Polish Journal of Veterinary Sciences Vol. 17, No. 2 (2014), 321–329

DOI 10.2478/pjvs-2014-0044

Original article

Motility activity, slime production, biofilm formation and genetic typing by ERIC-PCR for *Pseudomonas aeruginosa* strains isolated from bovine and other sources (human and environment)

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Abstract

The molecular-typing strategy, ERIC-PCR was used in an attempt to determine the genomic relationship of 28 *P. aeruginosa* strains isolated from faeces of healthy bovine, bovine *mastitis* and from faeces of hospital patients as well as from environment. ERIC-PCR fingerprinting revealed large molecular differentiation within this group of isolates. Twenty two out of 28 strains tested generated unique patterns of DNA bands and only three genotypes consisted of two isolates each were identified.

We also tested the *P. aeruginosa* isolates for their ability to form a biofilm on abiotic surfaces including polyvinylchloride and polystyrene. Different biofilm-forming abilities were demonstrated among strains; however, most of them (64.3%) showed moderate-biofilm forming ability. The strains with increased swimming and twitching motility displayed elevated biofilm formation. However, a negative correlation was found between slime and initial biofilm production. On the basis of the results obtained, we suggest that there are no major differences in phenotypic properties between *P. aeruginosa* strains isolated from different sources.

Key words: *Pseudomonas aeruginosa*, ERIC-PCR, biofilm, slime, twitching motility, swimming motility

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Inntroduction

Pseudomonas aeruginosa is a common inhabitant of soil, water, plants and other natural environments. It can multiply in almost any moist environment and has minimal nutritional requirements. Moreover it is tolerant to a wide variety of physical conditions. Consequently, *P. aeruginosa* can be found in the hospital environment and reservoirs such as sinks, floors, bath, bath soaps and also medical equipment. It is considered an opportunistic pathogen as it causes infection in immunodepressed subjects (Head and Yu 2004). *P. aeruginosa* has also been identified as an animal pathogen and as the occasional cause of bovine *mastitis* (McLennan et al. 1997, Malinowski et al. 2006). *Mastitis* caused by *P. aeruginosa* are frequently associated with the use of contaminated wash water, teat dips, or multidose *mastitis* antibiotic preparations (Erskine et al. 1987, Daly et al. 1999).

Further, evidence has suggested that there are no major differences in virulence between clinical and environmental isolates (Woods et al. 1997). In nature and clinical settings, *P. aeruginosa* are frequently found in surface-attached multicellular communities called biofilms. Forming of the biofilm is a highly regulated process that proceeds through a number of distinct stages. These stages can be divided into the initial attachment, microcolony formation, maturation and finally dispersion, which releases planctonic cells back into the environment (Sauer et al. 2002). The first stage of biofilm development involves initial contact, which is followed by irreversible attachment of bacteria to the surface. This process involves a multitude of different adhesins and extracellular appendages, the requirement of which is likely to depend on the surface and the environmental conditions. (O'Toole and Kolter 1998, Glessner et al. 1999).

In *P. aeruginosa*, the flagellum is recognized as a central component in the biofilm process. It provides the mobility needed to actively approach a surface. The next important factor in surface attachment and motility of *P. aeruginosa* are type IV pili. They are located at the pole of the cells and are involved in twitching motility, which is an unique type of movement across semi-solid surfaces. The movement results from the extension, tethering, and retraction of the pilus structure (O'Toole and Kolter 1998, Deziel at al. 2001, Skerker and Berg 2001). After surface colonization, the bacteria begin to communicate with one another in the process called "quorum sensing" and these signs guide the formation of microcolonies that will develop into mushroom-like pillars and give the mature biofilm an unique structure (Klausen et al. 2003, Kumar et al. 2009). Maturation of biofilms involves production of the extracellular matrix, the alginate polysaccharide. Alginate production was found to be a hallmark of *P. aeruginosa* chronic infection in the lungs of *cystic fibrosis* (CF) patients (Singh et al. 2000). The relationship between alginate production and biofilm formation is unclear, since alginate is not essential for biofilm formation *in vitro*. However, alginate shapes the structure of biofilms and protects the bacteria from the host immune response and from antibiotics and biocides (Hentzer et al. 2001).

