Signs of pathogenicity by *Pasteurella multocida* in different species of animals

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Abstract

A significant number of microorganisms in natural and artificial environments exist in a structured formation – biofilm. This formation attaches to a certain surface, particularly the epithelium. The ability to form a similar structure has been observed in *Pasteurella multocida*, the causative agent of anthropozoonoses that affect domestic and wild animals, birds, companion animals and humans. The spectrum of pathogenetic action of *P. multocida* is wide and associated with the development of respiratory and multisystemic pathology, bacteraemia and other manifestations. Timely detection of *P. multocida* and treatment of the diseases it causes in farm and domestic animals is important to limit economic losses and improve social security. The main objective of this study was to determine the pathogenicity of *P. multocida*, its ability to form a biofilm, its resistance to antibiotics, and to identify the genes responsible for the formation of dermonecrotic toxin and biofilm formation. The paper presents the results of a study of 11 isolates of *P. multocida*: six isolates (54.5%) from rabbits, two isolates (18.2%) from dogs, two isolates (18.2%) from cats, and one isolate from pigs (9.2%). In all isolates, the gene ptfA was detected. This gene encodes the formation of type 4 fimbriae and participates in the formation of the biofilm, and the studied cultures *in vitro* formed a biofilm of different densities. The genome of eight isolates (72.7%) included the *toxA* gene (provides the formation of dermonecrotic toxin), while 45.4% of isolates had a complete set of the studied signs of pathogenicity, both in phenotypic (biofilm formation, mortality for laboratory animals) and genotypic (presence of *toxA*, ptfA) traits, and three isolates (27.3%) showed signs of multidrug resistance. The virulence of the *toxA*-negative isolates of *P. multocida* was lower than in *toxA*-positive isolates. The culture with the highest virulence (0.5×10^1 CFU) and extreme resistance to antibiotics formed a biofilm of the highest density. The association of the gene in the biofilm-producing mechanism needs further evaluation, and further research is needed to identify the relationships between pathogens in *Pasteurella multocida* isolates from different species of animals and humans.

Key words: *biofilm; Pasteurella multocida; antibiotic resistance; toxA; ptfA*

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Introduction

The evolution of infectious diseases requires the study of the biological properties of pathogens: morphological, enzymatic, molecular genetics, pathogenic, susceptibility to antibacterial agents, and demands a search for new alternatives for their treatment and prevention. An important area of research is the development of measures and methods for their prevention, including zoonoses – diseases transmitted from animals to humans through direct contact or through food, water, and the environment (Bruchmann et al., 2021; Guan et al., 2021).

A large variety of microorganisms, in addition to their capacity to attach to surfaces, also produce an extracellular polymeric substance. This substance forms a thin layer around cells known as biofilm, a structure that comprises an extracellular polymeric substance and the bacterial cells within it. This ability to form biofilm leads to the major pathogenic factor of bacterial infections. Biofilm protects against attacks from the immune system and against antibiotic treatment, hindering the eradication of these infections (Donlan and Costerton, 2002; Coenye and Nelis, 2010; Petruzzi et al., 2018).

Bacteria in biofilm mode undergo conspicuous changes in their genetic and phenotypic expression by expressing many novel proteins, constituted by the outer membrane and heat shock proteins (Petruzzi et al., 2018; Guan et al., 2021). Biofilms could cause chronic and recrudescent infections, that are difficult to control by treatment (Welin, 2004). The increased resistance of biofilms is explained by several factors: 1) different rates of diffusion of substances; 2) the accumulation of extracellular enzymes in the matrix that have a destructive effect on antibiotics; 3) inaccessibility of bacteria due to adhesion; 4) the resistant properties of the cells themselves (Jamal et al., 2018; Guan et al., 2019; Petruzzi et al., 2020).

Various adhesins helps gram-negative bacteria in the colonisation of host tissues. One of these adhesins is type 4 fimbriae (pili). These structures allow gram-negative bacteria to colonise epithelial surfaces. This pili structure can be observed in *Pasteurella multocida* strains A, B and D. The type 4 fimbrial subunit protein (ptfA) was identified as an 18-kDa protein isolated from whole membrane fractions (Doughty et al., 2000).

