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Phenotypic and genotypic characterization of *Pseudomonas aeruginosa* strains isolated from mastitis outbreaks in dairy herds

Shlomo Sela[1], Orly Hammer-Muntz[1], Oleg Krifucks[2], Riki Pinto[1], Limor Weisblit[2] and Gabriel Leitner[2]*

[1] Microbial Food-Safety Research Unit, Department of Food Sciences, Agricultural Research Organization, The Volcani Center, PO Box 6, Bet Dagan 50250, Israel
[2] National Mastitis Reference Center, Kimron Veterinary Institute, PO Box 12, Bet Dagan 50250, Israel

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During 1998–2002 outbreaks of *Pseudomonas* sp. mastitis among more than 15 Israeli sheep and goat dairy herds were observed. The animals presented a wide spectrum of clinical signs ranging from subclinical to gangrenous udder. Ninety-five isolates of *Pseudomonas* sp. were isolated from clinical and subclinical mastitis of 47 sheep, 17 goats and 31 cows from 34 different farms. Biochemical and genetic analyses revealed that the all-causative organism was *Ps. aeruginosa*. Selections of isolates were further analysed on the bases of colony morphology, biochemical traits and capacity to form biofilm. All the strains displayed a wide heterogeneity in all the tested traits. No association between bacterial isolates, farm of origin and type of animal was found. Pulsed-field gel electrophoresis and cluster analysis showed no clonality among the tested strains. The present study revealed that a large variety of *Ps. aeruginosa* strains may cause mastitis outbreaks in sheep, goat and cattle in Israel.

Keywords: *Pseudomonas aeruginosa*, mastitis, sheep, goats, cattle.

Pseudomonas aeruginosa is an opportunistic pathogen that is frequently responsible for nosocomial infections in humans, particularly in cystic fibrosis (CF) and burn patients (Høiby, 1977). *Ps. aeruginosa* has also been identified as an opportunistic animal pathogen, occasionally involved in enzootic or epizootic outbreaks of mastitis in small ruminants (Bergonier et al. 2003). The microorganism is considered an environmental pathogen in mastitis occurrence in animals in clinical and subclinical cases. Additionally, it was detected in contaminated wash hoses in milking parlours and in water and spray nozzles (Kirk & Bartlett, 1984). From 1998 to 2002 several outbreaks of mastitis caused by a Gram-negative, non-coliform pathogen were reported in dairy herds in Israel. Preliminary laboratory investigation has identified the causative agent as *Ps. aeruginosa* (Yeruham et al. 2005). The morbidity of the animals reached 15–20%, including culling of animals with subclinical mastitis. Since such outbreaks impose a substantial economic burden on breeders, and might also contribute to the spread of highly virulent clones into the human population, a selected number of bacteria isolated from various dairy animals and farms were further characterized and subjected to phenotypic and genotypic analyses.

*For correspondence; e-mail: leitnerg@moag.gov.il

Materials and Methods

Bacteriological techniques

Ninety-five isolates of *Ps. aeruginosa* were isolated from clinical and subclinical mastitis of 47 sheep, 17 goats and 31 cows on 34 different dairy farms in Israel. In addition, *Ps. aeruginosa* ATCC 27853 was used as a reference strain. Duplicate quarter foremilk samples were taken aseptically according to the International Dairy Federation (IDF, 1985) procedures and submitted to the laboratory. Bacteriological analysis was performed according to accepted standards (Hogan et al. 1999). A 0·01-ml aliquot from each milk sample was cultured in defibrinated sheep blood (5%) agar (Bacto-Agar; Difco Laboratory, France) and incubated in aerobic conditions, at 37 °C for 18–42 h. Colonies suspected to be *Pseudomonas* were streak-isolated on *Pseudomonas*-isolation-agar (PIA) (Hy Lab. Ltd,

Rehovot, Israel) and putative *Pseudomonas* colonies were identified by means of the ID 20 NE Kit (API; Bio Merieux SA, France) and the BBL Crystal Identification System; Enteric ID Kit (BD BBL CRYSTAL; Becton Dickinson, Baltimore MD, USA). Isolates identified as *Pseudomonas* genera were submitted to 0·3% agar tube, oxidase (Oxidase Identification Stick; OXOID, Hampshire, UK), carbohydrate fermentation (lactose, glucose and sucrose), indol (Difco), urease, and reduction of NH_3 to NH_2.

