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GENOTYPING OF *ESCHERICHIA COLI* STRAINS ISOLATED FROM BIOAEROSOLS AND SEWAGES ON MUNICIPAL WASTEWATER TREATMENT PLANT

GENOTYPOWANIE SZCZEPÓW *ESCHERICHIA COLI* WYIZOLOWANYCH Z BIOAEROZOLI I ŚCIEKÓW NA TERENIE OCZYSZCZALNI ŚCIEKÓW KOMUNALNYCH

Keywords: bioaerosols, wastewater treatment, *Escherichia coli*, genotyping, Chromocult ES.
Słowa kluczowe: bioaerozole, oczyszczanie ścieków, *Escherichia coli*, genotypowanie, Chromocult ES.

Bioaerozole generowane przez oczyszczalnie ścieków zawierają do 100 różnych rodzajów mikroorganizmów, między innymi bakterie i grzyby. Normy mikrobiologiczne dla powietrza atmosferycznego obejmują analizę liczby bakterii heterotroficznych, Pseudomonas fluorescens, hemolizujących gronkowców, promieniowców i ogólną liczbę grzybów. Pałeczka okrężnicy (E. coli) zajmuje ważne miejsce w grupie wskaźników biologicznych stanu czystości środowisk zanieczyszczonych działalnością człowieka. Ścieki bytowo-gospodarcze poddawane oczyszczeniu zawierają duży ładunek bakterii pałeczki okrężnicy dyfundującej do powietrza, stanowiąc potencjalne ryzyko dla zdrowia człowieka. Wyniki przedstawione w pracy zawierają dane z analizy mikrobiologicznej powietrza metodą sedymentacyjną i zderzeniową, na płytkach z pożywką agarową Chromocult ES, jak również dane z testów molekularnych. Genotypowanie z analizą pochodzenia E. coli przeprowadzono, stosując 3 startery klasy RAPD-PCR. Wstępne badania 37 ze 118 szczepów E. coli potwierdziły umiarkowanie wysoki poziom zróżnicowania genetycznego Nei'a (H_T=0,4; Gst=0,1; P=100%; G=100%) i wysokie podobieństwo genetyczne szczepów (I_N=0,93). Ponadto wyniki analiz molekularnych ujawniły możliwość zanieczyszczenia krzyżowego prób powietrza pomiędzy stanowiskami technologicznymi. Należy zatem

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przewidzieć, że część bakterii w danym punkcie poboru prób może być generowana na innych, oddalonych stanowiskach technologicznych. Zrozumienie mechanizmów biologicznych powietrza i jego dynamiki pozwoli na dokładniejsze wykonanie analiz mikrobiologicznych bioaerozoli na terenie obiektów oczyszczania wód i ścieków. Markery RAPD wykazały zadowalającą rozdzielczość, pomocną podczas typowania szczepów i śledzenia dróg rozprzestrzeniania się E. coli na terenie biologicznej oczyszczalni ścieków. Uwzględnienie proponowanych badań jako obligatoryjnych normatywnie wymaga dalszego testowania.

1. INTRODUCTION

Escherichia coli is one of the main microbiological indicator among other bacteria, fungi, algae and protozoa that applied during monitoring of municipal wastewater treatment plants. As a model bacteria for genetic research, *E. coli* has well known genome, population structure, ecological preferences and biology [Blattner et al. 1997]. This colon bacteria is natural settler of the human digestive tract (gut) and valuable indicator of human impact on environment, especially by faecal pollutions. Some of strains can be etiological factor of strong health disorders such as hemorrhagic diarrhoea. In Polish standards bacterium of *E. coli* is an important indicator for microbial pollution of water, food and air.

Many types of genotyping methods were composed for *E. coli* based on genomic area of special interest such as genes (virulence, drugresistance), transposable genetic elements (Tn, IS) or dispersed and aggregated repetitions (VNTR) by PCR and/or restriction techniques [Fratamico and DebRoy, 2010, Hu et al. 2008]. The easiest methodological conception is to applied simple amplification methods by RAPD-PCR markers that work as scanning-like genome [Wiliams 1990].

