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# ANTIBIOTIC RESISTANCE OF *ESCHERICHIA COLI* ISOLATED FROM UKRAINIAN *BETULA VERRUCOSA* EHRH. POLLEN AFTER MICROBIOLOGICAL ANALYSIS

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**ABSTRACT**

Seven samples of silver birch pollen from different habitat of Ukraine were investigated in order to estimate their contamination with the *Enterobacteriaceae* family, anaerobic bacteria and fungi. Also resistance of 108 strains of *Escherichia coli* isolated from seven samples of Ukrainian *Betula verrucosa* Ehrh. pollen against 5 antibiotics: ampicillin, chloramphenicol, meropenem, ceftriaxone and ofloxacin were determined. Disc diffusion method was used for antibiotic suceptibility testing according to EUCAST 2012. It is established the concentrations of enterobacteria ranged from 0.00 to 4.16 log cfu/g, of anaerobic bacteria – 2.48 to 4.90 log cfu/g and concentration of fungi ranged from 2.48 to 4.14 log cfu/g. Degree of pollen contamination is different depending on the habitats. The resistance of *E. coli* isolates was determined against ampicillin, chloramphenicol, meropenem and ofloxacin. But intermediate resistance in the 33.3% of *E. coli* isolates and susceptibility in the 8.3% to ceftriaxone was found out. Antibiotic resistance was evaluated for all samples of pollen in whole.

**Keywords:** Microflora, antibiotic resistance, pollen, *Betula verrucosa* Ehrh., *E. coli*, disk diffusion method

## INTRODUCTION

Pollen of wind-pollinated plants is allergenic **(Shamgunova *et al.*, 2010)**. It is promoted by its physical (size and shape of pollen grains, copious amounts at flowering) and chemical characteristics (presence of allergenic proteins) **(Berzhec *et al.*, 2007; Rodinkova, 2005)**. The combination of these factors and also weather conditions lead to development of allergic reactions in susceptible humans **(Shamgunova *et al.*, 2010; Reid *et al.*, 2009)**. Also pollen is a carrier of fine particles of anthropogenic origin, which makes it even more active object **(Schäppi *et al.*, 1997; Behrendt *et al.*, 2001)**. It is also known that allergic and immunotoxic reactions can cause by microscopic fungi and their products, similarly to bacteria (**Aleshina, 2006; Belaja, 2012; Spiewak *et al.*, 1996)**. For example, fungi belonging to the genera *Penicillium*, *Alternaria* and *Cladosporium* possess strong allergenic properties **(Spiewak *et al.*, 1996)**. It cannot be excluded that a part of allergic symptoms caused by exposure to pollen may be due to the presence of microorganisms and their products on pollen grains. The presence of those on the pollen of different species is confirmed by **Lacey *et al.* (1988), Śpiewak *et al.* (1996)** studies. So far, it is not clear how and when the pollen can be contaminated by these microorganisms: before leaving the pollen sac or during its falling or by interaction with other bioaerosols. As a result bacteria can be moved together with the pollen over long distances. Getting to human respiratory system, pollen irritates the mucous membranes of the respiratory tract and causes an allergic reaction. Bacteria are transmitted together with pollen at inhalation. They get to favorable conditions for their growth and reproduction. If these bacteria contain the resistance genes, there is the possibility of transferring resistant bacteria and their genes to bacteria of digestive system of humans or animals **(Brovarskij *et al.*, 2010; Konov, 2002)**.

Pollen of silver birch (*Betula verrucosa* Ehrh.) is characterized by above mentioned characteristics **(Severova, 2009)**. Birch pollen is the most powerful and frequent source of allergens of *Fagales*, the main source of spring hay fever in Europe **(Mothes *et al.*, 2004; Erler *et al.*, 2011; Jato *et al.*, 2007; Shamgunova *et al.*, 2010)**. In Ukraine the dominance of distribution of birch pollen confirmed by aeropalynologic observations **(Savitsky *et al.*, 1996; Rodinkova, 2005; Vityk, 2008)**.

