УДК 579.842.61:616-092.8

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PLANT-ISOLATED *PANTOEA AGGLOMERANS* – NEW LOOK INTO POTENTIAL PATHOGENICITY

Pantoea agglomerans strains have been isolated from the surface of different edible plants which are major ingredients of traditional foods of the Black Sea region countries. Bacterial strains did not possess their pathogenic properties when tested routinely in vitro, but evinced the resistance to broad-spectrum antibiotics. Experiments on murine model (BALB/c mice) have demonstrated the ability of P. agglomerans to penetrate into internal organs and provoke the distinct dose-dependent physiological changes in the intestine and gut associated lymphoid tissues (GALT).

Key words: edible plants, Pantoea agglomerans, potentially pathogenic bacteria, opportunistic infections, quality of food, food borne pathogens.

Being the essential source of vitamins, bioactive compounds, starches, etc., vegetables, fruits, greens and berries take a positive effect on health. By contrast, the uncooked edible plants can be a source of the food-borne infections, caused by pathogenic and opportunistic microorganisms. The urgency of the food safety abidance is evident from the recent numerous outbreaks related to contaminated plant foods.

Pantoea agglomerans (former – *Enterobacter agglomerans*) is the typical resident of the plants' leaves and fruits' surface but also known as an opportunistic pathogen in the immunocompromised, causing wound, blood and urinary-tract infections. This bacterium is successfully used in agriculture as a postharvest biological fungi antagonist [7]. In the last years the number of the articles concerning the problem of *Pantoea*-associated opportunistic diseases (abscesses [9], arthritis [4], nosocomial outbreaks [1] etc) has increased. And this tendency is expected to rise within increasing *Pantoea*-related diseases, mostly in agricultural countries [10].

It is proved that *Pantoea* includes *hrp* genes that control the ability to cause diseases [6] and might possess virulence potential [10]. Despite this no significant gene differences have been found between clinical and non-clinical isolates toward human pathogenicity [11] and no marker uniquely associated to clinical strains was identified [8].

The lack of data available concerning the mechanisms which are involved in potential negative influence of *P. agglomerans* on the human host initiates our current study aimed to clarify the *P. agglomerans* pathogenic properties *in vitro* and *in vivo* on the animal model following to microbiological detection of contamination of plant ingredients with *P. agglomerans* of the traditional foods in the Black Sea region countries.

Materials and Methods. The plant samples collected in Georgia, Bulgaria, Russia, Romania, Ukraine and Turkey have been examined for the persistence of representatives of *Enterobacteriaceae* family. About 60-70 % of isolated strains were identified as *P. agglomerans*.

In current study four strains have been used: from carrot (Bulgaria), green beans (Turkey), parsley (Ukraine) and carrot (Ukraine).

In vitro pathogenicity was investigated via: (1) test of activity to coagulate the serum; (2) their haemolytic properties and (3) activity to utilise lecithin. For the first test 24-hour bacterial culture inoculates were introduced into the tube with dissolved rabbit plasma and incubated at a temperature of 37 °C for 1, 4, 18 and 24 hours. Positive reaction was observed as visualised cloudy contents in experimental vs. clean tubes (negative control). To detect haemolytic activity of the tested strains the latter were inoculated on the agar with 5% defibrinated human donors' blood and incubated at a temperature of 37 °C for 18 hours. The detection of visualised haemolytic zones (α or β) was performed, correspondingly. Finally, the ability to utilise the lecithin strains of *P. agglomerans* has been detected by opalescence zones of colonies growth on lecithin containing agar.

All the samples were tested for sensitivity to 7 antibiotics using Kirbi-Bauer method according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) recommendation: ampicillin (10 mg), amoxycilline/clavunate (20/10 mg), cefotaxime (10 mg), ceftazidime (30 mg),

ciprofloxacin (5 mg), amikacin (30 mg), gentamycin (10 mg) (HiMedia Laboratories Pvt. Limited, India). Inoculum was prepared by suspending of isolated 24-hour colony from non-selective agar medium in PBS with density of 0.5 by McFarland turbidity standard. Visible growth zone inhibition measurement was conducted in 24 hours of incubation.

P. agglomerans isolated from green beans (Turkey) which were characterised by the highest level of resistance to tested antibiotics had been chosen for *in vivo* experiment. Three groups of the BALB/c mice (9 animals per each group) of the same age, sex, weight, feed and drink (diet consumption) were formed and used in our study [2]. Suspension of the 24-hour *P. agglomerans* culture had been prepared in potassium-buffer solution (PBS) in three different concentrations ($1.4 \cdot 10^5$, $2.5 \cdot 10^6$ and $4.5 \cdot 10^8$ in 200 µl/mouse) and mice were orally inoculated.