Biofilm formation of *P. multocida* has become a new perspective of its virulence study, since it is a respiratory zoonotic pathogen and its ability to form biofilm could possibly be one of its virulence factors for survival inside the host (Steen et al., 2010; Rajagopal et al., 2013; Peng et al., 2017; Guan et al., 2020).

The main objective of this study was to describe biofilm formation of *P. multocida*, methods for its detection and the presence of genes responsible for dermonecrotic toxin and biofilm formation.

Materials and methods

This study was performed at the Ukrainian Laboratory of Quality and Safety of Agricultural Products and the Department of Epizoology, Microbiology and Virology of the National University of Life and Environmental Sciences of Ukraine (Kyiv, Ukraine) during 2019–2020. Research conducted with the use of animals in accordance with the requirements of the Ukraine Law On Protection of Animals from Cruelty, and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

We used a total of 11 cultures of *P. multocida*, isolated from various sick and clinically healthy animals. Cultures of *P. multocida* were isolated as a result of bacteriological examination of pathological material (from heart and liver blood) from 24 dead rabbits, 5 piglets, and the pharyngeal and tonsil smears from 12 clinically healthy dogs and 9 cats (veterinary clinic patients). Bacteriological studies were performed in accordance with the current requirements for bacteriological diagnosis of pasteurellosis in animals and the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

LD_{100} was determined by subcutaneous injection of test cultures in doses of $0.5x10^1$–$0.5x10^9$ CFU, the results were recorded for 72 hours. The LD_{100} was taken as the minimum amount of the studied culture, which caused 100% death of experimental animals.

A total of 11 animal *P. multocida* isolates were examined for virulence-associated and biofilm-associated genes (*ptfA* and *toxA*) using various polymerase chain reaction (PCR) methods reported elsewhere (Curtis, 1985).

Antibiotic susceptibility of *P. multocida* cultures was determined using the disc-diffusion method using discs produced by HiMedia (India), and Mueller-Hinton II agar with 5% horse blood produced by GRASO (Poland). Studies and the interpretation of results were performed according to EUCAST recommendations (version 12, available at: https://www.eucast.org), which provide specific recommendations for determining the susceptibility of *P. multocida* (Magiorakos et al., 2012).

The ability to form biofilms in the derived isolates was determined and the results interpreted (Kukhtyn and Krushelnytska, 2014). This study was performed using sterile polystyrene Petri dishes (Greiner Bio-One GmbH, Germany) of *d*=100 mm, in which 10 mL brain-heart infusion broth (HiMedia, India) and 1 mL inoculum with a cell content of 0.5 were added to the MacFarland daily culture of studied *P. multocida* isolates. The plates were cultured in a thermostat at a temperature of 37°C for 24 hours, the residues of the nutrient medium were carefully removed, the planktonic forms were washed three times with a sterile phosphate buffer solution ($KH_2PO_4 \cdot Na_2PO_4 \cdot H_2O$), pH 7.2–7.4. The Petri dishes were air-dried and 10 mL 96% ethanol was added to fix the formed biofilms. The fixation exposure was 10 minutes. The fixing liquid was drained and then the Petri dishes were divided into two parts: the first was stained with 0.1% alcohol solution of crystal violet for 10 min, and the second was stained with a mixture of saturated aqueous Congo red for 15 min. Plates were washed three times with sterile phosphate buffer solution (pH 7.2) and dried. Contributed to 10,0 mL 96% ethanol and placed on a shaker for shaking for 30 min, were then pipetted transferred to a cuvette and determined the optical density spectrophotometricall on an Evolution 300 spectrophotometer (Thermo Fisher Scientific, USA) at a wavelength of 570 nm for Petri dishes with solution of crystal violet and 495 nm for Petri dishes with solution of Congo red. The density of the formed biofilm was determined by measuring the adsorption level of the dye with ethanol measured in units of optical density (OD) using a spectrophotometer.

When the value of optical density is less than 0.1, strains were not considered to form a biofilm. At optical densities from 0.1 to 0.49 the ability to form a film was considered low, from 0.5 to 1.0 medium with medium density, and at values above 1.0 high with high density (Ewers et al., 2000).