It is interesting that pollen birch possesses useful properties. It is beneficial to microbes, bacteria and various enzymes in the human intestine. It promotes development of useful bacteria by eliminating harmful. Birch pollen is a perfect antibiotic. It has the ability to stop the growth of many bacteria, which are difficult to destroy and are the causative agents of many typhoid diseases. Due to the presence of antibiotics, birch pollen is a regulator in the intestinal tract **(Danikov, 1993; Mironenko, 2002)**. Previously, we had been investigated the antibacterial properties of the aqueous and water-salt extracts of seven birch pollen samples against three test cultures: *E. coli* M-17, *Ralstonia solanacearum 8202* and *Pseudomonas syringae* **(Shevtsova *et al.*, 2012)**. It was revealed Ukrainian *Betula verrucosa* Ehrh. pollen had antibacterial properties against *R. solanacearum* and *P. syringae* strains, but was not effective against *E. coli* that may be the result of possible synergism effect of microflora of birch pollen and *E. coli* like test culture. *E. coli* has been shown to exchange genetic material with other bacterial species and it is possible that this organism may pass antibiotic resistance genes to transient bacterial pathogens that cause disease in humans **(Alexander *et al.*, 2010)**. After examining the microflora of Ukrainian samples of silver birch pollen, we have decided additionally to isolate *E. coli* from samples and examine preliminary its resistance to antibiotics.

## MATERIAL AND METHODS

### Collection of samples

In total, 7 samples of *Betula verrucosa* Ehrh. pollen has been prepared prior to the beginning of anthesis in Kyiv and Rivne region of Ukraine, namely: 1 – Kyiv (pollen was collected from the birches growing in the park zone); 2a – Pereyaslav-Khmelnytsky, Kyiv region (pollen was collected from the birches growing near the road and housing estate) and sample 2b was collected from the birches growing in the park; 3 – Hotsky, Kyiv region (pollen was collected from the birches growing on separate glades among wood); 4 – Ivankiv, Kyiv region in III Chernobyl zone (according to definition of the territory by the Ministry of Ukraine on questions of extreme situations and on affairs of protection of the population from consequences of Chernobyl accident) **(Holoshi, 2008)** (pollen was collected from the birches growing near the highways and housing estate); 5 – Kuznetsovsk, Rivne region in IV Chernobyl zone (located in 380 km to the West from Kyiv) (pollen was collected from birches growing near the wood, and near the highways); 6 – Borodyanka, Kyiv region (pollen was collected from the birches growing near airdrome, IV Chernobyl zone). The samples were collected aseptically, placed in sterile plastic bags and brought to the laboratory.

**Determination of microflora**

The concentration of bacteria in the pollen samples was determined by dilution plating. One gram of each sample was suspended in 200 ml of distilled water. After vigorous shaking, 10-fold serial dilutions were made up to $10^{-3}$. The 1 ml aliquots of each dilution were spread on duplicate sets of media appropriate for determination of enterobacteria, anaerobic bacteria and fungi. The serial dilutions were inoculated on nutrient media. The plates with enterobacteria were incubated on Endo agar for 24 hours at 37°C, anaerobic bacteria were incubated on anaerobic agar for 36 hours at 30°C and fungi on Sabouraud's medium for 54 hours at 25°C respectively. The data were reported as colony forming units (cfu) per 1 g of pollen. The colonies were counted and fungi were determined using the «Dictionary of the Fungi» **(Kirk *et. al.*, 2001)**.

**Determination of antibiotic resistance**

**Cultivation and isolation of *E. coli***

For cultivation of bacterial strains Mac Conkey agar (Biomark, Pune) was used. Cultivation of *Enterobacteriaceae* genera was done at 35±2 °C during 24 hours. After the first incubation was need recultivation to obtain pure culture of *E. coli* in the same conditions. For recultivation and probably indetification of *E. coli* strains Chromogenic coliform agar (Oxoid, UK) was used. For obtaining the pure culture of *E. coli* four-ways streak plate method was used. Every these steps of recultivation was done in the same conditions.

**Identification of *E. coli* strains**

Initial identification of *E. coli* strains were done on Chromogenic coliform agar (Oxoid, UK) and Triple sugar iron agar (Biolife, Italy). Biochemical indetification of *E. coli* was done by ENTEROtest 24 (Erba Lachema, Brno). Working procedure for biochemical testing is describe into the manufacturer manual. Evaluation of biochemical test was done by identifying computer program TNW Lite 7.0 software (Erba Lachema, Brno). For accurate identification of *E. coli* strains were used MALDI-TOF MS Biotyper (Brucker Daltonics GmBH, Germany) and method for prepare of samples to identification was done by Kmeť and Drugdová **(Kmeť *et al.*, 2012)**.