PCR-based genotyping methods have played an important role in bacterial typing schemes (Wolska and Szweda 2012). One of the PCR-based methods, is Rep-PCR. This method is used for typing purposes of repetitive DNA elements, especially the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) (Versalovic et al. 1991). They contain a highly conserved central inverted repeat located in extragenic regions (Houlton et al. 1991). The position of ERIC elements in bacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species (Versalovic et al. 1991).

To date, there have been no reports in the literature comparing the clinical and environmental *P. aeruginosa* strains, through employment ERIC sequence. Therefore, the aim of this study was to examine the genetic relatedness of *P. aeruginosa* isolated from the following sources: faeces of healthy bovine, bovine mastitis and bovine environment as well as strains isolated from the hospital in Siedlce (Poland), through employment of ERIC-PCR and to evaluate the phenotypic properties of these strains such as swimming and twitching motility activity, slime production and biofilm formation.

Materials and Methods

Bacterial strains

A total of 28 strains of *P. aeruginosa* were originally isolated from a variety of sources. Fifteen isolates were obtained from faeces of 400 (3.75%) healthy bovine in 9 different dairy herds of the east-north region of Poland. Three bovine environmental strains were isolated from litter (1 and 2 isolates) and straw (isolate 14).

In the first farm two following strains were obtained: 1 and 17, in the second farm – 5 strains (2, 7, 8, 9, 21), the third one – three strains (3, 6, 13), the fourth one – three strains (5, 10, 12), the fifth one – two strains (14, 20), the sixth one – one strain (4), seventh – without isolation strains, the eigth one – one strain (16) and in the last farm – one strain (18). Faeces and environmental cultures were done on

blood agar plates and MacConkey agar plates (Difco, USA).

A selection of 10 control *P. aeruginosa* organisms were obtained from the Veterinary Laboratory and Depertment of Medical Microbiology at the Municipal Hospital in Siedlce for comparison. These controls included *P. aeruginosa* isolated from *mastitis* milk samples (n = 4) and isolates from faeces (n = 5) of patients with a variety of clinical cases and *P. aeruginosa* isolated from hospital sink (n = 1). The reference strains of *P. aeruginosa* NCTC 6749 and ATCC 27853 were also investigated.

All isolates were identified phenotypically employing a combination of conventional identification methods (e.g. oxidase, production of green pigment, characteristic odor, growth at 42°C), as well as the API identification scheme (API 20NE) (bioMerieux, France). We also identified *P. aeruginosa* by PCR amplification of fragment of gene coding for 16S rRNA (Kingsford and Raadsma 1995). All isolates resulted in a positive reaction. Stock cultures were stored in triptic soy broth (TSB, Difco, USA) containing 20% (v/v) glycerol at -80°C.

DNA extraction

Isolates were grown in TSB at 37°C for 24 h and DNA was extracted by using the Genomic DNA Pre Plus (A&A Biotechnology, Poland).

PCR procedures

The two oligonucleotide primers were pairs used to amplify the regions in the bacterial genome positioned between the ERIC sequences. The sequences were: Primer 1; 5' – CACTTAGGGGTCCTCGAATGTA – 3'; Primer 2; 5' – AAGTAAGTGACTGGGGTGAGCG – 3' (Versalovic et al. 1991). The PCR reaction mixtures contained: 10 µl of 2 x PCR Master Mix High GC containing optimized composition of Taq DNA polymerase, PCR reaction buffer and mixture of dNTPs (A&A Biotechnology, Poland), 1 µl of each of the two required primer solutions (10 µM), 1 µl of DNA solution (prepared as described above) and deionized sterile water to complete the mixture volume to 20 µl.

