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The relationship between biofilm formation, genes of virulence and iron metabolism in *Escherichia coli*

Introduction

Urinary tract infections (UTIs) are the most prevalent infectious diseases, and very problematic worldwide (Navidinia et al., 2018). Uropathogenic *Escherichia coli* T. Escher. (UPEC), which can colonise successfully in the urinary tract, is the primary etiologic agents associated with UTI (Peerayeh et al., 2018). Avian pathogenic *E. coli* (APEC) cause septicemia, polyserositis, aerosacculitis and other mainly extraintestinal diseases in chickens and other avian species. APECs are found in the intestinal microbiota of healthy birds, and most of the diseases associated with them are secondary to environmental and host predisposing factors (Dho-Moulin, Fairbrother, 1999). The common presence of a set of virulence-associated genes among as well as similar disease patterns and phylogenetic background indicate a genetic relationship between APEC and UPEC isolates (Kaper et al., 2004; Moulin-Schouleur et al., 2006; Ron, 2006). The success of *E. coli* in colonising such a wide range of hosts and environments is basically due to a noticeable ductility in exploiting the available resources. It is becoming increasingly clear that biofilms have an enormous impact on medicine (Mah, O'Toole, 2001; Wang et al., 2017), since 65% of human microbial infections involve biofilms (Labbate et al., 2004). Microbial biofilm formation is now recognised as a principle virulence factor in many localised chronic infections (Hyun Koo et al., 2017), and their role in infecting the biological devices among hospitalised patients is a universally accepted fact (Vasudevan, 2014). In addition, recent experimental evidence indicates a role of biofilm formation in acute infections (Hannan et al., 2012; Kumagai et al., 2011). Understanding biofilm formation to find effective ways to prevent biofilms is important for combating disease.

The primary aim of this study was to detect *E. coli* strains with a biofilm formation from animals and the detection of APEC virulence genes presence in these strains in compared to the ability of production a biofilm.

Material and methods

Escherichia coli strains were isolated from broilers rectal swab coming from farms of Eastern Slovakia. Samples were resuscitated overnight at 37°C in buffered peptone water (Oxoid, Basingstoke, UK) and subcultured on Mac Conkey agar (Oxoid) and UriSelect agar (Bio-Rad Laboratories, Hercules, CA, USA) again overnight at 37°C. The colonies were isolated, identified, and confirmed as *E. coli* by commercial identification microsystem ENTEROtest24 (ErbaLachema Brno, Czech Republic) and by using the MALDI-TOF MS biotyper (Bruker Daltonics, Bremen, Germany). Nineteen strains were selected for further testing.

Biofilm formation

The ability of biofilm formation was assessed in a quantitative assay using a micro-titer-plate test (Nunc, Roskilde, Denmark). Strains grown on BHI agar and colonies were re-suspended in BHI broth (Oxoid, UK) to reach the 0.5 suspension McFarland's standard, and volumes of 200 μ l of these cell suspensions were transferred to wells of the microplate. After incubation (24 h/37°C), adherent cells were washed three times using a saline solution and stained with a 0.1% crystal violet solution (Mikrochem, Pezinok, Slovakia) for 15 min. Afterwards, excess stain was rinsed off by filling the wells with sterile distilled water. The adhering dye was dissolved with 30% acetic acid for 15 minutes and the optical density was measured at 570 nm in Synergy HT Multi-Mode Microplate Reader (BioTek, USA) (Čuvalová, 2018). We divided isolates of *E. coli* into four classes based on Stepanovic et al. 2007. For classification, we used average optical density (OD) value and cut-off value (ODc) (defined as three standard deviations (SD) above the mean OD of the negative control). The final OD value of a tested strain was expressed as the average OD value of the strain reduced by the ODc value. For interpretation of the results, strains were divided into the following categories: $OD \leq ODc$ = non-biofilm producer; $ODc < OD \leq 2 \times ODc$ = weak biofilm producer; and, $2 \times ODc < OD \leq 4 \times ODc$ = moderate and $4 \times ODc < OD$ = strong biofilm producers.

