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A CONTRIBUTION TO THE IDENTIFICATION OF PSEUDOMONAS AERUGINOSA

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Abstract

M r á z O.: *A Contribution to the Identification of Pseudomonas aeruginosa.* Acta vet. Brno,56,1987:115-122.

A total of 70 *Pseudomonas aeruginosa* strains of both human and animal origin were studied with respect to their differential characteristics and methods of their identification.

The results obtained in 28 aberrant strains are in reasonable agreement (± 2.3%) with published surveys (B e r g a n 1981), but in tests for haemolysis, gelatinase and esterase the numbers of positive cases were 31.6 to 56% higher.

An original medium for identification, namely nitrite broth (0.1% KNO_2) with an inserted Durham tube and covered with a 1 cm layer of paraffin oil, proved very useful.

Among 17 strains showing reduced virulence or complete innocuity for the white mouse upon intraperitoneal administration of 0.2 ml volumes of broth culture aberrant strains were involved 7 % more frequently than typical *P. aeruginosa* strains.

Fed to white mice in equal volumes of culture, all the strains proved innocous.

Pseudomonas aeruginosa, bacteriological identification.

The species *Pseudomonas aeruginosa* was known already in the last century, mainly under the name of *Bacterium aeruginosum* (S c h r o e t e r 1872) or *Bacterium pyocyaneum* (L e h m a n n and N e u m a n n 1896). The first descriptions including the official characteristics (M i g u l a 1900) are very brief. They concern only cellular morphology including the polar flagellum, colonial type on gelatine including its liquefaction, growth on nutrient agar including fluorescence, potato -grown culture including greyish to greenish--blue stain of the medium, and coagulation with following peptonization of milk. Pyocyanin production, which was not mentioned by M i g u l a (1900), had been reported earlier by G e s s a r d (1981).

Later investigations revealed the other 6 to 7 pigments (K i n g et al. 1948; M e a d e r et al. 1925; P u l v e r e r and K o r t h 1962; a. o.) and hydrolytic activity in carbohydrates, higher alcohols and proteins (S a l v i n and L e w i s 1946; L i u 1952; P u l v e-

r e r and K o r t h 1962; S t a n i e r et al. 1966; a, o.) and led to the adoption of a larger number of chemical tests, of particular differential value among them being those for acetamide hydrolysis (B ü h l m a n et al. 1961), denitrification (D o u d o r o f f and P a l l e r o n i 1974) and arginine dihydrolase (T h o r n l e y 1960).

All these advances could not conceivably do away with difficulties arising from those 10% possible deviation in each characteristic that are currently taken into account in identification manuals (C o w a n and S t e e l 1965) including Bergey's Manual (K r i e g and H o l t 1984). With *P. aeruginosa* this difficulty is well documented by major published systems (B e r g a n 1981) listing, among other things, 3 to 7% non-motile strains, 46% non-haemolysing strains, 5 to 14% strains without pyocyanin production, 42% gelatinase-negative strains, 6 to 41% strains with the absence of gas upon denitrification and 1 to 4% arginine-negative strains.

Varying results may also be due to the use of different procedures (media, incubation temperatures, observation periods), and attempts at optimization along this line will, no doubt, continue. Some of these aspects are considered in the present paper.

Materials and Methods

Seventy strains of *P. aeruginosa* were included in the study[+]. Their origin was as follows:

Man	pathological material	2 strains
Man	clinical material	7 strains
Man and water	(Habsová 1957)	14 strains
Horse	uterine cervix	1 strain
Cattle	mastitis	17 strains
Cattle	semen	18 strains
Pig	semen	1 strain
Rat	rectum	1 strain
Mouse	pharynx	4 strains
Insects	Galleria mellonella, Dacus oleae	2 strains
Soil		2 strains
Salt		1 strain

In our laboratory their species identity was confirmed on the basis of their aerobic nature, rod-like shape, Gram-negative staining, ability to grow at 41°C, pigment production (pyocyanin, pyoverdin) and on the basis of their being oxidase-positive.

Further characteristics examined were in the first place deviations in motility, haemolytic activity, hydrolysis of gelatine, arginine and acetamide as well as in nitrate reduction and denitrification. Added to this was examination for catalase, type of carbohydrate metabolism in glucose, maltose and starch, for milk peptonization as well as esterase activity in Tween 80.

[+] Most of the strains were obtained through the courtesy of [+] RNDr.O. Lysenko, CSc., curator of the Culture Collection of Entomogenous Bacteria (CCEB), Entomological Institute of the Czechoslovak Academy of Sciences, Prague, and MVDr. J. Mazurová, State Veterinary Institute, Pardubice.