Three mice from each group were sacrificed with CO_2 inhalation on the 24, 48 and 72nd hour of the experiment in accordance with ethical permit requirements. The following examinations had been carried out: (1) macroscopic observation of the changes of lungs, heart, liver, spleen, small and large intestine, caecum, Payer patches (PPs) and mesenteric lymph nodes (MLNs); (2) microbiological analyses of persistence of inoculant in the trachea, lung, heart, liver, spleen and other tissues in order to examine their potential to translocate into such parenchymal organs and detection of key intestinal representatives of host microbiota in content of colon – to estimate their influence on gut homeostasis.

Macroscopic assay was performed immediately after mice autopsy according to standard procedure, microbiological investigation was conducted by sterile instrument in laminar box with maintenance of aseptic conditions. During microbiological experiment the following media for the dissemination investigation were used: meat infusion agar, MacConkey agar, potato agar, Streptococcus- and Enterococcus agar, MRS agar, URI-select media. Final identification was carried out using commercial standard biochemical tests systems (API-tests and ENTERO-tests, correspondingly produced by bioMérieux, France and LACHEMA, Brno).

Results and Discussions. All of four tested strains of *P. agglomerans* were not able to coagulate serum, or demonstrate haemolytic and lecithin utilising properties. The strains of *P. agglomerans*, isolated from different countries, were characterized by approximately identical resistance to the tested antibiotics, in particular, to ampicillin, amoxycilline/clavunate and cefotaxime, which partly may indicate this feature as a species not strain-specific resistance. Two isolates were susceptible to ceftazidime and all the examined strains were sensitive to fluroquinolone and aminoglycosides (Table 1). Antibiotic-sensitivity was detected according to the EUCAST recommendation for *Enterobacteriaceae* in the last Clinical Breakpoint Table [3].

Table 1

Antibiotics relation of strains of *P.agglomerans* isolated from different plants

№	Antibiotics	*P.agglomerans*				EUCAST Zone diameter breakpoint, mm
		Strain 1, carrot, Bulgaria	Strain 2, green beans, Turkey	Strain 3, parsley, Ukraine	Strain 4, carrot, Ukraine	
1	Ampicillin (10 mg)	**Res**	**Res**	**Res**	**Res**	14 mm (Sens ≥, Res <)
2	Amoxycilline/ clavunate (20/10 mg)	*Sens*	**Res**	**Res**	**Res**	17 mm (Sens ≥, Res <)
3	Cefotaxime (10 mg)	*Sens*	**Res**	**Res**	**Res**	20 mm (Sens ≥), 17 mm (Res <)
4	Ceftazidime (30 mg)	**Res**	**Res**	*Sens*	*Sens*	22 mm (Sens ≥), 19 mm (Res <)
5	Ciprofloxacin (5 mg)	*Sens*	*Sens*	*Sens*	*Sens*	22 mm (Sens ≥), 19 mm (Res <)
6	Amikacin (30 mg)	*Sens*	*Sens*	*Sens*	*Sens*	16 mm (Sens ≥), 13 mm (Res <)
7	Gentamycin (10 mg)	*Sens*	*Sens*	*Sens*	*Sens*	16 mm (Sens ≥), 13 mm (Res <)

Notes: "Res" – resistant to antibiotic, "Sens" – sensitive to antibiotic

All tested doses of *P. agglomerans* inoculant did not result in the death of experimental animals. Macroscopic examination of internal organs at the same time have revealed the dose-dependent morphological alteration of some internal organs of experimental animal. Particularly, mice which received the highest dose of bacterial suspension were characterized by early (within first 24 hours) reaction to the inoculation: PPs and MLNs' hypertrophy, moderate hepatomegalia and flatulence. A group of mice, which received inoculums in lower doses had the same alterations, but later on (72 hours after bacterial gavage). The results of macroscopic examination are shown in Table 2.

Table 2

The results of macroscopic investigation of internal organs and lymph nodes of mice inoculated with different doses of *P. agglomerans*.

Dose (CFU/mouse)	Hours	Changes in internal organs
$1.4 \cdot 10^5$	24	No visible pathological changes observed
	48	Moderate hepatomegalia, cholecele
	72	Lungs – with intense vascular pattern; Liver – hepatomegalia, cholecele; Spleen – with intense vascular pattern; Small intestine – inflated, with intense vascular pattern and intestinal distension; Large intestine – proximal parts are inflated and overfilled by faeces, distal one is empty; Submandibular and subaxillary lymph nodes - enlarged
$2.5 \cdot 10^6$	24	Liver – enlarged, cholecele; Small intestine – moderately inflated; Large intestine – no visible pathological changes; Submandibular lymph nodes- enlarged
	48	Liver – enlarged; Small intestine – inflated; Large intestine - no visible pathological changes, thin faecal masses
	72	Hepatomegalia; Small and large intestine – inflated, thin fecal masses; Submandibular and subaxillary lymph nodes – enlarged; Mesenteric lymph nodes – enlarged
$4.5 \cdot 10^8$	24	No visible pathological changes
	48	Spleen – moderately enlarged; Small intestine –inflated; Large intestine – no visible pathological changes, thin faecal masses; Submandibular and axillary lymph nodes – enlarged
	72	Lungs - with intense vascular pattern; Hepatomegalia, cholecele; Spleen - with intense vascular pattern; Small intestine and large intestine – inflated; Submandibular and axillary lymph nodes – enlarged
Control mice		No visible pathological changes