Mechanical and biological treatment of municipal sewages on different stages generates gas-liquid particles consisting bacteria, viruses, moulds, their metabolites, toxins, parts of proteins and nucleic acids (bioaerosols). Varied in size bioaerosols states potential hazards for human and animal health especially for alimentary and respiratory tracts [Forcier, 2002]. Treating municipal sewages consist many of pathogenic bacteria among others *E. coli* is dominating [Miyanaga et al. 2006]. From this reason estimation of *E. coli* concentration in bioaerosols generated from municipal wastewater treatment plant is main problem in many standards of air monitoring.

Microbiological influence of sewage treatment on environment is estimating by traditional methods during 10-30 minutes of air sedimentation on petri plates with solid medium [Filipkowska and Korzekwa 1999]. Another impact method of air monitoring rely on mechanical aspiration of air on petri plates with solid medium through perforated cover of MAS-100 ECO apparatus. Both of methods we applied for testing selective medium Chromocult ES highly selective for *E. coli*, *Citrobacter* sp. and *Salmonella* sp.

General aims of this research were:

1) test of Chromocult ES utility for bioaerosols monitoring;
2) estimate genetic diversity of *E. coli* isolated from bioaerosols and sewages;
3) specify possibilities of RAPD-PCR markers for revealing of cross contamination between air samples originated from different technological devices.

2. MATERIALS AND METHODS

Bacteria were isolated from Municipal Wastewater Treatment Plant "Łyna" in Olsztyn. Plant works in biological system based on activated sludge, supported by mechanically raked bars, sand/grit cannels, primary and secondary sedimentation tanks, sludge fermenters and lagoons. Air samples were aspirated from three technological devices: raked bar chamber (I), primary sedimentation tank (II) and activated sludge tanks (III). Moreover four *E. coli* reference strains were applied during analysis as positive control (ATCC 25922, ATCC 11303, ATCC 8739, LMG 2092) and two of *Citrobacter freundii* as negative one.

Field analysis of air were done sedimentation method according to [PN-89, Z-04008/08] and by MAS-100 ECO apparatus with standard Petri plates (90 mm) poured by Chromocult ES medium. Samples of the air (1 and 3 m^3) were collected on the end of October (19-26.10.2010) on two places of each technological devices: upwind and downwind in triplicates. During field sample collection weather conditions were controlled (wind, humidity, temperature). Sample of sewages were collected into 50 ml Falcon tubes with caps and held in ice-boxes at about 6-8°C until lab analysis.

Media for preliminary culturing and inoculation of strains were triptic soy broth and agar. Main testing medium was Chromocult ES (ChromoCult, Coliform Agar ES, 1.00850, Merck KGaA, Darmstadt, Germany). Standard plates (90 mm) were filled out by medium in the same day before field analysis. Inoculation, passaging and culturing of bacteria were realised by 24 h at 37°C. If necessary, another 12-24 h were cultured to obtain dark blue to violet colour of colony indicating the presence of *E. coli*. Further biochemical analysis were applied by API 20-E tests to confirm taxonomic membership of strains. Ten strains per plate were selected and digested for DNA isolation by CTAB method [Korzekwa, 2004].

Molecular RAPD-PCR analysis of *E. coli* were conducted according Wiliams [1990] with own modifications. Operon primers kit A-04 and B-11 were applied and semi random ISJ4 about follow sequences 5'-3': AATCgggCTg, gTAgACCCgT, gTCggCggACAggTAAgT. PCR mix (20 µl) consisted ddH_2O, PCR-enhancer (10x), polymerase Buffet (10x), Mg (2 mM), nucleotides (200 µM), primers (10 ng), Tfl polymerase (0,9 U), DNA matrix (60 ng).