**Antibiotic susceptibility testing**

The pure inoculum of *E. coli* strains were prepared by suspending of colonies into the physiological solution from agar plates and every suspension was adjusted to equal a 0.5 McFarland standard. The sensitivity of all *E. coli* strains were tested against: ampicillin (AMP 10) 10 µg.disc$^{-1}$, chloramphenicol (C 30) 30 µg.disc$^{-1}$, meropenem (MEM 10) 10 µg.disc$^{-1}$, ceftriaxone (CRO 30) 30 µg.disc$^{-1}$, ofloxacin (OFX 5) 5 µg.disc$^{-1}$ (Oxoid, UK). For antibiotic susceptibility testing disc diffusion method (according to **EUCAST 2012** – European Committee on Antimicrobial Susceptibility Testing) was used. Incubation of *E. coli* strains were done at 35±2 °C on Mueller-Hinton agar (Biomark, Pune). Interpretation of inhibiotion zones around the disc was accordingy to EUCAST (Breakpoint tables for interpretation of MICs and zone diameters Version 2.0, valid from 2012-01-01). The inhibition zones were controlled with the references sensitivity of *Escherichia coli* CCM 3988.

**RESULTS AND DISCUSSION**

Table 1 presents the numbers of representatives of the *Enterobacteriaceae* family, anaerobic bacteria and fungi in examined pollen. It may be seen that the numbers of representatives of the *Enterobacteriaceae* family ranged from 0.00-4.16 log cfu/g. Anaerobic bacteria were more numerous than enterobacteria and their numbers ranged from 2.48–4.90 log cfu/g pollen. And the number of enterobacteria and the number of anaerobic bacteria prevailed in samples 5 and 6 of birch pollen. In samples 3 and 4 representatives of the *Enterobacteriaceae* family were absent.

**Table 1** Microflora of seven Ukrainian samples of *Betula verrucosa* Ehrh. pollen in log cfu/g.

| Pollen sample | Representatives of the *Enterobacteriaceae* family | Anaerobic bacteria | Fungi |
|---|---|---|---|
| 1 | 2.00 | 2.90 | 2.48 |
| 2a | 2.02 | 4.68 | 3.04 |
| 2b | 2.30 | 3.79 | 3.26 |
| 3 | 0.00 | 4.78 | 4.14 |
| 4 | 0.00 | 2.48 | 3.23 |
| 5 | 3.54 | 3.91 | 2.90 |
| 6 | 4.16 | 4.90 | 3.38 |

The concentration of fungi ranged from 2.48-4.14 log cfu/g (figure 1). The most contaminated with fungi was sample 3 from wood, the least contaminated – 1. It has been observed that fungi were present in those pollen samples where there were no other groups of examined microorganisms. Identified fungi belonging to the representatives of the *Penicillium*, *Alternaria*, *Aspergillus* and *Cladosporium* genera.

There are literary data about microflora of *Betula verrucosa* Ehrh. pollen, collected from a relatively ecologically clean region in Kraków Province, a housing estate and a polluted industrial area near a cokery in Kraków. It was determined the presence on pollen grains mixed microflora, consisting of Gram-positive and Gram-negative mesophilic bacteria, thermophilic actinomycetes and fungi (*Cladosporium herbarum, Alternaria alternata, Penicillium* sp., *Oidiodendron flavum, Cephalosporium charticola*) **(Spiewak *et al.*, 1996)**. Samples from ecologically clean region were also contaminated. Fifty four samples of *B. pendula* pollen were examined in Moscow and the Moscow region **(Antropova *et al.*, 2008)**. Twenty four species of micromycetes were isolated from 12 genera. *Aureobasidium pulullans* and species close to *Quambariaria cyanescens* were absolutely dominated. *A. pullulans* often found on the leaves of various plants. **Ahapkina (2007)** reports about mould and yeast seminal effect of *Betula pendula* pollen which averages 3.69 log cfu/g. Our results confirmed by these. Tree pollen may be contaminated with yeast and fungi, it is known that contamination of plants is widespread. Probably pollen is a nutrient medium for these microorganisms as well as a unique way to spread in the environment **(Ahapkina, 2007)**.