Phenotypic and biochemical data were obtained from all isolates, from which a selected number of isolates were further identified by 16S ribosomal DNA analysis. Genetic relatedness among 10 representative *Ps. aeruginosa* isolates representing various farms and animals were tested by Pulsed-field gel electrophoresis (PFGE). The capacity of these isolates to form biofilm was also studied.

Susceptibility test

The susceptibility test was performed with the disk diffusion technique on Mueller-Hinton agar (Difco), according to NCCLS guidelines (NCCLS) (NCCLS, 1999). Commercially available disks (Dispens-O-Disc, Susceptibility Test System, Difco) were applied according to the manufacturer's instructions, and the plates were incubated at 37 °C in aerobic conditions. The antimicrobials tested were: gentamycin (10 μg), sulphamethoxazole with trimethoprim (25 μg), polymyxin (300 i.u.), ciprofloxacin (5 μg) and amikacin (30 μg).

Formation of biofilm

The ability of *Ps. aeruginosa* strains to develop biofilm on polystyrene was tested according to O'Toole et al. (1999). The strains were grown overnight at 37 °C in Luria broth (LB) (Hy-lab, Rehovot, Israel), brought to an OD_{600} of 1·0 and diluted 1 : 100 in fresh LB. Samples (100 μl) were transferred into 96-well polystyrene tissue culture plates (Greiner, Germany) and incubated for 24 h at 26 °C or 37 °C. The plates were washed, stained with crystal-violet (CV), and the absorbance of the extracted CV was read at 595 nm. Results are presented as the average OD and SD of 3–5 replicates. To visualize the macroscopic nature of the biofilms, bacteria were also grown in borosilicate glass tubes (16 × 150 mm) with 4 ml of LB in a Lab-line shaker operating at 100 rpm for 24 h at 37 °C. Digital images of the tubes at 24 h are presented.

Identification of Ps. aeruginosa *by 16S ribosomal DNA analysis*

Chromosomal DNA was extracted from each isolate using the Alkali-lysis technique, as described by Hartas et al. (1998). Eubacteria-domain-targeted PCR primers were used to amplify a conserved region of euobacterial DNA coding for 16S rDNA (1392 bp), as described previously (Amann et al. 1995). The following primers, purchased from Sigma Genosys (Israel) were used: 11F (5′-GTTTG-ATCMTGGCTCAG-3′) and 1392R (5′-ACGGGCGGTG-TGTAC-3′). PCR was performed in a Biometra/Tgradient thermocycler. Reactions were carried out in a final volume of 40 μl, with 0·5 pmol of each primer and 20 μl of PCR reaction master mix (Fermentas, Ontario, Canada). PCR conditions were: 95 °C for 4 min followed by 36 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1·5 min. The final cycle was followed by extension at 72 °C for 10 min. PCR products were visualized on 1% agarose gel and, following a purification step, were sent for sequence determination (Hy Laboratories, Rehovot, Israel). Sequence identity was determined with the BLAST program (Altschul et al. 1997).

Pulsed-field gel electrophoresis

DNA preparation and electrophoresis conditions were essentially as previously described (Grundmann et al. 1995; Kaufmann, 1998) with minor modifications. Bacteria were grown overnight in Brain Heart Infusion (BHI) (Becton Dickinson, Sparks MD, USA). The bacteria were washed twice and the pellet was suspended in SE buffer (75 mM-NaCl, 25 mM-EDTA, pH 7·4) to a final concentration of $\sim 1 \times 10^9$ cfu/ml. The bacterial suspension was mixed with 2% low-melting-point agarose (Agarose III, pulse-field application; Amersco, Solon OH, USA), filled into plug moulds and solidified at room temperature. Bacterial lysis was performed with proteinase K (Sigma, St. Louis MO, USA) in a modified EC lysis buffer (6 mM-Tris–Cl, pH 7·6; 1 M-NaCl; 100 mM-EDTA, pH 8·0; 0·5% Brij-58; 0·2% deoxycholate; 0·5% N-lauroyl-sarcosine, and 20 μg/ml RNAse) at 56 °C, overnight. The DNA plugs were washed four times, each for 30 min, in TE buffer (10 mM-Tris–1 mM-EDTA, pH 7·5) at room temperature. The DNA plugs were then stored in TE buffer at 4 °C until use. The DNA plugs were cut up and immersed in 100 μl of restriction enzyme buffer, and digestion was performed with 10 U *Spe*I (Fermentas, Ontario, Canada) at 37 °C for 16 h. The DNA fragments were separated by gel-electrophoresis in 1·2% agarose gel (Amersco) in a modified Tris-borate–EDTA (0·25 × TBE) buffer (Amersco) using CHEF-DRII module (Bio-Rad Laboratories, Hercules CA, USA). Electrophoresis was performed at 14 °C for 22 h, and the switch interval was ramped from 5 s to 60 s. The gel was stained with ethidium bromide for 1 h and de-stained in distilled water for 2 h. Finally, the gel was photographed under transmitted u.v. illumination, and the pictures were saved as TIFF files. A 48·5–1000-kb lambda DNA ladder (Bio-Rad Laboratories) was used as a molecular weight marker.