A thin layer of paraffin oil was added to prevent evaporation. The DNA amplification was done in a thermal cycler (Biometra T-Gradient, Goettingen, Germany) using the following PCR cycles: the first denaturation step of 4 minutes at 94°C, followed by 35 cycles of denaturation (0.5 min at 94°C), annealing (0.5 min at 52°C) and extension for 1 min at 72°C, with the exception of the last cycle, which had a final extension time of 10 min. Amplifications were performed twice to confirm consistency of the method. The PCR products obtained were separated on 2% agarose for 60 min at 110 V and compared with Gene Ruler 100 bp or 1 kb ladder (Thermo Scientific).

All data analysis were performed using the computer software Unistat for Windows. Euclidean distance was calculated and compared to evaluate similarity among strains through the use of BIOGENE software.

Biofilm assay to microtiter plates

The biofilm formation protocol was adapted from that of O'Toole and Kolter (1998). Briefly, *P. aeruginosa* overnight culture was diluted 1:100 in fresh TSB medium (Difco, USA), dispensed (200 µl) to wells of a 96-well polystyrene microtiter plate (Sigma-Aldrich, USA) and grown for 24 h at 37°C (unless otherwise noted) with no aeration. Wells were stained with 200 µl of 1% crystal violet (CV) for 15 min at room temperature. Stain was discarded, and the plate was rinsed three times with phosphate buffer saline (PBS pH 7.2) and allowed to dry. Stained biofilm was solubilized with 200 µl of 95% ethanol for 10 min and read according to the optical density at 450 nm (OD_{450}). Each assay was performed in triplicate and repeated 3 times. The following values were assigned for biofilm determination: $OD_{450} = 0.050$, non-biofilm forming; $0.050 < OD_{450} \leq 0.1$, weak; $0.1 < OD_{450} \leq 0.15$, medium; $0.15 < OD_{450}$, strong.

Biofilm assay to Tiemann catheter

Prosser et al. (1987) developed a simple method to produce biofilm *in vitro* on catheter pieces. Sterile Tiemann's polyvinylchloride (PVC) catheter pieces of 1 cm were cut from the tubing (Maersk Medical, USA) and placed into Petri dishes (3 cm) containing 5 ml of bacterial suspension (overnight culture of *P. aeruginosa* – TSA, diluted 1:100 in TSB). Biofilm was allowed to develop under stationary conditions for 3 days by regularly changing the catheter piece to fresh medium after every 24 h. Each day catheter pieces in duplicate were removed and were placed in separate Petri dishes, rinsed thrice with PBS and transferred to the new Petri dishes containing 5 ml of fresh TSB. The last time, catheter pieces were washed again in PBS and transferred to Petri dishes with fresh TSB broth and 1 drop of 1% 2,3,5-triphenyltetrazolium chloride in PBS (TTC, Sigma-Aldrich, USA). The presence of red stained bacterial biofilm