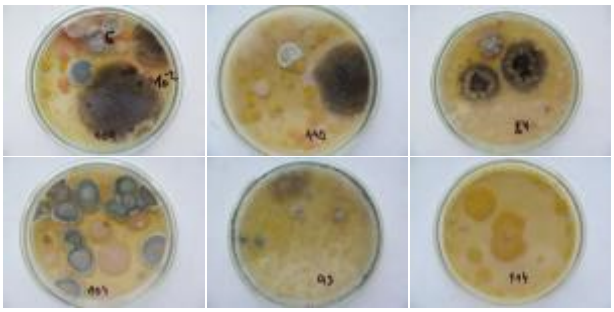

**Figure 1** Present fungi on *Betula verrucosa* Ehrh. pollen

The antibiotic resistance was studied in 108 *E. coli* strains isolated from six samples of *Betula verrucosa* Ehrh. pollen. Isolation of *E. coli* from sample 5 was not successful. It was determined that isolated *E. coli* were sensitive to all tested antibiotics. Although 8.3% of *E. coli* strains were resistant to ceftriaxone and 33.3% showed intermediate result. Results of antibiotic resistance of *E. coli* are shown in the figure 2.

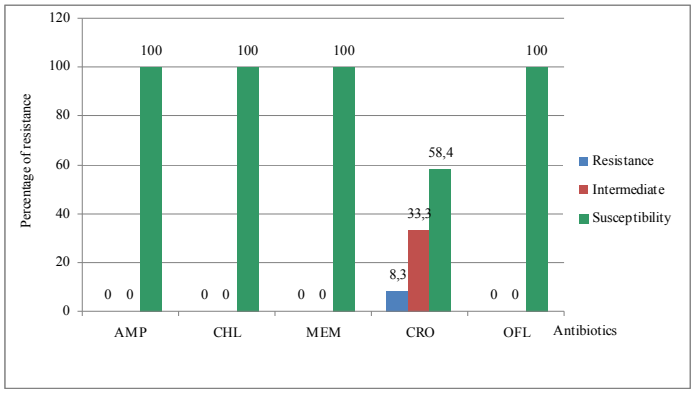


**Figure 2** Antibiotic resistance of *E. coli* from *Betula verrucosa* Ehrh. pollen

There are not much information about antibiotic resistance in bacteria isolated from pollen. Many papers are devoted to the study of microflora of bee pollen, a little less to microflora of pollen and pollen of anemophilous plants. So, it is difficult to compare our results with the results of other authors. We have chosen *Escherichia coli* because it is the main representative of the *Enterobacteriaceae* family. *Escherichia coli* is a common inhabitant of intestinal tract of humans and animals, and can be easily disseminated in different ecosystems through the food chain and water **(Costa *et. al.*, 2009)**. Besides, antibiotic resistance of *E. coli* has been studied in details. Some investigators have proposed the presence of lineages or even clones within the *E. coli* population carrying most of the widely spread resistance determinants. The most important mechanism for the acquisition of antibiotic resistance is horizontal gene transfer, in *Enterobacteriacae* mainly mediated by conjugation **(Sundqvist, 2010).** However, we have isolated and identified from samples of birch pollen and other bacteria that do not belong to the *Enterobacteriaceae* family. They are *Staphylococcus succinus* (resistant to ampicillin), *Moraxella osloensis* (resistant to piperacillin) and *Acinetobacter radioresistens* (not resistant to all tested antibiotics).

A bacterium will acquire antibiotic resistance as a response to the environment. Antibiotic resistant of *E. coli* have been found in almost every environment **(Sundqvist, 2010)**. Based on published data we can say that pollen of *Betula verrucosa* Ehrh. becomes allergenic under adverse environmental conditions but it retains useful properties. Reaction on birch pollen is individual. Our results showed susceptibility of *E. coli* isolates to tested antibiotics. Resistance to ceftriaxone is very low.

## CONCLUSION

Our research of *Betula verrucosa* Ehrh. pollen, collected in the territory of Ukraine from seven different habitats, has shown presence of representatives of the *Enterobacteriaceae* family, anaerobic bacteria and fungi. Most contaminated samples from roads, airdrome and housing estate.Evaluating the stability of *E. coli* isolates from Ukrainian *Betula verrucosa* Ehrh. pollen to antibiotics, we found out that *E. coli* were to all tested antibiotics sensitive. Resistance to ceftriaxone is 8.3%. It was the preliminary study.

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