Detection of genes by PCR

Screening of *E. coli* isolates for APEC virulence genes were carried out by polymerase chain reactions with the amplification of the following: the receptor for aerobactin – *iutA* (Johnson, Stell, 2000); colicin V – *cvaC* (Johnson, Stell, 2000); increased serum survival – *iss* (Foley et al., 2000); temperature sensitive haemagglutinin – *tsh* (Dozois et al., 2000); P fimbrial adhesion – *papC* (Le Bouguéneć et al., 1992); capsular polysialic acid virulence factor – *kps* (Johnson, Stell, 2000); iron-regulated gene a homologue adhesion – *Iha* (Johnson et al., 2000) and genes of iron metabolism – putative iron transport gene – *sitA* (Rodrigues-Siek et al., 2005); iron-related genes – gene which

mediates ferric iron uptake *feoB* (Rodrigues-Siek et al., 2005), encodes an iron-responsive element and putative siderophore receptor gene – *IreA* (Russo et al., 2001) and iron repressible gene associated with yersiniabactin synthesis – *irp2* (Schubert et al., 1998), yersiniabactin receptor for ferric yersiniabactin uptake – *fyuA* (Schubert et al., 1998), and the catecholate siderophore receptor gene – *IroN* (Johnson, Stell, 2000), and primers are listed in table 1.

Tab. 1. Primers used for detection of virulence genes and genes of iron metabolism

Gene	Primer sequences (5'–3')	Annealing [°C]	Size [bp]
<i>iutA</i>	F: GGCTGGACATGGGAACCTGG R: CGTCGGGAACGGGTAGAATCG	63	300
<i>cvaC</i>	F: CACACACAAACGGGAGCTGTT R: CACACACAAACGGGAGCTGTT	63	680
<i>iss</i>	F: ATCACATAGGATTCTGCCG R: ACAAAAAGTTCTATCGCTTCC	61	700
<i>tsh</i>	F: GGTGGTGCACCTGGAGTGG R: AGTCCAGCGTGATAGTGG	55	620
<i>papC</i>	F: GACGGCTGTACTGCAGGGTGTGGCG R: ATATCCTTTCTGCAGGGATGCAATA	61	328
<i>kps</i>	F: GCGATTTGCTGATCGTTG R: CATCCAGACGATAAGCATGAGCA	63	272
<i>Iha</i>	F: CTGGCGGAGGCTCTGAGATCA R: TCCTTAAGCTCCCGCGGCTGA	60	827
<i>sitA</i>	F: AGGGGGCACAACCTGATTCTCG R: TACCGGGCCGTTTTCTGTGC	59	608
<i>feoB</i>	F: AATTGGCGTGCATGAAGATAACTG R: AGCTGGCGACCTGATAGAACAATG	59	470
<i>IreA</i>	F: TGGTCTTCAGCTATATGG R: ATCTATGATTGTGTTGGT	55	415
<i>irp2</i>	F: AAGGATTCGCGTGAC R: TCGTCGGGCAGCGTTTCTTCT	59	287
<i>fyuA</i>	F: TGATTAACCCCGCGACGGGAA R: CGCAGTAGGCACGATGTTGTA	55	880
<i>IroN</i>	F: AAGTCAAAGCAGGGGTTGCCCG R: GACGCCGACATTAAGACGCAG	60	655

Results

The interpretation of obtained results requires a definition of the cut-off value that separates biofilm producing from non-biofilm-producing strains. We divided isolates based upon the previously calculated OD values, which was a modification of method and classification described by Stepanović et al. (2007): very weak 12/19 (63.0% of strains), weak 2/19 (10.5%), moderate 2/19 (10.5%) and strong 3/19 (16.0%) biofilm producers. The occurrences of 13 detected genes are presented in figure 1. Among 19 *E. coli* isolates, all isolates contained the *feoB* gene, 16 isolates contained the *sitA*