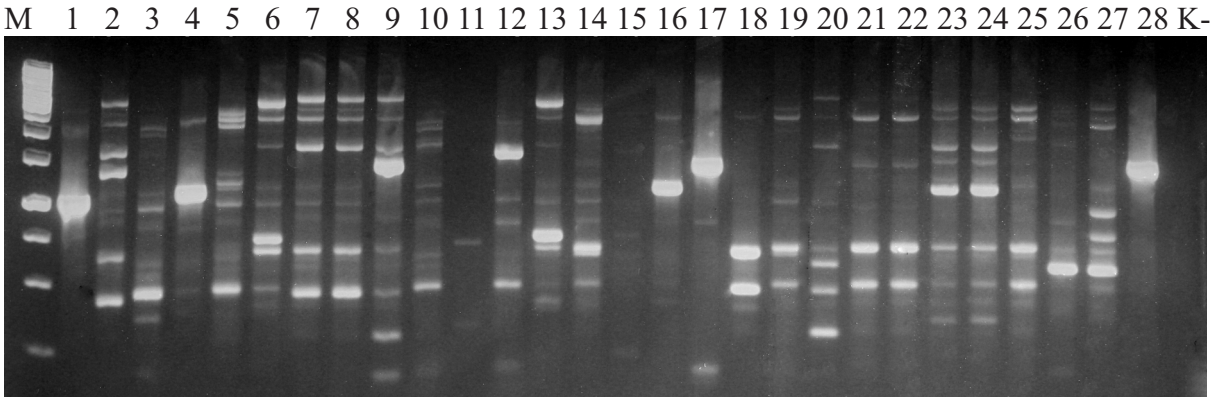

Fig. 1. ERIC-PCR fingerprint of *P. aeruginosa* strains. Lane M; molecular weigh marker (1000 bp); lane K; negative control; lane 1-28; *P. aeruginosa* strains isolated from faeces of healthy bovine (3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 16, 17, 18, 20, 21), bovine *mastitis* (11, 19, 22, 28), bovine environment (1, 2, 14), faeces of hospital patients (23, 24, 25, 26, 27) and hospital sink (15).

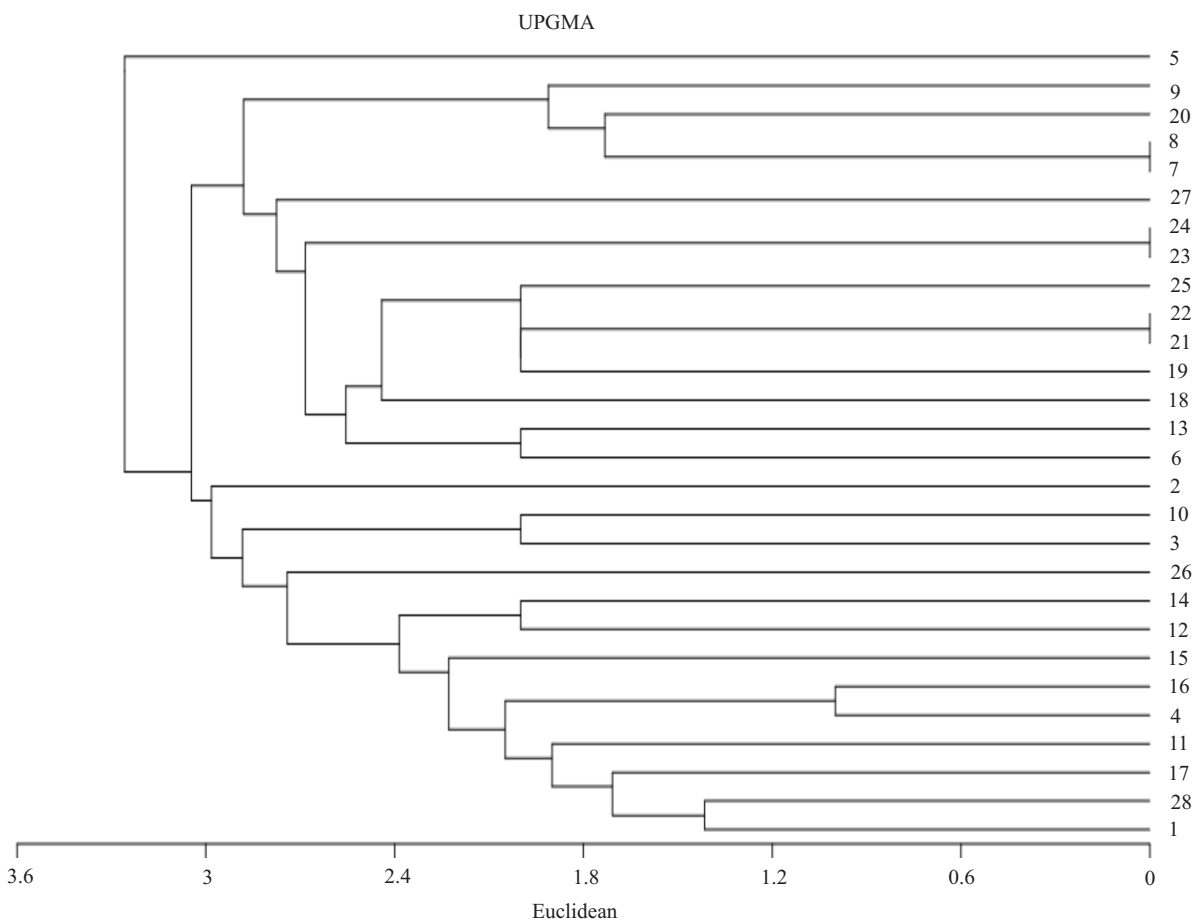

Fig. 2. The dendrogram indicating the genetic relatedness of the *P. aeruginosa* strains.