Genomic DNA from *P. multocida* cultures was isolated by the express method. For this, the lyophilized mass of

Table 1. Characteristics of primers that were used in the studies

Gene	Primer sequences 5` - 3`	Product size (bp)	Reference
toxA	F: TCTTAGATGAGCGACAAGG R: GAATGCCACACCTCTATAG	846	(Rajagopal et al., 2013)
ptfA	F: AAT GCC AGT CCA CTC GTT GT R: CGTCCTGAGGCAAGCGTGTT	435	(Doughty et al., 2000)

Table 2. Results of determination of *toxA* gene, virulence, antibiotic resistance, *ptfA* biofilm gene in isolates of *P. multocida*, and the results of phenotypic determination of biofilm formation (by crystal violet and Congo red staining)

№ з/п	Strain ID	Host species	toxA	Virulence LD_{100}, *CFU, white mouse (16–18 g) (n=10)	Antibiotic resistance	ptfA	Density of the formed biofilm (n=6)			
							Crystal violet		Congo red	
							λ 570	%	λ 495	%
1	P2	Rabbits	-	$0.5x10^6$	-	+	0.1683	100	0.1455	87.5
2	P5	Rabbits	+	$0.5x10^4$	MDR	+	0.8593	100	0.8217	95.6
3	P15	Rabbits	+	$0.5x10^4$	-	+	0.1344	92.1	0.1460	100
4	P16	Rabbits	+	$0.5x10^4$	-	+	0.5505	100	0.5046	91.7
5	P17	Rabbits	+	$0.5x10^6$	-	+	0.2162	95.7	0.2258	100
6	P50	Rabbits	+	$0.5x10^1$	XDR	+	1.7893	100	1.7301	96.7
7	PC	Dogs	-	$0.5x10^6$	MDR	+	0.3082	100	0.2014	97.8
8	P97	Dogs	+	$0.5x10^6$	-	+	0.5053	93.9	0.5381	100
9	P99	Cats	+	$0.5x10^4$	-	+	0.2485	100	0.2157	86.8
10	P99p	Cats	+	$0.5x10^4$	-	+	0.2458	82.8	0.3001	100
11	P69p	Swine	-	$0.5x10^6$	-	+	0.1801	100	0.1691	93.9

* CFU – colony forming units

the culture of *P. multocida* was dissolved in 1 mL sterile buffered peptone water (HiMedia, India) and centrifuged at 13,500 rpm for 2 min and the supernatant was removed. The bacterial pellet was resuspended in 200 µL TE buffer and incubated in a thermostat at 95°C for 5 minutes. Cell debris was precipitated by centrifugation at 5000 rpm for 2 min and 180 µL supernatant was taken for the PCR. The DNA concentration was measured on a Biofotomer spectrophotometer (Eppendorf, Germany) at a wavelength of 260 nm. The amplification reaction was performed in a reaction mixture with a volume of 25 µL, with the following composition: 1x PCR buffer, 2.5 mM $MgCl_2$, 2.0 mM each deoxynucleotide triphosphates, 10 pM each primers for detection and 1 unit DNA polymerase. DNA was added in an amount of 5.0 µL (100-150 ng). Studies were performed on a thermal cycler 2720 (Applied Biosystems, USA) with the temperature

profile given in the relevant literature source. The nucleotide sequences and other characteristics of the primers used in the study are given in Table 1. The amplification products were separated in 1.5% agarose gel.

Molecular genetic studies were performed to identify the presence of the genes *toxA*, which is responsible for producing a dermonecrotic toxin, and *ptfA*, which encodes production of the products that assemble to form type 4 fimbriae on the bacterial surface and are responsible for biofilm formation (Devi et al., 2018; Gong et al., 2020).

Results

This paper presents the results of a study of 11 isolates of *Pasteurella* isolated from pathological material from 24 rabbits and five piglets, and smears from 12 clinically healthy dogs and nine cats (Table 2): six isolates (54.5%) were from rabbits, two isolate (18.1%) from dogs, two (18.1%) from cats, and one (9.1%) from pigs.

The virulence factor of the *toxA* gene (Table 2, Figure 1), which is responsible for the formation of a dermatonecrotic toxin, was present in 72.7% of cases in *P. multocida* strains P5, P15, P16, P17, P50, P97, P99, and P99p.