Phylogenetic analysis

The relatedness among the DNA fragment patterns (fingerprints) of the various isolates was determined with the Molecular Analyst DST, version 1.6 software (Bio-Rad).

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Table 1. Phenotypic and biochemical characterization of *Ps. aeruginosa* strains according to farm origin and animal species

Isolate	Farm	Animal	Colony colour	Urease	NH_3 to NH_2	N_2	Mannose	Genetic ident.†
ATCC 27853	—	—	Green	+	–	+	+	ND‡
BI-6	1	Goat	Brown	+	–	+	+	ND
BI-40	1	Goat	Yellow	+	–	+	+	+
BI-43	1	Goat	Green	–	+	–	–	+
GA-13	2	Sheep	Yellow	+	+	–	–	+
GA-15	2	Sheep	Brown	–	+	–	–	+
GA-27	2	Sheep	White	+	–	–	–	ND
GA-30	2	Sheep	Brown	+	+	+	+	+
KA-52	3	Cow	Green	–	–	+	ND	+
MA-58	4	Cow	Brown	–	+	+	ND	+
GE-61	5	Sheep	Green	+	+	+	ND	+
GE-71	5	Sheep	Brown	–	–	+	–	ND
HC-93	6	Cow	Yellow	–	+	+	+	+
HC-56	6	Cow	Brown	–	+	–	–	ND
KZ-85	7	Cow	White§	–	+	–	–	+
AE-51	8	Cow	Brown	–	–	+	–	ND
KK-48	9	Sheep	Yellow	–	–	–	–	ND
KK-59	9	Sheep	Green	–	+	–	–	ND
AF-69	10	Cow	Green	+	+	+	–	ND
CO-106	11	Goat	Green	–	–	–	–	ND

† Genetic identification by 16S rDNA amplification and BLAST analysis
‡ ND, not done
§ Highly mucoid colonies

The size of the DNA fragments was normalized against the Lambda ladder. For comparisons among the DNA profiles a band tolerance of 2·0% was used. Cluster analysis was performed by the unweighted pair-group method, using arithmetic averages (UPGMA), and the calculation of the DNA relatedness was based on the Dice coefficient (Altschul et al. 1997).

Results and Discussion

All isolates were found to be Gram-negative rods, motile, oxidase-positive, positive for fermentation of glucose and negative for tryptophan fermentation to indol. Further biochemical identification, by means of the API and Crystal kits, revealed that the strains belonged to *Ps. aeruginosa*. Phenotypic and biochemical data from a selected number of isolates representing various farms and animals are presented in Table 1. Susceptibility studies revealed that all the isolates were sensitive to gentamicin, polymyxin, amikacin and ciprofloxacin and resistant to sulphamethoxazole-trimethoprim. Susceptibility of *Ps. aeruginosa* strains isolated from bovine mastitis cases, during a 7-year period was recently reported (Erskine et al. 2002). In contrast to our results, most strains displayed multiple antibiotic resistance, although all the 53 strains tested were still susceptible to gentamicin. This might reflect difference in antimicrobial usage in the two countries. Based on morpho-physiological characteristics, biochemical traits and the susceptibility test, no association was observed between isolates, farms and animal species (Table 1).

