells. It has been previously reported that aromatic hydrocarbons that are part of the aerosol generated in this production act as a ligand for it. The effect of B lymphocyte stimulation through the aryl hydrocarbon receptor has been demonstrated within the clinical study of the pathogenetic role of aromatic hydrocarbons in the development of autoimmune connective tissue diseases [21]. Not only chemical factors affect antibody production. Emergence of opportunistic pathogens leads to an increase in IgG synthesis, which is confirmed by its statistically higher level in coke oven workers carrying *S. pneumoniae* compared to non-carriers. The mechanism of increasing the IgG level in the latter may be associated with impaired neutrophil phagocytosis slowing down the clearance of haptens that enhance autoimmunization and sensitization [22]. Long-term persistence of pneumococci in the body has been often attributed to suppression of the phagocytic functions of neutrophils [23, 24].

Conclusions:

1. The workers of the basic oxygen furnace shop exposed to industrial aerosols containing iron, vanadium (V) oxide, manganese and their compounds, and the workers engaged in coke production and thus exposed to aerosols containing aromatic hydrocarbons, with the exposure levels 2.5 to 3 times higher than the permissible ones, have a significantly higher *Streptococcus pneumoniae* carriage rate and the bacterial load of *Streptococcus spp.* (as established by Ct values) compared to the controls.

2. In the steelmakers, including *Streptococcus pneumoniae* carriers, the load of determinants of resistance to macrolides (the *Mef* gene*)* established by the Ct value is statistically higher than that in the control group. The carriage is accompanied by a decrease in immunological reactivity that is confirmed by a statistical decrease in the count of mature T lymphocytes (CD3+), T helpers (CD4+), and cytotoxic T cells (CD8+), a decrease in the count and proportion of B lymphocytes (CD19+), a switch of IgM to IgG synthesis, and a decrease in neutrophil bactericidal activity demonstrated by the result of the NBT test compared to the control group.

3. We obtained no data confirming contribution of immunological reactivity to developing *Streptococcus pneumoniae* carriage in the coke production workers, except for the increased IgG level compared to control and reference values.

4. Our findings on immune response and the presence of macrolide resistance genes will be useful when choosing a regimen for specific prevention or treatment of pneumococcal infection.

The study has certain limitations related to the small size of the sample tested for *S. pneumoniae* carriage. Despite the epidemiological characteristics of the microorganism carried by 5–10 % of the adult population, studying its prevalence in the working population is of certain interest since it is a stage in the development of invasive and non-invasive pneumococcal diseases.

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