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PSEUDOMONAS AERUGINOSA IN ORAL INFECTIONS

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Twentynine strains of *Pseudomonas aeruginosa* were isolated from 2518 specimens from patients with oral infections and the following clinical diagnoses: periodontitis apicalis, necrosis pulpae, pulpitis and alveolitis maxillaris and mandibularis. All strains produced at least one haemolytic toxin and most of the following extracellular enzymes: lecithinase, protease, elastase, lipase and an esterase. The importance of these proteins for the development of tissue necrosis during infection is discussed. The antibiogram showed that most strains were sensitive to treatment with gentamicin, carbenicillin or streptomycin, but were resistant to several other antibiotics. The strains belonged to 9 different phage types and 15 strains were of the same serotype (Habs's group 1) which is remarkable, since Habs's group 6 is most frequently found among isolated strains from infections outside the oral cavity.

In recent years *Pseudomonas aeruginosa* has attracted more attention than before and there have been more reports on *Pseudomonas* infections during the last decade than in the preceding century (*Alexander,* 1970). *Pseudomonas aeruginosa* is a microorganism of low virulence for healthy patients but is an important pathogen for patients debilitated by other diseases (*Caselitz,* 1966; *Bodey,* 1970; *Alexander, Fisher & MacMillan,* 1971; *Schimpff et al.,* 1971). It often causes infections on the skin and in the urinary tract but also in other organs (*Caselitz,* 1966). However, it is often difficult to prove the clinical significance of colonization with *Pseudomonas aeruginosa* from different body tissues with special reference to the respiratory tract (*Crowder & White,* 1970; *Lerner & Federman,* 1971; *Myerowitz, Medeiros & O'Brien,* 1971). Strains of *Pseudomonas* are often multiresistant and respond poorly to antibiotic therapy (*Davies, Ianette & Wedgewood,*

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1971). Many symptomatic infections are refractory to treatment because of the natural resistance of the organisms to antibiotic therapy or disinfectant treatment (*Burdon & Whitby*, 1967; *Bruun & Digranes*, 1971). Successful chemotherapy of other pathogenic micro-organisms plays a part by allowing *Pseudomonas* to grow without competition.

At the present time little is known about the mechanism by which this organism exerts its damaging effects on the host. The endotoxin has a relatively low toxicity (*Homma*, 1968), but *Pseudomonas* also produces extracellular proteins which are toxic to human and animal tissues and tissue culture cells (*Caselitz*, 1966; *Heckly*, 1970). Recently, a partially purified elastase was found to be lethal upon intranasal injection in animals (*Meinke et al.*, 1970). Lecithin and other proteins, without known enzymatic activity, were also found to be toxic to animals (*Caselitz*, 1966; *Homma*, 1968; *Heckly*, 1970; *Meinke et al.*, 1970).

Pseudomonas has hitherto been found in few samples from oral infections, and until recently bacteria members of this genus were considered to be harmless saprophytes. No systematic study has been carried out to characterize *Pseudomonas aeruginosa* isolated from infections in the oral cavity. The purpose of the present investigation was to characterize oral *Pseudomonas* by biochemical tests, sensitivity to different antibiotics, phage typing, serotyping and the ability to produce different extracellular toxins and enzymes. A similar study on 148 strains of *P. aeruginosa* isolated from nonbacteriemic and bacteriemic infections in hospital patients was recently finished and will soon be published (*B. Wretlind, L. Sjöberg & T. Wadström,* in preparation). The extraordinary findings concerning the patients and the findings concerning the serotypes of the isolated strains in this study made it necessary to report the properties of these oral strains in a separate publication.

MATERIAL AND METHODS

In 2518 bacteriological specimens from patients with oral infections 29 isolates of *Pseudomonas aeruginosa* (17 men and 12 women, 18—51 years of age) were diagnosed. The sampling for microbiological examination was either taken from the apical region through the root canal of teeth or from the soft tissues and the alveolar bone. The samples were transported to the laboratory in a liquid storage medium (VMG II; *Möller*, 1966) and cultured over night at 37° on endo-agar (Difco). The diagnosis of the disease is given according to the International Statistical Classification of Diseases, Injuries and Causes of Death (ICD, 1965 revision, accepted by WHO 1966). The

following diagnoses were found among the 29 infections: periodontitis apicalis (acuta and chronica; 16 cases), necrosis pulpae (8 cases), pulpitis (3 cases), and alveolitis maxillaris or mandibularis (2 cases). The strains were identified as *P. aeruginosa* according to *Hugh* (1970) by the following criteria: catalase and oxidase production; oxidation of glucose; motility; production of pyocyanin and fluorescin; growth on Simmons citrate agar; growth on cetrimide agar; indole negative; H_2S negative; formation of nitrogen gas from nitrate; degradation of gelatin; production of acid from arabinose, fructose, mannitol, and xylose; no production of acid from lactose, maltose, sorbitol, sucrose and trehalose.