Thermal profile were initial denaturation (96°C, 3 min), 45 cycles of denaturation (96°C; 1 min), annealing (36°C; 1 min), elongation (72°C; 2,5 min). PCR reaction was finished by 5 minutes elongation at 72°C. Electrophoresis was conducted in 1,2% agarose gels at 10V/cm of voltage. For ISJ4 annealing at 48°C by 1 minute was applied.

Statistic software such as POPGENE ver. 1.32, STATISTICA ver 5.1, GenAlEx ver 6, STATISTI XL ver. 1.8 were applied for genetic diversity h, H_T, identity I_N, distance D_N dendrogram plotting and PCA analysis. Band on gel was considered as allele "1" (complementary region for primer). In case of lack of amplification n product such allele were signed as "0" (binary system).

Scheme of experiment and subsequently statistical processing consisted 3 parts with dividing of strains as follow: a) one population for all strains isolated from treatment plant, b) two populations (isolated from sewage and bioaerosols), c) six populations (isolated from sewage and bioaerosols on each technological device).

3. RESULTS AND DISCUSSION

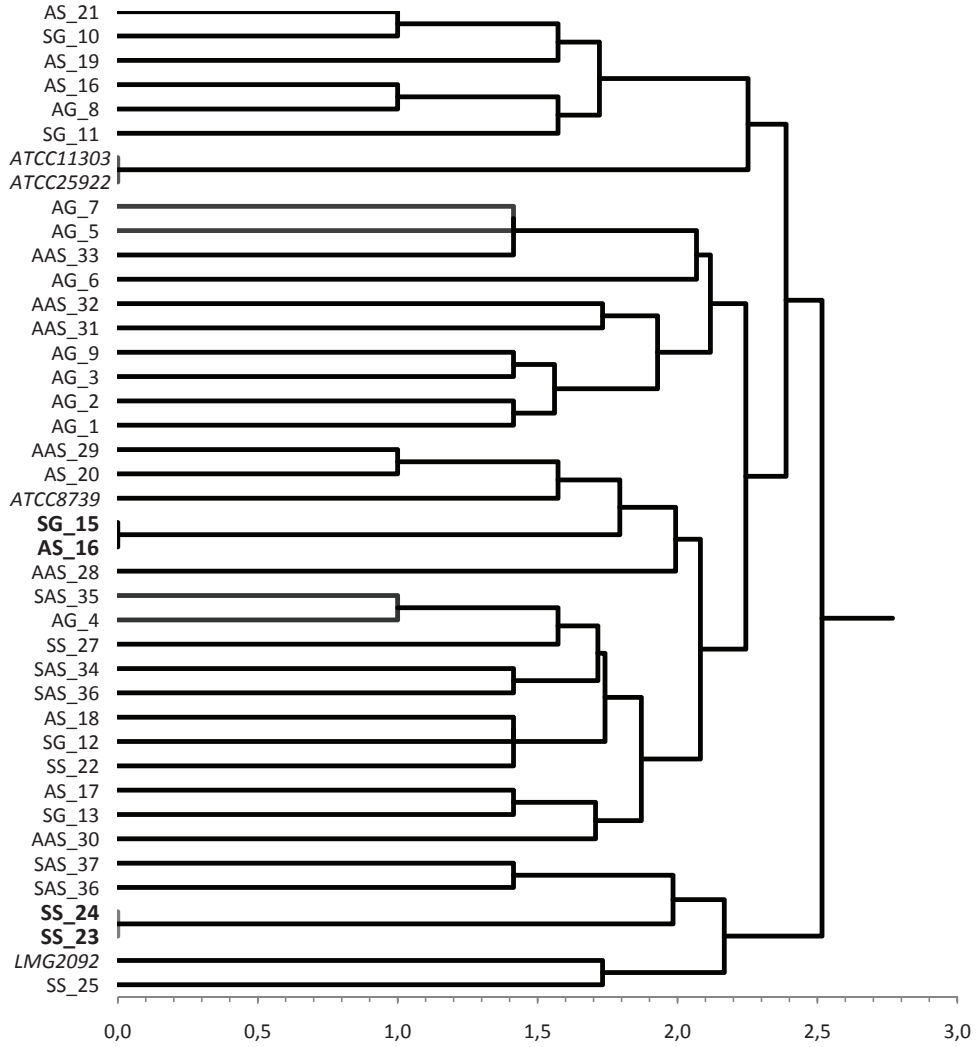
Based on results we revealed low level of *Escherichia coli* in bioaerosols (tab. 1) in given weather conditions. Number of bacteria in sewages were higher on 19.10 than 26.10 in opposite to number of *E. coli* in bioaerosols. Lower CFU for E. coli in bioaerosols were caused by lower amount of air that was aspirated by MAS-100 ECO. We tested standard 1 m^3 of air but it was 2-3 times lower than in case of second field analysis when MAS-100 ECO aspirated 3 m^3 of polluted air. Cause of varied wind directions number of isolated bacteria from plates was almost the same for upwind and downwind places for all technological devices.