The isolate *P. multocida* P50 had the highest virulence. At 6 hours after infection, the death of 100% of animals was recorded in all experimental groups, LD_{100} - 0.5×10^1 CFU (Table 2). LD_{100} for the isolates of *P. multocida* P5, P15, P16, P99 and P99p was 0.5×10^4 CFU, the death of experimental animals was recorded 14–18 hours after infection. LD_{100} for isolates of *P. multocida* P2, P17, PC, P97 and P69p was 0.5×10^6 CFU, and the death of experimental animals was recorded 24 hours after infection.

The sensitivity of *P. multocida* isolates to the antibacterial drugs penicillin, cephalosporin, fluoroquinolol and tetracycline groups was determined. The susceptibility to *P. multocida* antibiotics are shown in Table 3: 54.5% of isolates were resistant to benzylpenicillin, 45.4% to cefotaxime, 18.2% to ampicillin, and none of the studied isolates showed resistance to amoxicillin.

Examining the group of fluoroquinolones showed that 27.3% of isolates of *P. multocida* were resistant to ciprofloxacin and 54.4% to levofloxacin. Resistance to nalidixic acid (screening) was demonstrated at 18.2% for each drug. Screening for fluoroquinolone resistance for nalidixic acid sensitivity was positive for only four (36.4%) *P. multocida* isolates

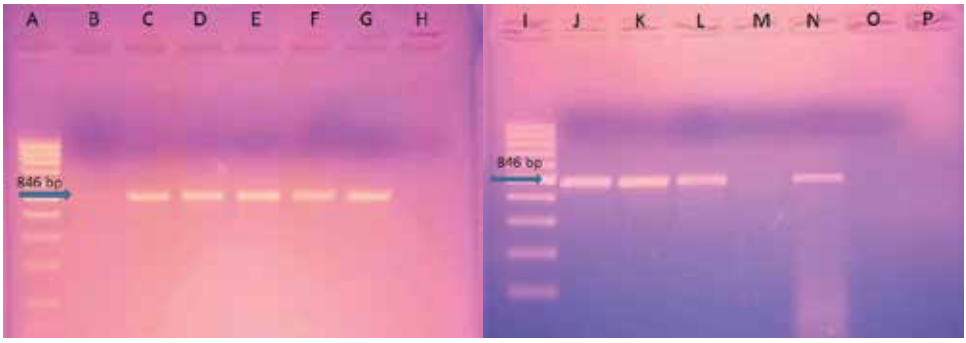

Figure 1. *P. multocida* polymerase chain reaction for the detection of the *toxA* gene (846 bp) of *P. multocida*. Lane A and I – marker lanes; lanes B, H, M: samples negative for *toxA* gene; Lanes C, D, E, F, G, J, K, L: samples positive for *toxA* gene; Lane N – positive control for *toxA* gene; Lane O – negative control for *toxA* gene

Table 3. Sensitivity testing to antibiotics in isolated isolates of *P. multocida*, according to the EUCAST recommendations, (mm)

Antibiotics	EUCAST S≥; R<	P2	P5	P15	P16	P17	P50	PC	P97	P99	P99p	P69p
Penicillins												
Benzylpenicillin	17	26 S	10 R	10 R	20 S	30 S	14 R	6 R	24 S	20 S	6 R	12 R
Ampicillin	17	22 S	6 R	28 S	22 S	34 S	9 R	30 S	20 S	26 S	25 S	22 S
Amoxicillin	17	24 S	26 S	28 S	10 S	24 S	20 S	21 S	24 S	20 S	19 S	22 S
Cephalosporins												
Cefotaxime	26	24 R	24 R	32 S	24 R	30 S	24 R	26 R	38 S	30 S	32 S	34 S
Fluoroquinolones												
*Nalidixic acid	23	**28 S**	26 S	32 S	29 S	36 S	**20 R**	31 S	10 R	**30 S**	29 S	**24 S**
Ciprofloxacin	27	**32 S**	30 S	30 S	31 S	36 S	**26 R**	21 R	32 S	**29 S**	26 R	**42 S**
Levofloxacin	27	**34 S**	24 R	20 R	24 R	22 R	**26 R**	26 R	30 S	**38 S**	38 S	**30 S**
Tetracyclines												
Doxycycline	24	20 R	26 S	31 S	28 S	24 S	16 R	14 R	18 R	21 R	26 S	22 R
Tetracycline	24	26 S	24 S	24 S	32 S	30 S	20 R	30 S	26 S	28 S	25 S	16 R

*Nalidixic acid susceptibility testing by disk diffusion can be used to screen for resistance to fluoroquinolones (EUCAST version 12.0).