Phage typing was performed with a standard set of 22 bacteriophages as recently described (*Sjöberg & Lindberg*, 1968). Petri dishes containing 1.5 % trypticase soy agar (Difco) were flooded with a 5 h culture of each strain in trypticase soy broth (Difco). The 22 phages were applied in routine test dilution (RTD) and 100 times RTD, incubated over night at 30° and the lysis reactions were then recorded.

Serotyping. Sera were produced in New Zeeland white rabbits using 14 different serotypes including 12 types described by *Habs* (1957). Tube agglutination was read after incubation over night at 50° (*L. Sjöberg & A. A. Lindberg*, to be published).

Antibiotic sensitivity testing. The resistance patterns of the 29 strains were determined on blood agar (Difco) (*Ericsson, Tunevall & Wickman*, 1960) by the disc method described by *Ericsson & Sherris* (1971). The zone of inhibition around the disc was measured after 18 hours' incubation at 37° and the minimum inhibitory concentration for the isolated strains was determined. Determination of antibacterial activities was repeated once for each strain with good reproducibility.

Enzyme and toxin analysis. The basic medium consisted of a blood agar base (Difco) in all kinds of plates. The ability to produce the various factors was determined from positive reactions on the different agar plates, i.e. as zones more than 2 mm around the streaks after growth at 37° for 48 h.

Haemolysin production was detected on plates containing nutrient broth (Difco) and erythrocytes from rabbit, sheep, human, horse and ox (5 % final concn w/v) washed once with saline.

The production of protease was studied on agar containing sterile skimmed milk (*Brown & Scott*, 1970). Elastase was assayed on elastin agar plates (*Sbarra, Gilfillan & Bardawil*, 1970).

Positive egg yolk reaction was determined on egg yolk agar (*Lundbeck & Tirunarayanan*, 1966). Lipase activity was assayed on tributyrin agar prepared after sonication of the substrate according to *Hugo & Beveridge*

(1962) and lecithinase on lecithin agar containing 5 g of lecithin per liter agar medium (*T. Wadström, P. Allestam & R. Möllby*, to be published). Esterase activity was detected on agar containing indoxylbutyrate (*Holt*, 1971).

DNase activity was determined on DNase test agar (BBL) according to *DiSalvo* (1958) and RNase activity on RNA agar plates (*Miller, Sandine & Elliker*, 1971). After 48 h at 37° the plates were flooded with normal hydrochloric acid and the positive culture showed a clear zone around the streak.

Bacteriolytic activity was determined on agar plates containing heat-killed *Micrococcus lysodeikticus*, strain N.C.T.C. 2665, (*Hawiger*, 1968) or *Staphylococcus aureus*, strain Copenhagen (*Schindler & Schuhardt*, 1964). Coagulase was assayed on reconstituted rabbit plasma (Bactocoagulase plasma, Difco) as prescribed by the manufacturer.

Hyaluronate lyase (hyaluronidase) activity was assayed on a medium containing umbilical cord hyaluronic acid (*Smith & Willett*, 1968) with a strongly positive *Staphylococcus aureus* strain as a control and by a 100 times more sensitive viscosimetric method (*Söder & Nord*, 1969) after cultivation in a liquid casein hydrolysate medium supplemented with yeast extract. This method was also used to measure chondroitinsulphatase activity.

Chemicals. Paper discs for antibiotic sensitivity testing were provided by AB Biodisk, Stockholm, Sweden. Casein hydrolysate, yeast extract and the agar bases were purchased from Difco. Tributyrin, egg lecithin, elastin powder from bovine neck ligamen, hyaluronic acid from umbilical cord grade III, ribonucleic acid from yeast grade II and chondroitinsulphate grade II were obtained from Sigma Chem. Comp., St Louis, Mo., USA. Indoxylbutyrate was purchased from Koch-Light, Colnbrook, Bucks, England.