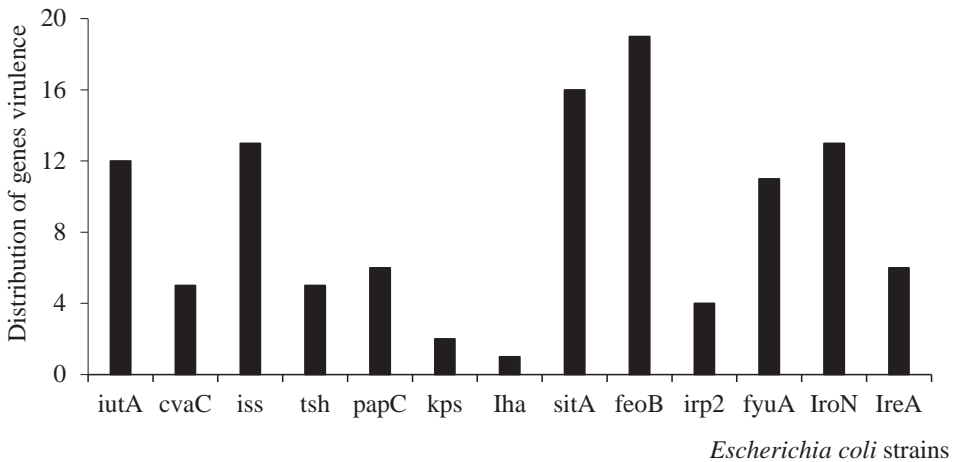


Fig. 1. Distribution of genes virulence in *Escherichia coli* strains: *iutA* (12/19), *cvaC* (5/19), *iss* (13/19), *tsh* (5/19), *papC* (6/19), *kps* (2/19), *Iha* (1/19), *sitA* (16/19), *feoB* (19/19), *Irp-2* (4/19), *fyuA* (11/19), *IroN* (13/19) and *IreA* (6/19)

gene, 13 isolates contained the *iss* and *iroN* genes, 12 isolates contained the *iutA* gene, 11 isolates contained the *fyuA* gene, 6 isolates contained the *papC* and *IreA* genes, 5 isolates contained the *cvaC* and *tsh* genes, 4 isolates contained the *irp2* gene, and 2 isolates contained the *kps* gene.

For better comparison of our results, we created two groups of strains (Fig. 2). The first of the two groups represented very weak biofilm producers and the second group represented weak, moderate, and strong formers. Representation genes of virulence were high in isolates from the first group – from seven genes were six highly, only *papC* was low. Genes of iron metabolism were different. Genes *sitA*, *fyuA*, and *ireA* were higher in the second group, and *feoB*, *irp2* and *iroN* were higher in the first group.

Discussion

Genes coding adhesins, toxins, or iron acquisition systems have been described to be of particular importance during the pathogenesis of septicemia (Gyles, 1994; Babai et al., 1997; Terlizzi et al., 2017; Robinson et al., 2018), and iron acquisition is a requirement for UPEC survival in an environment that is as iron-limited as the urinary tract (Skaar, 2010). Isolated *E. coli* strains were investigated for the presence of thirteen virulence genes that are associated with colibacillosis and iron metabolism.

Two genes, *fyuA* and *irp2*, coding proteins involved in iron acquisition, were described in *Yersinia* sp., and this iron acquisition determinant has been found in human septicemic and enteroaggregative *E. coli* isolates (Karch et al., 1999; Pelludat et al., 1998; Schubert et al., 1998). The sequences of the *irp2* and *fyuA* genes in *E. coli* are al-