(P2, P50, P99, P69p).

A study of the tetracycline group found that 54.5% of isolates were resistant to doxycycline and 18.2% of *P. multocida* isolates were resistant to tetracycline.

Isolate P5 showed resistance to the group of penicillins (benzylpenicillin, ampicillin), cephalosporins (cefotaxime), fluoroquinolones (levofloxacin), indicating that this isolate is multidrug-resistant (MDR). The isolate PC was also included in this category of resistance, showing resistance to the group of penicillins (benzylpenicillin), cephalosporins (cefotaxime), fluoroquinolones (levofloxacin, ciprofloxacin), tetracyclines (doxycycline). Isolate P50 with extreme multidrug-resistance (XDR) showed resistance to the group of penicillins (benzylpenicillin, ampicillin), cephalosporins (cefotaxime), fluoroquinolones (levofloxacin, ciprofloxacin), tetracyclines (doxycycline, tetracycline), and nalidixic acid.

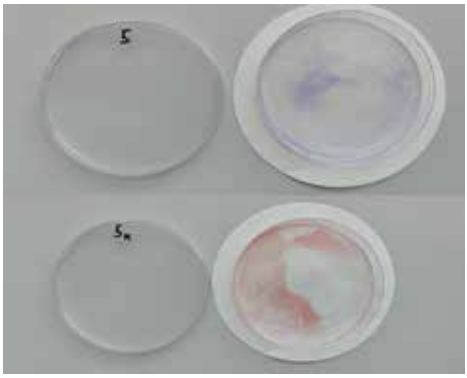

Figure 2. Biofilm formation of *P. multocida* stained with crystal violet (top) and Congo-red (bottom), dishes on the left – control

The density of the biofilm was determined by staining methods with crystal violet and Congo red (Table 2, Figure 2). The results showed that these staining methods provide close in value results, deviations of the optical density results were registered in the range of 2.2–17.2%, which did not exceed 15%. Therefore, in the study of the phenotypic biofilm formation, staining with Congo-red did not differ from the method with crystalline violet.

In the study of the phenotypic biofilm formation using crystalline violet and Congo-red staining methods It was found that one isolate (9.1%) formed a high-density biofilm (λ-1.7). The medium-density biofilm was formed by three (27.3%) isolates *P. multocida*: P5 - λ 0.8, P16 - λ 0.5, P97 - λ 0.5. The low-density biofilm (λ 0.13 - 0.3) was formed by 63.6% of the studied isolates *P. multocida*.

In three isolates (isolated from rabbits and pigs, LD_{100} $0.5x10^4$–$0.5x10^6$ CFU) the density of the biofilm ranged from λ 0.1344–0.1801. In four isolates, the optical density of the biofilm in vitro was λ 0.2162–0.3082. These isolates were

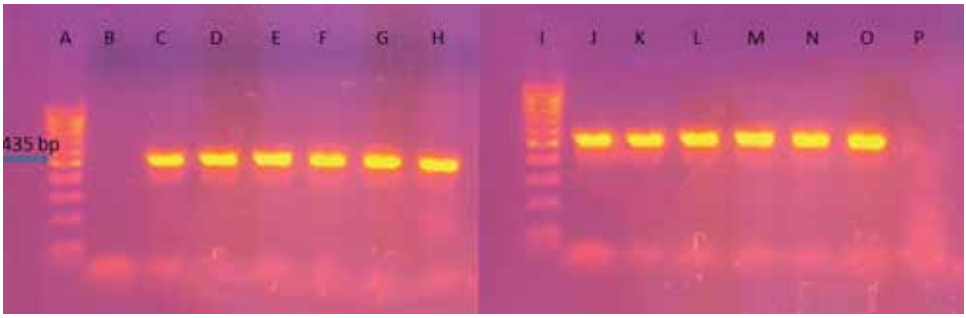

Figure 3. *P. multocida* polymerase chain reaction for the detection of the *ptfA* gene (435 bp) of *P. multocida*. Lane A and I - marker lanes; Lanes D, E, F, G, H, J, K, L, M, N, O: samples positive for *ptfA* gene; Lane C - positive control for *ptfA* gene; Lane B – negative control for *ptfA* gene