RESULTS

Infections with *Pseudomonas* most often occurred in patients with the diagnosis of periodontitis apicalis and necrosis pulpae. Almost all patients with *Pseudomonas* infection showed poor oral hygiene, decayed teeth and gingivitis and had a lower socioeconomic standard than the average Swedish population. No systemic chronic disease, such as diabetes, pulmonary and heart disease or cancer, was found among these patients during the therapeutic period. In nine of the cases mixed bacterial infections were shown. *Pseudomonas* together with streptococci dominated. No isolates of other *Pseudomonas* species, such as *P. fluorescens* or other gram negative rods, were found in the 29 bacterial cultures from which *P. aeruginosa* was isolated.

Table I

Sources and serotypes of the isolated strains. SA = spontaneous agglutination

Diagnosis	Tooth	Age	Sex	Serological type (Habs)
Peridonitis	46	23	F ♀	6
apicalis	17	25	F ♀	6
acuta	24	29	M ♂	6
	13	30	F ♀	6
	14	30	M ♂	1
	14	36	F ♀	1
	45	41	F ♀	6
	43	43	M ♂	1
	45	45	F ♀	6
	35	46	M ♂	1
	36	18	M ♂	6
Peridontitis	15	34	M ♂	1
apicalis	14	35	M ♂	1
chronica	23	37	F ♀	1
	46	40	M ♂	SA
	23	51	F ♀	1
	27	18	F ♀	SA
Necrosis	14	22	M ♂	1
pulpae	24	24	M ♂	1
	14	27	M ♂	2B
	15	27	M ♂	1
	23	35	F ♀	6
	34	40	F ♀	1
	46	51	F ♀	1
	16	22	M ♂	5C
Pulpitis	12	30	M ♂	1
	33	45	M ♂	1
Alveolitis maxillaris		19	M ♂	6
Alveolitis mandibularis		20	M ♂	2B

Distribution of the strains according to phage type. Nine different phage types were found but 16 of the isolated strains belonged to one phage type (21/44/1214/109/F8). The reproducibility of the typing was very good and all strains were typable by the standard set of phages.

Table II

Production of various enzymes and haemolysin by 29 strains of Pseudomonas aeruginosa isolated from oral infections

Haemolysin	29
Protease	29
DN-ase	29
Esterase	22
Lecithinase	23
Lipase	22
Elastase	20
RN-ase	18

Distribution of the strains according to serological type. Fifteen of the twentynine strains were found to belong to Habs's serotype 1, nine strains to group 6, two strains to group 2B and one to 5C (Table I). Two strains were untypable due to spontaneous agglutionation. Fourteen of the strains of phage type 21/44/1214/109/F8 were grouped by serological typing in Habs's group 1. Cross reactions between different serological groups were only found for two strains.

Production of toxins and enzymes. The analysis of the 29 strains for production of different enzymes and haemolysins is shown in Table II. All strains produced haemolysin, protease and nuclease (DNase) but only 18 strains produced RNase; 23 gave a positive egg yolk reaction and 22 produced lipase and esterase. Twentythree strains were lecithinase producers, 20 strains produced elastase, and 9 a staphylococcolytic enzyme. No strains showed positive reaction on plates used for analysis of hyaluronate lyase and lysozyme and no strains were able to coagulate rabbit plasma. *P. aeruginosa* was reported to produce a hyaluronate lyase (*Caselitz*, 1966) but none of the strains degraded either hyaluronic acid or chondroitinsulphate as measured by the very sensitive viscosimetric assay.

Susceptibility of Pseudomonas in vitro to different antibiotics. All strains of *Pseudomonas aeruginosa* were resistant to most of the antibiotics studied (Table III). All strains but one were sensitive to streptomycin and carbenicillin and about half the number of strains were sensitive to sulphonamide. Eighteen of the 29 strains were also sensitive to therapeutic doses of gentamicin. All strains showed a high resistance to all other antimicrobial agents tested except colistin.

Table III.