Table 1. Results of field research for bioaerosols and sewages on wastewater treatment plant.
Tabela 1. Wyniki badań bioaerozoli i ścieków na terenie oczyszczalni ścieków

Parameter	Technological device*		
	I	II	III
Mean CFU of *E.coli* in 1 ml of sewages	8 x 10^5 (5 x 10^5)	6 x 10^5 (1 x 10^5)	7 x 10^5 (5 x 10^5)
Mean CFU of *E.coli* in 1 m^3 of bioaerosols	6 (14)	0 (22)	0 (6)
Temperature	10.8 °C (8.7)	10.4°C (6.6)	9.8°C (7.4)
Humidity	57% (68)	61% (82)	66% (74)
Wind	0 m/s (0)	2.5 m/s (2.2)	2.3 m/s (3.3)

Note: I – raked bar chamber; II – primary sedimentation tank; III – activated sludge tanks; * – values for 19.10.2010 are before parenthesis and inside for 26.10.2010.

After incubation of 118 bacterial strains 37 were randomly selected and identified as *E. coli* then genotyped and statistically processed. Both 10-nucleotide operon primers revealed 26 complementary places (loci) in *E. coli* genome and ISJ primer – 17 loci. All loci were polymorphic (P=100%). For statistical analysis of species divided into subpopulations percent of polymorphic loci was from 35 up to 82. All electrophoresis patterns were unique but two pairs of strains that were identical: SG_15, AS_16 and SS_23, SS_24 (fig.).



Note: AAS – bioaerosols from activated sludge; AG – bioaerosols from bars; AS – bioaerosols sedimentation tank; SAS – sewage from activated sludge; SG – sewahe from bars; SS – sewage from sedimentation tank; **bold** – strain with I=1; *italic* – reference strains of *E. coli*.

Fig. Euclidean distance by average grouping cluster method

Rys. Euklidesowa odległość genetyczna metodą analizy skupień

The most similar were reference strains ATCC 11303 with 25922, isolated from sewage of sedimentation tank and isolated from bars with sedimentation tank. It is clear that genotypes of two randomly isolated strains originated from the same source of sewages treated in one device may by the same. Whereas, high genetic similarity (I_N=1) of two un-

related strains may suggest possibility of cross-contamination between raked bar building and area of air near primary sedimentation tank. But it could be possible that given bacterial strains about given genotype migrated with municipal sewages then part of them evaporated in raked bar building then remain strains migrated with sewages to primary sedimentation tank and evaporated to bioaerosols. Together with such fingerprinting should be realising additional analysis of one selected gene, mobile genetic element or repetitive sequence may help for providing a proof for cross-contaminations. We applied rep-BOX A1 sequence in such primary experiment and revealed as good additional molecular tool together with RAPD-PCR (prepared for publication).

Mean number of allele for each RAPD loci (N_a) was 2, mean frequency of allele 1 for whole population (A_1 = 0.34) and genetic diversity of species was about 0.38 (tab. 2).