Translocation of *P. agglomerans* into the spleen, lungs and liver was observed in the mice inoculated with the lowest dose of suspension; translocation into the heart – in the mice inoculated with the middle and the highest dose; and into the trachea in each experimental group on the 48th hour of the experiment (Table 3).

Table 3

Translocation of *P. agglomerans* into parenchymal organs of experimental animals in different inoculated doses in the dynamic

Amount of isolated *P. agglomerans* , CFU/g					
Time of experiment, hours	**24**	**48**			**72**
Experimental group, dose	1 $1.4 \cdot 10^5$	1 $1.4 \cdot 10^5$	2 $2.5 \cdot 10^6$	3 $4.5 \cdot 10^8$	1 $1.4 \cdot 10^5$
Trachea	$(6.5 \pm 0.3) \cdot 10^4$	$(5.7 \pm 0.27) \cdot 10^3$	$(1.8 \pm 0.1) \cdot 10^4$	$(1.8 \pm 0.12) \cdot 10^4$	$(4.4 \pm 0.47) \cdot 10^4$
Heart	0	0	$(5.5 \pm 0.24) \cdot 10^3$	$(4.5 \pm 0.27) \cdot 10^3$	0
Lungs	$(1.8 \pm 0.13) \cdot 10^6$	0	0	0	0
Liver	0	$(3.2 \pm 0.32) \cdot 10^3$	0	0	0
Spleen	$(5.8 \pm 0.45) \cdot 10^4$	0	0	0	0
Kidney	0	0	0	0	0

Notes: "0" means absence of bacterial growth

Changes of key microorganisms in content of the colon of all the experimental mice were not dependent statistically on the oral dose of *P. agglomerans* suspension.

The presence of all strains-inoculants in the gut of all experimental groups was confirmed; the insufficient decreasing of *Lactobacteriaceae* and moderate declining of lactose-positive *Escherichia coli* had been detected; the sufficient diminishing until to complete absence of *Enterococcus spp.,* which proportionally depended on concentration of inoculated strains suspension, had been also observed.

The summarized results of alteration of the gut microbiota homeostasis with oral gavage of different doses of *P. agglomerans* in all experimental groups of mice on the 72th hour after inoculation are presented in Table 4.

Table 4

The shift of key microbial isolates in colon in different groups of mice inoculated with *P. agglomerans* (72 hours) vs. to control group

Group of mice, number	Group 1	Group 2	Group 3	Control
P. agglomerans	$(2.1\pm0.2)\cdot10^3$	$(2.1\pm0.1)\cdot10^3$	$(6.3\pm0.3)\cdot10^4$	0
***Lactobacillus* spp.**	$(5.3\pm0.4)\cdot10^5$	$(3.3\pm0.3)\cdot10^4$	$(2.7\pm0.2)\cdot10^5$	$(2.6\pm0.3)\cdot10^5$
***Staphylococcus* spp.**	$(4\pm0.2)\cdot10^6$	0	$(4.76\pm0.4)\cdot10^4$	$(7.2\pm0.54)\cdot10^4$
***Streptococcus* spp.**	$(5.3\pm0.3)\cdot10^5$	$(1.3\pm0.1)\cdot10^5$	$(2\pm0.2)\cdot10^5$	$(4.3\pm0.7)\cdot10^3$
***Enterococcus* spp.**	$(9.47\pm0.5)\cdot10^2$	0	0	$(4.3\pm0.36)\cdot10^3$
***E. coli lactose* +**	$(2.1\pm0.12)\cdot10^6$	$(3.2\pm0.3)\cdot10^4$	$(3.17\pm0.2)\cdot10^4$	$(3\pm0.17)\cdot10^6$
***E. coli lactose* -**	$(2.2\pm0.2)\cdot10^3$	$(2.8\pm0.2)\cdot10^4$	$(5.6\pm0.37)\cdot10^4$	$(3.8\pm0.26)\cdot10^2$