In view of a possible relation between potential atypism of the strains and their virulence (G a b y and L o g a n 1961) our experiments were extended to include bioassay on mice (orally and intraperitoneally) and, in 5 randomly chosen strains also determination of LD_{50}.

Only the procedures of major importance are described.

Ability to Grow at 41°C. Inoculated meat-peptone broth was incubated at 41°C for 4 days (B ü h l m a n n et al. 1961; J o h n s and T i s c h e r 1973).

Relation to Molecular Oxygen. Semi-solid agar containing 0.05% sodium thioglycolate and resazurine as indicator of O_2 was inoculated by stabbing and incubated at 37°C for 3 days.

Motility. Semi-solid agar (same as described above) containing 0.1% KNO_3 was inoculated by stabbing and incubated at 37°C for 3 days. In dubious cases examination was also made of the hanging drops from 18-th broth cultures grown at 20, 30 and 37°C (B e r g a n 1981).

Haemolysis. Meat-peptone agar containing 5% defibrinated sheep blood was inoculated using the isolation technique and incubated at 37°C for 2 days.

Hydrolysis of Gelatine. Inoculated agar according to F r a z i e r (1926) was incubated at 37°C for 3 days and the result was evaluated using gelatine precipitant (acid $HgCl_2$).

O-F test. The type of carbohydrate metabolism was examined in glucose, maltose and soluble starch using the method H u g h and L e i f s o n (1953). After inoculation by stabbing, semi-solid medium was incubated at 37°C for 7 days.

Denitrification Ability. Meat-peptone broth containing 0.1% KNO_2, with an inserted Durham gas tube, and covered with a 1 cm layer of paraffin oil was inoculated by means of a loop and incubated at 37°C for 5 days to 10 days. If no visible amount of gas was detected, the experiment was repeated, but not before a 24-hour adaptation incubation of the strain in nitrate broth (P a l l e r o n i and D o u d o r o f f 1972).

Arginine Hydrolysis. The medium was prepared according to M ö l l e r (1955) except that bovine extract was left out and the amount of Proteose peptone was reduced to 0.2%. After heavy inoculation of the medium and its storage at 37°C in an incubator positive results were generally observed by the end of 24 hours.

Esterase Activity in Tween 80. Agar prepared according to S i e r r a (1957) was inoculated, incubated at 37°C for 3 days and then examined for the presence of precipitate round the colonies.

Acetamide Hydrolysis. Liquid medium prepared according to A r a i et al. (1970) was inoculated using the heavy inoculation technique, incubated at 35°C for 18 hours and then checked for the development of ammonia by means of Nessler's reagent.

Pyocyanin Production. Glycerol-peptone water prepared according to G e s s a r d (1891) using Neopeptone Difco was inoculated and incubated at 30°C for 5 days. The resultant pigment was shaken into chloroform and transformed to red sulphate of pyocyanin (M a c C o m b i e and S c a r b o r o u g h 1923).

For quantitative assessment of pyocyanin antibious activity, inoculation was made on special agar A prepared according to K i n g et al. (1954). After 24-hour preincubation at 30°C Bacillus subtilis ATCC 6633 test strain was applied perpendicularly to the line of growth and incubation was continued at the same temperature for another 24 hours.

Pyoverdin Production. Special agar B prepared according to the aforementioned authors was inoculated and incubated at 30°C for 48 hours. The cultures were examined for fluorescence with UV light at 365 nm.

Serotyping of the strains according to O-Antigen. This was performed by slide agglutination using diagnostic sera 01 - 012 obtained from the Institute of Sera and Vaccines, Prague.

Bioassay. Pairs of white mice were inoculated i.p. with 0.2 ml volumes of 18- to 24-hour broth culture and observed for 10 days. In separate experiments the same volumes of culture were administered per os. LD_{50} was determined according to R e e d and M u e n c h (1938) in groups of 4 white mice treated i.p. with 0.2 ml volumes of various dilutions of 18- to 24-hour broth culture.

Results

The strains under study were gram-negative aerobic rods that grew at 41°C. They haemolysed blood agar containing sheep erythrocytes, were catalase- and oxidase-positive, acidified glucose, oxidatively peptonized skim milk, hydrolysed arginine and acetamine but did not utilize maltose and starch and proved innocuous to white mice upon oral administration. The other characteristic are given in Table 1 which gives an insight into the variability within the species. It can be seen that 6 strains (8.6%) were non-motile, 5 strains (7.1%) were gelatinase-negative, 2 strains (2.8%) showed delayed nitrate reduction, 2 strains (2.8%) showed denitrification without producting visible gas, 1 strain (1.4%) was esterase-negative, 8 strains (11.4%) showed the absence of pyocyanin, 8 strains (11.4%) showed the absence of pyoverdin and 3 strains (4.3%) exhibited no antibious activity towards B. *subtilis*.