(reduction by live bacteria of colorless TTC to the red insoluble formazan crystals) was examined after 1, 2, 5 and 24 h of incubation at 37°C. Following categories of biofilm were determined: the complete absence of catheter color, non-biofilm producting; light spot color of the catheter surface after 1, 2, 5 hours, and additionally pink medium color after 24 h of incubation, weak; redness of the surface and the interior part of the catheter and the red color of medium after 24 h of incubation (after 5 h – pink medium color), medium; redness of the whole surface and the interior of the catheter and the deep of medium after 24 h of incubation (after 5 h – red medium color), strong. All experiments were performed in triplicate.

Twitching motility assay

Cells from fresh culture plates were stab-inoculated with a toothpick a thin (approximately 3 mm) Luria-Bertani (LB) agar layer (1.0% agar) (Difco, USA) to the bottom of the Petri dish. After incubation for 24 at 30°C, a hazy zone of growth at the interface between the agar and the Petri dish surface was observed (Deziel et al. 2001).

Swimming motility assay

Tryptone swim plates (1% tryptone, 0.5% NaCl, 0.3% agar) (Difco, USA) were inoculated with a sterile toothpick and incubated for 24 h at 26°C. Motility was then assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation (Deziel et al. 2001).

All motility experiments were performed in triplicate.

Slime production

The procedure was made as described by Christensen et al. (1982). Two to three colonies of the isolates were inoculated into 5 ml of TS broth in a plastic white tube and incubated statically for 24 h at 37°C. The contents of the tubes were removed and then stained with 1% safranin. An adherent film on the surface of the glass tube was considered as evidence of slime formation. The absence of a film or the mere presence of a ring at the liquid-air interface was interpreted as a negative result (-). In the study, positive results were recorded as strong (+++), moderate (++), weak (+). Each test was interpreted by three different observers.

Statistical analysis

Comparison of biofilm formation with swimming and twitching motility activity as well as slime production was made using the ratio of correlation (r_{xy}). The p values of < 0.05 were considered significant.

Results

ERIC-PCR

ERIC-PCR patterns were detected in each sample (Fig. 1). The genetic patterns were consisted of one to 10 bands. The average amplicons per gel lane was 6. The size of generated PCR products varied mainly between 200 and 3000 bp and the most frequently found products of PCR amplification were the following: about 600 bp and 2500 bp. ERIC-PCR demonstrated a high discriminatory ability, only three genotypes consisting of two isolates were distinguished (strains with numbers: 7 and 8, 21 and 22, 23 and 24, respectively). The first genotype consisted of the strains isolated from faeces of healthy bovine in the same farm. The second one was represented by the strains obtained from healthy and sick bovine. And the last one consisted of the pair of strains isolated from humans. The other 22 isolates generated unique patterns of amplified DNA fragments. (Fig. 1,2).

Biofilm production

Next, all isolates were tested for biofilm-forming ability using an adaptation of a polystyrene 96-well microtiter dish method. The strains ATCC 27853 and NCTC 6749 were included in every data to determine biofilm formation. A significant variation in biofilm formation was found among all isolates tested (A = 0.080 to 0.217, the average A = 0.126) (Table 1). The majority of these isolates (18/28; 64.3%) formed medium-strength biofilms (the average A = 0.127) like the controls (Table 1). Only 10/28 (35.7%) of the samples varied in relation to ATCC27853 and NCTC6749. Four (14.3%) isolates among them two from faeces of healthy bovine, one strain isolated from bovine environment and one strain obtained from faeces of hospital patients, showed increased amounts of biofilm compared with controls. And only six (21.4%) of the samples formed less biofilm than ATCC27853 and NCTC6749. They were isolated from bovine *mastitis* (3/4) and from the healthy bovine (3/15; 20%). Considering place of isolation of strains tested, we demonstrated that the highest amounts of biofilm formed strains isolated from bovine environment (average 0.153). The strains isolated from faeces of healthy bovine, faeces of human and hospital sink formed moderate biofilms (the average A = 0.125, A = 0.131, A = 0.125, in particularly) and the strains isolated from bovine *mastitis* formed a weak biofilm (the average A = 0.103).