Notes: "0" means absence of bacterial growth

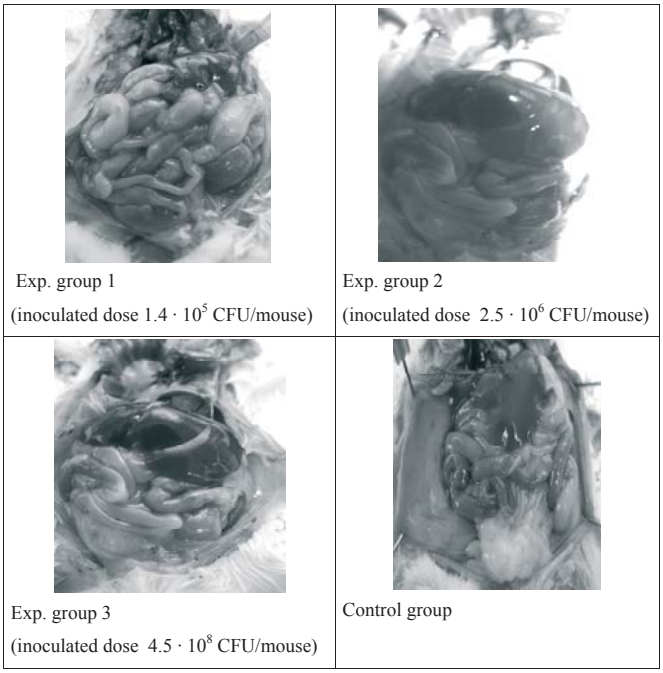

Fig. 1. Macroscopic changes of the abdominal cavity organs detected in mice, orally inoculated with different doses of *P. agglomerans* suspension and in control group

Finally, we can conclude that resistance of the investigated *P. agglomerans* strains to the most frequently used antibiotics (ampicillin, amoxicillin, cephalosporines) illustrates their importance as causative organisms in case of either food-borne or nosocomial infections. The absence of pathogenic properties *in vitro* could cause one to come to a false conclusion about *P. agglomerans* harmlessness, but detrimental effect on the organism could be proved within *in vivo* investigation. Different orally inoculated doses of bacterial suspension leave the life-threatening functions of the immunocompetent organism unaltered. But *P. agglomerans*-associated diseases could be crucial for immunocompromised organisms due to the bacteria's ability to provoke lymph nodes hypertrophy; to translocate into different internal organs as a precondition for the sepsis beginning; to change microbial content of faeces via the beneficial bacteria suppression up to the total absence.

Our results indicate the necessity of *in vivo* research in order to clarify the pathogenic properties of microorganisms with obligatory detection of their potential influence on microbial homeostasis of the intestine and then on the local (mucosal) immune response (data not shown here). This research is important to develop or improve the national and EFSA regulation to food safety and quality issues especially in case when vegetable products are fresh-eaten.

This work is funded under the EU FP7 Theme 2: Food, agriculture, fisheries, and biotechnology, Grant Agreement No: 227118.

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ІЗОЛЬОВАНА З ПОВЕРХНІ РОСЛИН *PANTOEA AGGLOMERANS* – НОВИЙ ПОГЛЯД НА ПРОБЛЕМУ ПОТЕНЦІЙНОЇ ПАТОГЕННОСТІ

Р е з ю м е

Штами *P. agglomerans* були ізольовані з поверхні різних їстівних рослин, які є основними компонентами традиційних страв країн чорноморського регіону. У даних ізолятів не виявлено факторів патогенності *in vitro*, однак штами характеризувались стійкістю до антибіотиків широкого спектру дії. Експерименти на мишах (BALB/c лінія) продемонстрували здатність *P. agglomerans* проникати в деякі внутрішні органи мишей і провокувати дозозалежні фізіологічні зміни в кишечнику та в асоційованій лімфатичній тканині кишечнику (КАЛТ).

К л ю ч о в і с л о в а: їстівні рослини, *Pantoea agglomerans*, потенційно патогенні бактерії, опортуністичні інфекції, якість харчових продуктів, харчові патогени.

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ИЗОЛИРОВАНАЯ С ПОВЕРХНОСТИ РАСТЕНИЙ *PANTOEA AGGLOMERANS* – НОВЫЙ ВЗГЛЯД НА ПРОБЛЕМУ ПОТЕНЦИАЛЬНОЙ ПАТОГЕННОСТИ

Р е з ю м е

Штаммы *P. agglomerans* были изолированы с поверхности различных съедобных растений, которые являются основными компонентами традиционных блюд стран черноморского региона. Нами не выявлено у них факторов патогенности *in vitro,* однако данные штаммы характеризовались устойчивостью к антибиотикам широкого спектра действия. Эксперименты на мышах (линия BALB/c) продемонстрировали способность *P. agglomerans* проникать во внутренние органы мышей и провоцировать дозозависимые физиологические изменения в кишечнике и в ассоциированной лимфатической ткани кишечника (КАЛТ).

К л ю ч е в ы е с л о в а**:** съедобные растения, *Pantoea agglomerans*, потенциально патогенные бактерии, оппортунистические инфекции, качество пищевых продуктов, пищевые патогены.

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Отримано 15.08.2011