Since in some cases the aberrations cumulated, the total number of atypical strains was 28 (40%). As to their origin, the atypical strains included 15 (55%) out of the 27 CCEB strains, 7 (38.8%) out of the 18 strains isolated from bull semen and 1 (5.8%) out of the 17 strains isolated from bovine mastities.

The density of broth cultures in 10 randomly chosen strains ranged from $315 \cdot 10^6$ to $1.07 \cdot 10^9$ microbial cells per ml, the mean being $813 \cdot 10^6 \cdot ml^{-1}$. In half of these strains determination was also made of LD_{50} for the white mouse. This was found to be 0.2 ml of broth culture diluted 1 : 1 414 to 1 : 5 032, with the corresponding number of microbial cells fluctuating from $30\ 007 \cdot 10^6$ to $99\ 717 \cdot 10^6$.

Bioassay on white mice using intraperitoneal administration of the cultures (Table 1) revealed a total of 17 strains showing reduced virulence or even complete innocuity for the white mouse. These 17 strains included 9 (21.4 %) out of the 42 typical strains and 8 (28.5 %) out of the 28 aberrant strains. As to the origin of the strains, low virulence or innocuity was shown by 5 (18.5%) out of the 27 CCEB strains, 7 (38.8%) out of the 18 strains isolated from bull semen and by 3 (17.6%) out of the 17 strains isolated from mastitis. The likelihood that a test strain will be of reduced virulence is 7 % higher in aberrant than in typical strains.

Antibious activity was found in 67 (95.7%) strains without showing any relation to their virulence. The inhibition zone with B. *subtilis* ranged from 4 to 11 mm, the mean being 7 mm.

Antigenic structure could be determined in 54 (76%) strains. All the serovars (01 - 012) were represented but the following ones were involved most frequently: 06 (14 strains, i.e. 25.9%), 05 (10 strains, i.e. 18.5%) and 01 (8 strains, i.e. 14.8%). The least represented serovars were 09 (4 strains, i.e. 7.4%) and 012 (2 strains i.e. 3.7%) and both of them were associated with reduced virulence to complete innocuity for the white mouse. In the other serovars no direct relation to virulence was observed.

Table 1. Variable results in strains of Pseudomonas aeruginosa

Strain	Motility	Gelatinase	Nitrate reduction	Denitrification	Hydrolysis of Tween 80	Pyocyanin	Pyoverdin	Antibious activity	Pathogenicity[1] for mice (i.p.)
1	+	+	+	+	+	+	+	+	2/2
2	+	+	+	+	+	+	+	+	1/4
23	+	+	+	+	+	+	+	+	2/2
28	+	+	+(72 h)	+	+	+	+	+	0/4
29	+	+	+	+	+	+	+	+	2/2
30	+	+	+	+	+	+	+	+	2/2
31	+	+	+	+	+	+	+	+	2/2
32	+	+	+	+	+	+	+	+	2/2
33	+	+	+	+	+	+	+	+	3/4
40	+	+	+	+	+	+	+	+	2/2
43	+	+	+	+	+	+	+	+	2/2
45	+	+	+	+	+	+	+	+	0/4
47	+	+	+(48 h)	+	-	+	+	+	2/2
48	+	+	+	+	+	+	+	+	2/4
49	+	+	+	without gas	+	+	+	+	2/2
51	+	+	+	+	+	+	+	+	2/2
52	+	+	+	+	+	+	+	+	2/2
53	+	+	+	+	+	+	+	+	2/2
54	+	+	+	+	+	+	+	+	2/2
56	+	+	+	+	+	+	+	+	3/4
75	+	+	+	+	+	+	+	+	2/2
76	+	+	+	+	+	+	+	+	3/4
77	+	+	+	+	+	+	+	+	0/4
78	+	+	+	+	+	+	+	+	1/4
80	+	+	+	+	+	+	+	+	2/2
81	+	+	+	+	+	+	+	+	2/2
82	+	+	+	+	+	+	+	+	2/4
83	+	+	+	+	+	+	+	+	2/2
84	+	+	+	+	+	+	+	+	3/4
86	+	+	+	+	+	+	+	+	2/4
87	+	+	+	+	+	+	+	+	2/2
89	+	+	+	+	+	+	+	+	0/4
91	+	+	+	+	+	+	+	+	1/4
108	+	+	+	without gas	+	+	+	+	2/2
109	+	+	+	+	+	+	+	+	1/4
115	+	+	+	+	+	+	+	+	3/4
117	+	+	+	+	+	+	+	+	3/4

[1] dead animals/animals used

Discussion

Aberrant strains of *P. aeruginosa* occur rather frequently and their identification requires a good knowledge of the whole genus.