Table 2. Values of parameters obtained by statistical process of amplicons
Tabela 2. Wartości parametrów otrzymanych po statystycznej obróbce amplikonów

Populations of *E. coli*	N	A_1	h_{min}	h_{max}	h_S	H_S	G_{st}	H_T
Whole	37	0.34	0.05	0.50	x	0.38	x	
Bioaerosols	21	0.37	0.09	0.50	0.37	0.35	0.07	
Sewages	16	0.29	0.0	0,49	0.34			
Boaerosols:bars	9	0.40	0.0	0.49	0.29	0.31	0.19	0.38
Sewages bars	6	0.26	0.0	0.50	0.27			
Bioaerosols sedim. tank	6	0.27	0.0	0.50	0.31			
Sewages sedim. tank	6	0.36	0.0	0.50	0.32			
Bioaerosols activated sludge	6	0.44	0.0	0.50	0.32			
Sewages activated sludge	4	0.35	0.0	0.50	0.33			

Note: N – number of strains; $h_{min/max}$ – minimum or maximum value of genetic diversity in locus; h_S – mean genetic diversity on locus into population; H_S – mean genetic diversity on locus on population; G_{st} – proportion of populations genetic diversity in whole species genetic diversity.

Genotypnig of colon bacterium is one of the interesting and many time realising research problem over the world science. In every stage of modern biotechnology, medicine, epidemiology, food processing, agriculture and environmental protection we need information about origin, sources and routs of *E. coli* strains. Typing of genetic patterns is valuable and common molecular technique for bacteria of all species including that habit in sewages and bioaerosols, for example *Haemophilus influenzae Rd* [Fleischmann et all. 1995], *E. coli* K-12 [Blattner et al. 1997], *E. coli* O157:H7 [Hayashi et al. 2001; Perna et al. 2001], *E. coli* DH10B and W [Durfee et al. 2008; Archer et al., 2011], *Salmonella Typhimurium* LT2, SGSC1412, CT18 [McClelland et al. 2001], *Carsonella ruddii* [Nakabachi 2006], *Micrococcus luteus, Kocuria rhizophila* [Young et al. 2010, *Pseudomonas aeruginosa* PAO1 [Stover et al. 2000], *Pseudomonas putida* KT2440 [Nelson et al. 2002]. Molecular DNA typing or fingerprinting is

especially useful during microbial source tracking (MST) by PFGE, AFLP, RAPD, RFLP, rep-PCR and 16S rDNA-PCR methods (U.S. Environmental Protection Agency 2005). In spite of well known genome of colon bacterium relatively small data is recorded about genetic diversity of bacteria transmitted by bioaerosols generated during wastewater treatment. The most of research work is concentrated to drug resistant strains isolated from natural environment (rivers, lakes) under anthropogenic pressure. Many microbiological standards of water and sewage quality do not include molecular markers or cultivation-independent methods. That is why we tested one of such random and semirandom amplified DNA markers for comparison of *E. coli* genotypes isolated from sewages and air, among others for answer the question: if we isolated bacteria from this technological device treating sewages or may be from another device working in wastewater treatment plant? Second aspect of work should help answer the question if chromogenic medium is suitable for bioaerosols investigations and could substitute traditional ENDO suggested by Polish standards [PN-89, Z-04111/01, PN-89, Z-04008/08, PN-89, Z-04111/02, PN-89, Z-04111/03]. We did not tested ENDO simultaneously with Chromocult ES what has been estimating in our second presently conducting work (data not published). Although, after work we suggest Chromocult as more sensitive, handy, easy to interpretative medium with some major limitation related to more air that should be aspirated. It means changing in standards or additional regulations what may our results make difficult to applicate, interpretate and compare with others.

In conclutions wastewater treatment plant in Olsztyn did not generate hazard level of *Escherichia coli* in bioaerosols. Strains of colon bacterium isolated from bioaerosols originated from sewages treated in given technological device but one case of cross-contamination of air by strains from building of bars. Chromocult ES is very useful and lab handy medium for bioaerosol examination but in case of low contamination of the air by *E. coli*, more air should be aspirated by MAS-100 ECO, up to 3 cubic meters (3 m^3).

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