Furthermore, these isolates were also tested for biofilm formation on Tiemann's polyvinylchloride catheter surface. The dynamics and trend of biofilm formation on this surface were identical to those observed with polystyrene microtiter dish method (Table 1).

Table 1. ERIC-PCR typing, twitching and swimming motility, production of slime, and formation of biofilm of *Pseudomonas aeruginosa* strains.

Isolates	Derivation of strains	ERIC-PCR typing	Twitching motility (mm)	Swimming motility (mm)	Slime	Biofilm to				
						Microtitrate plate 24 h (A 450 nm)	Tiemann catheter			
							1 h	2 h	5 h	24 h
3V	faeces	1	20	17	–	0.136	–	1+	1+	2+
4V	faeces	2	23	20	–	0.200	1+	1+	2+	3+
5V	faeces	3	26	20	1+	0.129	1+	1+	2+	2+
6V	faeces	4	20	10	3+	0.091	–	–	1+	1+
7V	faeces	5	25	25	–	0.217	1+	1+	3+	3+
8V	faeces	5	21	22	–	0.118	–	–	1+	2+
9V	faeces	6	20	20	1+	0.103	–	–	1+	2+
10V	faeces	7	20	12	1+	0.107	–	–	–	1+
12V	faeces	8	22	17	1+	0.123	–	1+	2+	2+
13V	faeces	9	11	12	2+	0.080	–	–	1+	2+
16V	faeces	10	12	15	–	0.150	–	–	1+	2+
17V	faeces	11	20	20	–	0.113	–	–	1+	2+
18V	faeces	12	20	18	–	0.106	–	1+	1+	2+
20V	faeces	13	18	16	–	0.098	–	–	1+	2+
21V	faeces	14	15	17	1+	0.107	–	–	1+	2+
11V	milk	15	8	15	1+	0.098	–	–	1+	2+
19V	milk	16	12	17	–	0.120	–	–	–	1+
28V	milk	17	6	10	1+	0.096	–	–	–	1+
22V	milk	14	16	12	–	0.098	–	–	–	1+
1EV	litter	18	28	25	–	0.124	–	1+	1+	2+
2EV	litter	19	30	22	–	0.205	1+	1+	2+	3+
14EV	straw	20	28	20	–	0.130	–	–	1+	2+
23H	faeces	21	28	24	1+	0.128	1+	1+	2+	2+
24H	faeces	21	25	20	1+	0.117	–	–	1+	2+
25H	faeces	22	29	25	1+	0.165	1+	1+	2+	3+
26H	faeces	23	19	20	1+	0.120	–	–	1+	2+
27H	faeces	24	25	17	1+	0.124	–	–	1+	2+
15EH	sink	25	20	24	1+	0.125	1+	1+	2+	2+
Total	28 strains	12 of types	20.25± 6.24	18.3± 4.39	13 (1+), 46,4% 1(2+)- 3.6% 1(3+)- 3.6% 13(-)- 46.4%	0.126± 0.03	7(1+)- 25% 21(-)- 75%	11(1+)- 39.3% 17(-)- 60.7%	16 (1+)-, 57.1% 7(2+)- 25% 1(3+)- 3.6% 4(-)- 14.3%	5 (1+)-, 17.8% 19(2+)- 67.9 4(3+)- 14.3%
NCTC 6749		nt	15	12	1+	0.116	–	–	1+	2+
ATCC 27853		nt	17	14	1+	0.120	–	–	1+	2+

V – veterinary strains, H – human strains, EV – environmental veterinary, EH – environmental human, (-) – negative result