isolated from rabbits (1), dogs (1) and cats (2), and virulence was $0.5x10^4$–$0.5x10^6$ CFU. In two isolates, the optical density of the biofilm was λ 0.5053–0.5505. These isolates were isolated from rabbit and dog, and LD_{100} was $0.5x10^4$ and $0.5x10^6$ CFU, respectively. One culture of *P. multocida* isolated from rabbits with a virulence of LD_{100} $0.5x10^4$ formed a biofilm *in vitro* with an optical density λ 0.8593. The isolate with the highest virulence LD_{100} $0.5x10^1$ formed a biofilm with the highest optical density λ 1.7893.

Finally, it should be noted that all isolates possessed the *ptfA* gene (Table 3, Figure 3), which is responsible for the formation of special adhesives (type 4 pili).

Discussion

In this study, 11 isolates of *P. multocida* were used. *P. multocida* isolates were pathogenic for white mice, LD_{100} ranged from $0.5x10^1$ to $0.5x10^6$ CFU. The virulence index LD_{100} $0.5x10^6$ CFU was found in isolates from rabbits (2), dogs (2), pigs (1). The virulence index LD_{100} $0.5x10^4$ CFU was found in five isolates from rabbits (3) and cats (2), while the virulence index LD_{100} $0.5x10^1$ CFU was found in the isolate from rabbit.

Among the studied strains, eight (72.7%) were *toxA* positive (formed dermonecrotic toxin), while five (45.4%) had a complete set of phenotypic (biofilm formation, lethality) and genotypic (*toxA*) traits. Three strains (27.3%) showed significant signs of multidrug resistance. All studied isolates possessed the gene *ptfA* that encodes the formation of fimbriae type 4, which participates in the formation of the biofilm. Virulence in *toxA*-negative *P. multocida* was lower compared to *toxA*-positive isolates.

It should be noted that the determination of biofilm density obtained by staining crystal violet and Congo red did not show a deviation of optical density results exceeding 15%.

As a result of determining the sensitivity of *P. multocida* isolates to antibacterial drugs, it was found that the cultures P5 and PC showed signs of multiple drug resistance MDR, and culture P50 exhibited extensive drug resistance (XDR).

The ability to form a biofilm and the presence of the gene for the adhesive ability of cultures was found in all strains of *P. multocida*, though there was a tendency to increase the optical density of the biofilm at a higher strain virulence. This indicates the ability to form a biofilm in *P. multocida* as another possible sign of bacterial virulence.

It should also be noted that two strains of *P. multocida* that showed signs of polyresistance (P5 and P50), were isolated from rabbits from a single farm, and this indicate that the antibiotic-resistant strains of *P. multocida* are confined to this farm. It is also important to distinguish MDR-Pasteurella from a dog as a companion animal, which can make this a medical issue, since *P. multocida* can cause pathological processes in both animals and humans.

The results indicate a possible relationship between the level of *P. multocida* virulence and the density of the formed biofilm; we believe that an in-depth study of this issue will be the subject of our future research.

It should also be noted that the phenotypic manifestation of the ability to form biofilms in this study did not coincide with the results of the relevant genetic marker detection. Indirectly, this fact indicates the need for in-depth study of this phenomenon to identify previously unknown genetic loci that determine the phenotypic manifestation of resistance and the ability to form biofilms, which is important for understanding the pathogenesis of pasteurellosis and their effective detection. In addition, data on the prevalence of virulence factors will be the scientific basis for improving the specific prevention of animal pasteurellosis.

Conclusion

As a result of the study of 50 samples of biological material from animals, 11 isolates of *P. multocida* were isolated: six from rabbits, two from dogs, two from cats and one from pigs.

Isolated isolates of *P. multocida* were sensitive to amoxicillin; one isolate was assigned to XDR, and two isolates to MDR. Screening of susceptibility of *P. multocida* isolates to fluoroquinolones by the disc-diffusion method, sensitivity to nalidixic acid did not coincide in 63.6% of cases, which gives grounds for further studies.