Examination for motility of a large number of strains is time-consuming and tedious and therefore the method using semi-solid agar with 0.1% KNO_3 is to be preferred. Dubious strains, the proportion of which does not exceed 10%, can then be examined in the hanging drops from broth cultures. With the strains examined in the present study 18-hour incubation at 37°C proved sufficient since no further positive results were obtained at either 20°C or 30°C.

The natural capacity of *Pseudomonas* spp. to alkalize the base of a variety of sugars makes it necessary to reduce the peptone content to as little as 0.2%. Considering that the oxidative type of carbohydrate metabolism requires in addition more access of free oxygen, the most suited procedure is the OF test in semi-solid agar according to H u g h and L e i f s o n (1953).

The same caution as is needed with a variety of sugars is required in testing for arginine dihydrolase. As indicator of the pH mixture of phenol and bromphenol red proved very useful. A check on the correctness of the procedure is a negative result in the control tube without arginine.

The medium for acetamide hydrolysis according to A r a i et al. (1970) contains no peptone so that the aforementioned considerations are unnecessary in this case. It should be pointed out that this test is of great diagnostic value since out of *Pseudomonas* spp. growing at 41 °C it is only some strains of *P. cepacia* (S c h u b e r t et al. 1975) that give positive results besides *P. aeruginosa*.

The results of nitrate reduction are evaluated after 8-hour incubation in meat-peptone broth containing 0.1 % KNO_3 but as far as denitrification sensu stricto is concerned decomposition to NO and O_2 or even to gaseous N is required. According to J e t e r and I n g r a h a m (1981) the only substrate with which conclusive results can be obtained is nitrite, an observation by which we were guided in formulation our procedure. The fact that 97 to 100 % of our results were positive provides evidence for the right choice of the procedure.

Pyocyanin production is specific for *P. aeruginosa* but its frequency hovers only about the decisive 90%. In our experience, a point of particular importance here is again the choice of optimum medium, an approach to which can be seen in Gessard's liquid medium (1891) and special agar A according to K i n g et al. (1954).

In conclusion it can be said that the results of our biochemical tests with aberrant strains are in reasonable agreement (± 2.3%) with the data tabulated by B e r g a n (1981) but the proportion of our positive cases upon examination for gelatinase, haemolysis and esterase was 31.6 to 56% higher.

No demonstrable relation exists between antibiosis or atypism of a strain and its virulence for the white mouse upon i.p. administration so that where needed bioassay is to be included.

Příspěvek k diagnostice druhu Pseudomonas aeruginosa

Autor prostudoval 70 kmenů *P. aeruginosa* humánní i animální provenience po stránce diferenciačních znaků a příslušných metod.

Výsledky u 28 aberantních kmenů souhlasí (± 2,3%) s publikovanými pře-

hledy (B e r g a n, 1981), ale u zkoušek na hemolýzu, želatinázu a esterázu byly zjištěné počty kladných případů o 31,6 - 56% větší.

Během práce se osvědčilo orig. médium pro denitrifikaci, a to nitritový bujón (0,1% KNO_2) s vloženou plynovkou, pokrytý 1 cm vrstvou parafinového oleje.

Na 17 případech menší virulence až úplné neškodnosti pro bílou myš (0,2 ml bujónové kultury i.p.) se podílely aberantní kmeny o 7 % častěji než kmeny typické.

Při zkrmení stejné dávky kultur myším byly všechny kmeny neškodné.

К диагностике вида Pseudomonas aeruginosa

Автор проводил исследования 70 штаммов P. aeruginosa человеческого и животного происхождения с точки зрения различительных признаков и соответствующих методов.

Результаты 28 аберрантных штаммов совпадают (± 2,3%) с опубликованными обзорами (Bergan, 1981), однако в ходе испытания на гемолиз, желатиназу и эстеразу были установлены положительные случаи на 31,6 - 56% больше.

В ходе работы оправдалась оригинальная среда для денитрификации, а именно нитритный бульон (0,1% KNO_2),с "газовкой",покрытый слоем парафинового масла толщиной 1 см.

В 17 случаях меньшей вирулентности даже безвредности для белый мышей (0,2 мл питательного бульона)участвовали аберрантные штаммы на 7 % чаще типичных штаммов.

В ходе крмления мышей одинаковой дозой культур все штаммы отличались безвредностью.

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