Swimming and twitching motility

The formation of biofilms is a multistep process that requires participation of structural appendages, such as flagella and type IV pili. Variations in biofilm formation, as presented in Table 1 could be due to altered activities of these structural appendages. To test this, we measured the motility and twitching abilities of the isolates, the results of investigation are presented in Table 1. The flagellum activity of 28 of *P. aeruginosa* strains ranged from 10.00 to 25.0 mm. The overall mean value was 18.3 mm with a standard deviation of 4.39. Half of the studied strains demonstrated higher activity of swimming motility, among them 6 (40.0%) of the strains isolated from faeces of healthy bovine, 3 (100%) of the strains from bovine environment, 5 (100%) of the strains isolated from faeces of hospital patients and one strain isolated from hospital sink. To facilitate comparison of the results obtained we included also reference strains *P. aeruginosa* NCTC 6749 and ATCC 27853 and determined their swimming motility activity to be 12 mm and 14 mm, respectively. In our study the majority (23/28, 82.1% isolates) of isolates exhibited better swimming motility activity than the reference *P. aeruginosa* strains NCTC 6749 and ATCC 27853.

The quantitative correlation between swimming motility and biofilm production was tested among isolates ($r = 0.59$, $p < 0.05$).

Twitching was also visible in all samples, however the isolates varied greatly in their activity. As shown in Table 1, the twitching activity of the reference strains NCTC 6749 and ATCC 27853 was smaller (15 and 17 mm, respectively) as compared to that of the studied strains (faeces of healthy bovine – 19.5 mm, faeces of hospital patients – 25.2 mm, hospital sink – 20 mm, bovine environment – 28.7 mm) without strains isolated from bovine *mastitis* (10.5 mm).

Isolates forming more biofilm coupled with increased twitching activity. Thus, the quantitative correlation was seen between twitching and biofilm formation ($r = 0.53$, $p < 0.05$).

Slime production

Mucoidy is an important virulence factor in chronic *P. aeruginosa* infections. As seen in Table 1, the slime producers (15/28, 53.6% isolates), 46.7% of the strains isolated from faeces of healthy bovine, 50% of the strains isolated from bovine *mastitis*, 100% of the strains isolated from faeces of human, the strain from hospital sink and references strains NCTC 6749 and ATCC 27853, did not lead to the largest amounts of biofilm formed in this study (the average $A = 0.114$). Conversely strains without slime production (13/28, 46.4%) generated higher amounts of biofilms (the average $A = 0.140$). Therefore, a negative correlation was seen between slime production and initial biofilm production (-0.44, $p < 0.05$).

Discussion

P. aeruginosa is an ubiquitous environmental bacterium that is capable of infecting humans, a wide variety of animals and plants. Source of infection or colonization remains to be environment (Head and Yu 2004). The first stage of the infection is adherence, which can initiate the formation of biofilm (Deziel et al. 2001).

Bacterial adhesion and biofilm formation is a complex process. A study by O;Toole and Kolter (1998) revealed that type IV pili and flagella mediated motility in *P. aeruginosa* was required for biofilm formation on biotic and abiotic surfaces. In our study, we report that all the tested strains demonstrated swimming and twitching activities, the overall mean value was 18.3 mm and 20.25 mm, respectively. The vast majority of isolates exhibited better twitching and swimming motility than *P. aeruginosa* NCTC 6749 and *P. aeruginosa* ATCC 27853. The strains with great motility activity (isolated from faeces of healthy bovine, hospital patients and bovine environment) showed a higher biofilm-forming ability to polystyrene and polyvinylchloride surfaces. Similarly, in previous study (Wolska and Kot 2013), we found a correlation between biofilm production and twitching motility activity of clinical *P. aeruginosa* strains with the mean value of motility of 24.4 mm.

Such a relationship is not found, when taken into account the production of slime by the strains. Probably, the extracellular matrix including alginate is not required for the initial phase of biofilm formation by *P. aeruginosa* (Doig et al. 1987).