To verify that the strains identified according to biochemical characteristics belonged to the species *Ps. aeruginosa*, we employed common prokaryotic primers to amplify a region (~1300 bp) of the 16S rDNA gene in 10 selected strains, which represented isolates from different farms and animals. Sequence analysis with the BLAST program revealed 98–100% identity, in ~700 bp, with *Ps. aeruginosa* in all the tested strains (Table 1), confirming the identity of the isolates.

The capacity of bacterial pathogens to form biofilm is recognized as an important virulence factor to facilitate colonization and persistence both outside and within the host (Costerton et al. 1999; Parsek & Fuqua, 2003; Hall-Stoodley & Stoodley, 2005). Comparison among the biofilm masses developed by *Ps. aeruginosa* strains showed high heterogeneity at each of the temperatures tested, with some strains forming greater biofilm mass at lower and others at higher temperatures (Fig. 1). It is hypothesized that in-vitro biofilm formation, at the air-liquid interface, has environmental relevance to colonization of surface and volume, thus, the ability of *Pseudomonas* strains from divers ecological niches to form biofilm (pellicle) at the air-liquid interface was recently studied (Ude et al. 2006). Under static conditions at temperatures of 18–22 °C, 76% of the isolates formed pellicles (Ude et al. 2006). In concurrence with these results, the majority of *Ps. aeruginosa* strains in our study also generated a clearly visible pellicle

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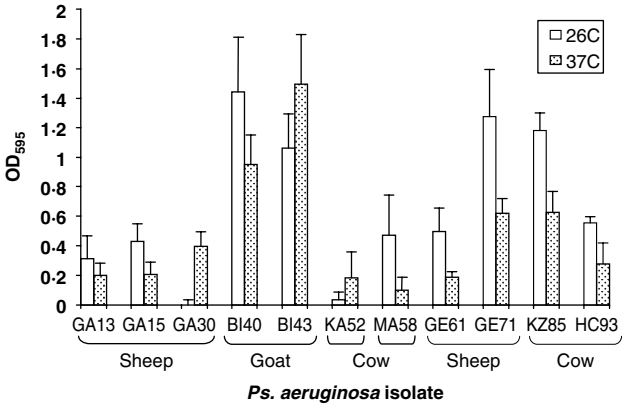

Fig. 1. Quantification of *Ps. aeruginosa* biofilm, assayed by the crystal violet staining method, according to the average OD_{595} and SD of four replicate wells.

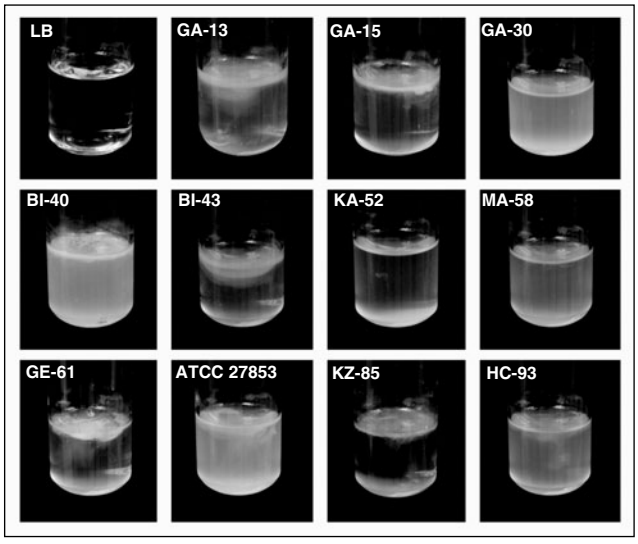

Fig. 2. Image of *Ps. aeruginosa* growth in borosilicate glass tube. Strains were grown in LB for 24 h at 37 °C with gentle agitation. Glass tubes were imaged by digital camera. For strain designations, see legend to Fig. 1.