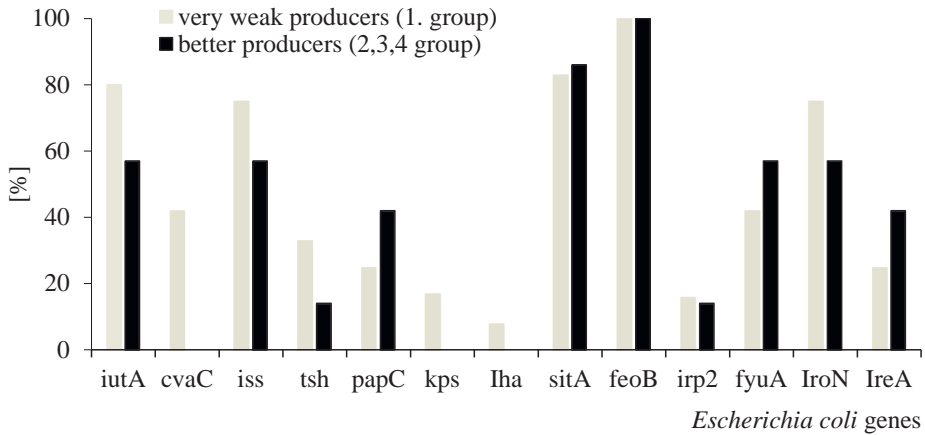


Fig. 2. Presence of detected genes expressed as a percentage; divided in two groups: first group = first column of data (very weak producers) and second group = second column of data (weak, moderate and strong biofilm producers)

most identical to those of *Yersinia* spp. (Germon et al., 2005) and have been described in APEC isolates by Gophna et al. (2001), Subedi et al. (2018). From nineteen isolates in our study, we detected *irp-2* gene in four and *fyuA* in eleven strains. To increase survival and resistance, *E. coli* strains also form biofilm, but published data is variable, depending on the strain origin, different types of surfaces, culture medium, and the methodology used for quantifying biofilm. In our study, the presence of genes of virulence was low in second group – better biofilm formers and only *papC* was higher. To compare with another study (Naves et al., 2008; Pavlickova et al., 2017), *papC* was also determined in a strain with better forming biofilm, and *tsh*, which was similar to our results, was detected in weak biofilm formers. *IutA* was represented in both groups of strains with weak and strong production of biofilm. Genes of iron metabolism shows that *sitA*, *fyuA*, and *ireA* were represented higher in the second group (weak, moderate and strong). Naves et al. (2008) recorded the presence of *fyuA* in all strong biofilm producers, but *iroN*, unlike our study, was higher in low biofilm producers. The literature contains only a few papers correlating the virulence factors investigated in this study with the ability of pathogenic *E. coli* to form biofilms *in vitro*. Further studies involving larger numbers of clinical strains are needed to corroborate our data concerning the interaction between biofilm formation and virulence factors.

Conclusion

Biofilms are of particular interest in the poultry industry and public health, because these films can harbour pathogenic microorganisms. In this study, *Escherichia coli* strains were identified and analysed for the presence of genes of iron metabolism,

virulence-associated genes, and biofilm-forming abilities. All 19 *E. coli* strains evaluated were able to form biofilms, with the majority exhibiting very weak biofilm-forming potential. The prevalence of the virulence-related genes was higher in low biofilm producers, where the presence of the siderophore-related genes was variable, but no significant differences were observed between strong and weak biofilm producers. Results provide a basis for the further study of the pathogenesis of APEC and its abilities of formation biofilms.

Acknowledgement

This study was supported by the Slovak project VEGA 2/0085/18.

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Abstract

Escherichia coli is known as one of the bacterial species with the widest adaptability to a variety of niches either within organisms or outside in environment. Most strains of *E. coli* are of low virulence and associated with opportunistic infections, whereas others are highly virulent. The success of *E. coli* in colonising such a wide range of hosts and environments is basically due to a noticeable ductility in exploiting the available resources. It is becoming increasingly clear that biofilms have an enormous impact on medicine, because 65% of animal and human bacterial infections involve biofilms. In the present study, we isolated strains of *E. coli* from animals. 19 interesting isolates were selected and tested by PCR amplification to virulence – *iutA*, *cvaC*, *iss*, *tsh*, *papC*, *kps*, *iha* and iron metabolism genes – *sitA*, *feoB*, *irp2*, *fyuA*, *iroN*, and *ireA*. The ability of biofilm formation was assessed in a quantitative assay using microtiter-plate tests. Bacterial strains were grown on BHI. We divided isolates of *E. coli* into four classes: very weak (63.0%), weak (10.5%), moderate (10.5%), and strong (16.0%) biofilm producers. Representation genes of virulence were high in isolates from very weak biofilm producers – from 7 genes were 6 highly and only *papC* (P fimbrial adhesin) was low. Genes of iron metabolism were different. Genes – *sitA*, *fyuA*, and *ireA* in strong isolates producing biofilm and *feoB*, *irp2*, and *iroN* in weak producers were most represented. The results show a possible relation between presence virulence factors and low biofilm formation.