Our data support the results of other authors, who have indicated that there is no clear line between clinical and environmental strains. Kus et al. (2004) reported that over 71% of non-CF rectal and clinical isolates and 95.8% environmental isolates exhibited twitching motility. The high prevalence of twitching motility observed amongst *keratitis* isolates in comparison to other clinical isolates (Kus et al. 2004) supports the notion that such activity is a requirement for successful *P. aeruginosa* corneal pathogens. Also Woods et al. (1997) were not able to differentiate both clinical and environment isolates on the basis of correlation of production of extracellular enzymes and LD50. While the results of Kumar et al. (2009) indicate that pathogenicity potential of *P. aeruginosa*

varies depending on the source of strain. Despite similar abilities of biofilm formation and production of quorum sensing signal molecules by environmental and clinical isolates, clinical *P. aeruginosa* isolates were found to have selective advantage in establishing and causing pathology in the urinary tract of mice. Different *in vitro* biofilm forming capacities and reduced ability of lung colonization was observed in environmental isolates as compared to those exhibited by clinical isolates by Head and Yu (2004). Similarly, Sela et al. (2007) displayed a wide heterogeneity in capacity to form biofilm among *P. aeruginosa* strains isolated from *mastitis* outbreaks.

To improved our understanding of the genetic link between *P. aeruginosa* isolates, we studied 28 *P. aeruginosa* strains isolated from different ecological origins. The relationship between isolates was calculated by numerical analysis of genetic features determined by ERIC-PCR. This molecular genotyping is much faster and more cost-effective; furthermore, it possesses a higher discriminatory ability than other typing techniques (Wolska and Szweda 2012). ERIC-PCR was used successfully, for example, for typing of clinical *P. aeruginosa* (Wolska and Szweda 2008) and to determine the link between pathogenic and non-pathogenic isolates (Yang et al. 2005).

Our study has suggested that there is low level of correlation between *P. aeruginosa* strains isolated from healthy bovine and bovine environment as well as strains isolated from hospital patients. Interesting, thought, is that the two strains, one isolated from faeces of healthy cattle, and the second strain isolated from a case of *mastitis*, were identical to the basis of dendrogram. This is to confirm that the source of infection of the mammary gland in cattle may be endogenous microflora of the gastrointestinal tract.

Similar results were obtained by Hamouda et al. (2008), who demonstrated high genetic diversity of *P. aeruginosa* strains isolated from food-producing animals and those of clinical origin. However, other authors observed some relationship between clinical and environmental strains. Typing of 18 human and animal (bovine, calves, chicken) isolates by random amplified polymorphic DNA and amplified fragment length polymorphism analysis Osman and colleagues (2010) indicated the similar groups. Romling et al. (1994) demonstrated that 19% of *P. aeruginosa* strains isolated from various clinical cases and from the aquatic habitats were found to belong to the same clonal group. Similar homogeneity was found among *P. aeruginosa* strains isolated from cases of either clinical infection and from gasoline sources (Foght et al. 1996).

The genetic studies of strains isolated from cows with *mastitis* and strains isolated from clinical infections in humans have not confirmed the close relationship of these microorganisms. However, the use of one or a few strains to represent animal, human and environment habitat may have limited the conclusion of these studies. Similar results were obtained by Daly et al. (1999). Thirty six isolates *P. aeruginosa* from *mastitis* outbreaks were tested and compared to fourteen clinical isolates. Eight of the clinical isolates gave the same ClaI ribotype pattern as the *mastitis*-causing strains. However, PwuII proved more discriminatory, with only the outbreak isolates producing identical pattern. Similar results were obtained with RW3A-primed DNA amplification fingerprinting, with all outbreak isolates except one displaying the same fingerprint array. The clinical strains produced several fingerprint patterns, all of which were different from those of the *mastitis*-causing isolates (Daly et al. 1999).

In conclusion, the genetic relationship among the groups of *P. aeruginosa* strains tested here showed that neither healthy and sick bovine nor hospital patients and environment was caused by specific genotypes within this species. The evolution of *P. aeruginosa* strains has not allowed the establishment of a clear phylogenetic separation between pathogenic and non-pathogenic strains.

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