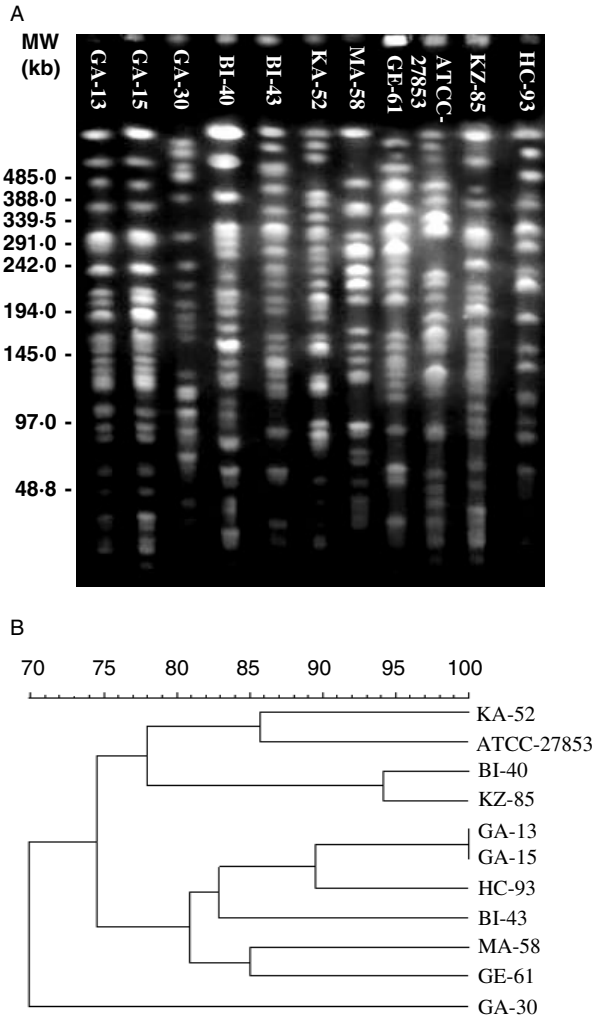

Fig. 3. Genotypic analysis of *Ps. aeruginosa* strains by pulsed-field gel-electrophoresis (PFGE). (A) DNA fingerprints obtained with PFGE. (B) A dendrogram showing percentages of similarity among the isolates, based on the PFGE fingerprint data. Strains were clustered by the unweighted pair-group method of arithmetic averages. Strain details are presented in Table 1.

(Fig. 2). Notably, most strains displayed a distinct combination of growth pattern and pellicle morphology. Since all strains were grown under identical conditions, these findings support the presence of genetic heterogeneity among the *Ps. aeruginosa* strains isolated from mastitis cases in Israel. Further studies should be performed to examine possible correlations between biofilm formation and capacity of a strain to cause clinical or subclinical masitits.

Genetic relatedness among Ps. aeruginosa *strains*

Phenotypic characterization of the isolates from the outbreaks suggested the presence of distinct strains in different farms and different animals. PFGE is a commonly employed fingerprinting method that has become the gold standard for typing *Ps. aeruginosa* strains (Ojeniyi et al. 1993; Grundmann et al. 1995; Jalal et al. 2000; Spencker et al. 2000; Bertrand et al. 2001; Douglas et al. 2001; Rementeria et al. 2001). To further delineate the relationship between the 10 clones that were genetically identified as *Ps. aeruginosa*, the strains were subjected to PFGE analysis (Fig. 3a). The variations observed in the PFGE profiles suggest that several different strains, rather than one clone, were involved in the various outbreaks. It is interesting to note that strains GA-13, GA-15, which were derived from the same sheep farm, represent a single clone (similarity coefficient 100%), while strain GA-30, isolated also from the same farm was only distantly related

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(similarity coefficient 70%). If we use the >90% criterion for closely related strains, then strains BI-40 and KZ-85 seem to form a distinct cluster. Interestingly, the two strains were derived from different animals and farms in distinct geographical regions. Our findings are similar to those reported by Las Heras et al. (2002), who found 14 different pulsotypes among 32 *Ps. aeruginosa* strains derived from clinical and subclinical cases of mastitis in 12 sheep flocks. Whether strains associated with outbreaks in animals may also cause human infection in susceptible individuals remains to be studied.

In conclusion, our findings support the evidence that mastitis outbreaks of *Ps. aeruginosa* in sheep, goat and cattle in Israel were caused by different virulent clones and isolates coming from various dairy farms. *Ps. aeruginosa* is classified as an environmental pathogen in animal mastitis, a relatively rare disease, associated with infections. The occurrence of this microorganisms as a mastitis pathogen in Israeli dairy farms remained unclear. Others studies will be performed in order to evaluate the predisposal factors or epidemiological data that facilitate the occurrence of *Ps. aeruginosa* infections in mammary glands in sheep, goat and cattle in Israeli dairy animal herds.

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