Sensitivity testing of 29 strains of oral Pseudomonas aeruginosa

Group	Sulphonamide	Phenoxymetyl-penicillin	Ampicillin	Carbenicillin	Cephalosporin	Erythromycin	Streptomycin	Gentamicin	Chloramphenicol	Tetracycline	Colistin
1	2	0	0	0	0	0	28	2	0	0	
2	13	0	0	28	0	0	—	16	3	1	
3	2	0	0	0	0	15	—	11	—	8	
4	12	29	29	1	29	14	1	0	26	20	
S											12
R											17

Group 1 = sensitive
Group 2 = moderately sensitive
Group 3 = moderately resistant
Group 4 = resistant
S = sensitive
R = resistant

DISCUSSION

Pseudomonas aeruginosa was isolated from 29 oral infections from 2518 bacteriological cultures. However, it must be remembered that aerobic gram negative rods constitute only about 5 per cent of the total micro-organisms isolated in oral infections (*Möller,* 1966).

All patients in this investigation, where *Pseudomonas* was isolated, showed symptoms of infection and were probably not contaminants as has been reported for deeper parts of the respiratory tract where *Pseudomonas* might colonize without giving clinical symptoms or pathological effect. (*Lowbury et al.* 1970; *Crowder & White,* 1970). Most patients in this study had poor oral hygiene, decayed teeth and gingivitis and probably a lower resistance to infections. They did not have infections in other parts of the body, however. In none of the case histories was diabetes, leukemia, cancer or another debiliating chronic disease found, which have been reported to give increased susceptibility for *Pseudomonas* infections in other organs (*Bodey,* 1970; *Alexander,* 1971; *Lerner & Federman,* 1971; *Myerowitz, Medeiros & O'Brien,* 1971). No development of systemic

infections from these oral focal infections was found, in spite of the fact that *Pseudomonas* septicemia can develop from pneumonia, skin or urinary infections (*Alexander*, 1970, 1971).

The high resistance of *Pseudomonas* to most antibiotics often makes treatment very difficult and both systematic and focal treatment with various antibiotics have been tried (*Alexander*, 1970, 1971). However, since all patients in this study probably had focal affections these should be treated and not only looked upon as secondary invaders as in the cases with tracheostomas (*Lowbury et al.*, 1970). Before gentamicin and carbenicillin became available, streptomycin and sulphonamides were the drugs most often used (*Forkner*, 1960; *Caselitz*, 1966). The ototoxicity and nephrotoxicity in patients with impairment of renal function, make streptomycin and gentamicin less attractive for common use (*Garrod*, 1971). Several studies on single and combined antibiotics in the treatment of *Pseudomonas* infections have recently been reported. Gentamicin was found to act synergistically with carbenicillin which facilitated treatment in most cases (*Smith et al.*, 1970; *Schimpff et al.*, 1971; *Garrod*, 1971), in spite of the fact that rapid development of resistance to carbenicillin has been reported (*Lowbury, Kidson & Lilly*, 1969). No systematic study on the efficiency of the various treatments has hitherto been presented (*Davies, Ianette & Wedgewood*, 1971). *Pines et al.* (1970) reported successful treatment with gentamicin and carbenicillin of patients with *Pseudomonas* in sputum. Carbenicillin is also distinguished from other penicillins by its highly selective activity against *Pseudomonas* (*Bodey et al.*, 1971).

Habs's serotype 6 was found in about 30 per cent of different types of infections in hospital infections (*Mikkelsen*, 1970; *Wretlind et al.*, in preparation). Thus it is quite remarkable that in this investigation serotype 1 was most frequently found in oral infections. These isolates were also found to belong to one phage type, while 148 strains from hospital infections showed more than 100 different phage types and none was found in more than a few of all the isolated strains (*Wretlind et al.*, in preparation).

Liu (1966) showed that *Pseudomonas* lecithinase and protease were toxic and produced necrosis in different organs upon intravenous infection. More recently *Homma* (1968) and *Meinke et al.*, (1971) reported that a nonenzymatic protein and a partially purified elastase were toxic in animal experiments. The plate assay analysis used in this study to discriminate between the production of various extracellular proteins revealed that different enzymes and haemolysin are produced by nearly all the isolated strains. *Carnery* and *Jones* (1968) reported that protease, elastase, lecithinase and haemolysin were more frequently produced by virulent than by avirulent

strains of *P. aeruginosa*. It is quite reasonable that these proteins, which can cause tissue necrosis, are important invasive factors also in oral infections by this organism.

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