The isolated cultures were pathogenic to white mice. The LD_{100} of isolate P50 was $0.5x10^1$ CFU in the lightning course of the disease. In five isolates, LD_{100} was $0.5x10^4$ CFU, and in five others was $0.5x10^6$ CFU. No association was established between virulence and the species of animals from which *P. multocida* has been isolated. The *toxA* gene was detected in eight cultures with LD_{100} $0.5x10^1$–$0.5x10^6$ CFU isolated from rabbits, dogs and cats; in three isolates with LD_{100} $0.5x10^6$ CFU this gene was not detected.

In the study of phenotypic biofilm formation using Congo-red staining, the results did not differ from the method with crystalline violet. The genome of all studied isolates of *P. multocida* contained the *ptfA* gene; the studied cultures *in vitro* formed a biofilm of varying density. The culture with the highest virulence ($0.5x10^1$ CFU) and extreme resistance to antibiotics formed a biofilm of the highest density.

From this study, it can be concluded that the *toxA* gene is an important marker gene for defining the pathogenic potential of *P. multocida* strains in various animals. However, other virulence genes are also found to be widely distributed among pathogenic strains of *P. multocida*. The association of the gene in the biofilm-producing mechanism needs further evaluation. Further research is also needed to identify the relationships between pathogens among *P. multocida* isolates from different species of animals and humans.

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Studija znakova patogenosti kod *Pasteurella multocida*, izolirane iz životinja različitih vrsta

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Znatan broj mikroorganizama u prirodnim i umjetnim okruženjima postoji u obliku strukturirane formacije – biofilma i ta se formacija može vezati i na određenu površinu, posebice na epitel. Mogućnost formiranja slične strukture zamijećena je kod *Pasteurella multocida*, koja je uzročnik antropozoonoza, a pogađa i ljude, kućne ljubimce, ptice i domaće i divlje životinje. Spektar patogenih učinaka *P. multocida* prilično je širok i povezan je s razvojem respiratorne i multisistemske bolesti, bakterijemije i drugih manifestacija. Pravovremeno otkrivanje *P. multocida* i liječenje bolesti koje ova bakterija prouzroči u životinja na farmi i domaćih životinja važno je za ograničenje ekonomskih gubitaka i sigurnost društva. Glavni je cilj ove studije bio utvrditi patogenost *P. multocida*, sposobnost formiranja biofilma, otpornost na antibiotike, ali i identificirati gene odgovorne za formiranje dermonekrotičnog toksina, odnosno formiranje biofilma. Ovaj rad predstavlja rezultate studije 11 izolata *P. multocida*: 6 izolata (54,5 %) izoliranih iz zečeva, 2 (18,2 %) iz pasa, 2 (18,2 %) iz mačaka te 1 izolat izoliran iz svinja (9,2 %). U 100 % proučavanih izolata, otkriven je gen (*ptfA*) koji kodira formiranje fimbrija tipa 4 i sudjeluje u formiranju biofilma. Ispitane su kulture *in vitro* formirale biofilm različitih gustoća. U genomu 8 ispitanih izolata (72,7 %) otkrivena je prisutnost *toxA* gena (koji omogućuje formiranje dermonekrotičnog toksina). 45,4 % ispitanih izolata pokazalo je cijeli set proučavanih znakova patogenosti – fenotipskih (formiranje biofilma, smrtnost za laboratorijske životinje) karakteristika i genotipskih (prisutnost *toxA*, *ptfA*) karakteristika. Tri izolata (27,3 %) pokazala su otpornost na više lijekova. Otkriveno je da je kod *toxA*-negativnih izolata *P. multocida* virulencija bila niža u usporedbi s *toxA*-pozitivnim izolatima. Kultura s najvišom virulentnošću (0,5 x 10^1 CFU) i ekstremnom otpornošću na antibiotike formirala je biofilm najveće gustoće. Asocijacija gena u mehanizmu proizvodnje biofilma zahtijeva dodatnu procjenu, a potrebno je i dodatno istraživanje za identifikaciju odnosa između patogena među izolatima *P. multocida* izoliranima iz ljudi i različitih vrsta životinja.

Ključne riječi: *biofilm, Pasteurella multocida, otpornost na antibiotike, toxA, ptfA*