Key words: biofilm, virulence genes, iron metabolisms genes

Received: [2018.05.30]

Accepted: [2018.12.29]

Związek pomiędzy tworzeniem się biofilmu, genami wirulencji i metabolizmem żelaza u *Escherichia coli*

Streszczenie

Escherichia coli znana jest jako jeden z gatunków bakterii o najszerzej zdolności adaptacji do różnych nisz w organizmach lub w środowisku zewnętrznym. Większość szczepów *E. coli* ma niską wirulencję i wiąże się z infekcjami oportunistycznymi, podczas gdy pozostałe szczepy są wysoce wirulentne. Sukces *E. coli* w kolonizowaniu tak szerokiego zakresu żywicieli i środowisk wynika przede wszystkim z zauważalnej ciągłości w wykorzystywaniu dostępnych zasobów. Staje się jasne, że biofilmy mają ogromny wpływ na medycynę, ponieważ 65% zakażeń bakteryjnych zwierząt i ludzi dotyczy biofilmów. W obecnych badaniach izolowano szczepy *E. coli* ze zwierząt. Wybrano 19 interesujących izolatów i testowano je przez amplifikację PCR pod względem wirulencji – geny metabolizmu *iutA*, *cvaC*, *iss*, *tsh*, *papC*, *kps*, *iha* oraz żelaza – *sitA*, *feoB*, *irp2*, *fyuA*, *iroN*, *ireA*. Zdolność tworzenia biofilmu oceniano w teście ilościowym, stosując test płytki mikrotitracyjnej. Szczepy bakteryjne hodowano na BHI. Izolaty *E. coli* podzielono na cztery klasy producentów biofilmu: bardzo słabe (63,0%), słabe (10,5%), umiarkowane (10,5%) i silne (16,0%). Geny reprezentacyjne wirulencji były w większości izolowane od bardzo słabych producentów biofilmu – z 7 genów było 6 wysoce wirulentnych; tylko *papC* (adhezyna fimbrialna) była niska. Geny metabolizmu żelaza były różne pod względem wirulencji. Najbardziej reprezentowane były geny – *sitA*, *fyuA*, *ireA* w silnych izolatach produkujących biofilm oraz *feoB*, *irp2*, *iroN* u słabych producentów. Wyniki pokazują możliwą zależność pomiędzy obecnym czynnikiem zjadliwości, a niską formacją biofilmu.

Słowa kluczowe: biofilm, geny wirulencji, geny metabolizmu żelaza

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The main area of her interest is genetic ecology and the spread of antibiotic resistance genes. She studies the resistance occurrence in small mammals, which could serve as a reservoir of antibiotic resistance (ESBL, plasmid encoded chinolone resistance, carbapenemases) in indicator bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus* spp.

Anna Čuvalová

She is interested in anti-biofilm activities of natural compounds using the static and dynamic biofilm models with resistant staphylococci (MRSA and MRCoNS), *Escherichia coli* (ESBL and cefotaximases) and *Pseudomonas aeruginosa* on various surfaces (plastics, catheters and food grade stainless sheet).

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He is interested in biofilm and anti-biofilm activities of natural compounds using the static and dynamic biofilm models with *Escherichia coli*, resistant staphylococci (MRSA and MRCoNS). The area of his interest is genetic ecology and gene encoding factors of virulence, metabolism, and the spreading of these genes. He studies the resistance occurrence in animal, which could serve as a reservoir of antibiotic resistance in indicator bacteria.