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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

ON THE CHARACTERISTICS OF RECENTLY FOUND BACTERIAL STRAINS
TENTATIVELY DESIGNATED AS "PASTEURELLA X"

[Following is translation of a German-language article by Werner Knapp, Institute for Hygiene of Tübingen University and Ernst Thal, Institute for Veterinary Medicine, Stockholm in Zentralblatt für Bakteriologie, Parasitenkunde, Infektions-Krankheiten und Hygiene (Central Journal for Bacteriology, Parasitology, Infectious Diseases and Hygiene), No. 190, 1963, pp 472-484, Stuttgart.]

Summary: The authors report on the cultural, biochemical, serological and immunological properties as well as on animal experimentation of 40 strains of a bacterial species tentatively designated as "pasteurella X" of which two are of human and 38 of animal origin. They were isolated and independently identified as *pasteurella pseudotuberculosis* in Denmark, Germany, Holland, Sweden and Switzerland.

From common morphological, cultural, biochemical and serological characteristics, the authors conclude that all these strains belong to a species different from *pasteurella pseudotuberculosis*. They differ primarily by the fermentation of saccharose and the absence of fermentation of aesculin, rhamnose and adonitol. In contrast to *pasteurella pseudotuberculosis*, ornithine-decarboxylase reaction is positive. "Pasteurella X"-strains are not dissolved by PST-phages. An antigen or immunogen relation to *P. pseudotuberculosis* type I-V could not be demonstrated. In mice, guinea pigs and rabbits, "Pasteurella X"-strains were apathogenic or only mildly pathogenic and symptoms of disease were not observed clinically.

It should be noted that infection with "Pasteurella X" in man presents the same syndrome as infection with *P. pseudotuberculosis* and takes a septicemic-typhoid or enteral course. The "Pasteurella X" strains so far primarily found in chinchillas are also pathogenic to man which is of importance epidemiologically to veterinaries.

Introduction: During 1962 and 1963, we received from various sides either directly or on request bacterial strains for differentiation and/or further investigation which were isolated with few exceptions during 1962-1963 from chinchillas affected by "pseudotuberculosis". According to early information (Becht, 1962; Daniels, 1963), these strains corresponded to *Pasteurella pseudotuberculosis* only in their morphological and cultural but not in their biochemical, serological, pathogenic and immunological characteristics. A search of the literature for corresponding strains indicated that Hässig et al (1949) described two strains as *P. pseudotuberculosis* although they differed in their biochemical and animal-experimental characteristics from typical strains of *P. pseudotuberculosis*. Both strains had been isolated from patients who died from a clinically not clarified septic condition. In both cases, the pathological findings showed a pseudotuberculosis of the liver with large nodules. Serological examination, together with Knapp, demonstrated antigen relation between these strains and the chinchilla strains from Holland (Daniels et al; Daniels).

Personal communications from Dr. Siegmann (Celle) and Dr. Frederiksen (Copenhagen) informed us that additional strains had been isolated from chinchillas infected with "pseudotuberculosis". Dr. Winblad (Malmö, 1963) isolated a "pseudotuberculosis strain" from the mesenteric lymph node of a youthful patient suffering from mesenteric lymphadenitis and ileitis terminalis. Detailed examination by Thal showed that the cultural and biochemical characteristics of the strain also corresponded to the strains described by Hässig et al and agglutinated in dilutions of as much as 1:1,280 of the immune serum (No. 81) produced with a chinchilla (No. 268). The patient (No. 897) and/or chinchilla (No. 268) strains were also agglutinated in dilutions of as much as 1:160 and/or 1:640 of the patient serum. The repeated isolation of these strains triggering especially in chinchilla the clinical and pathological manifestations due to *P. pseudotuberculosis*, the demonstration in two patients with the clinical symptoms of septic-typhoid and/or enteric human pseudotuberculosis of two strains apparently identical or very similar in their bacteriological and serological characteristics and finally the findings that the data reported by Becht and/or Daniels in regard to the strains investigated by them did not entirely agree, induced us to investigate these strains in greater detail since they would appear to be of equal importance to human and veterinary medicine.

Our investigation attempted to answer the following questions:

1. Are the strains of human origin described by Hässig et al related to or identical with the strain isolated by Winblad?
2. Are the strains isolated from chinchilla in Denmark, Germany, Holland, Sweden (the demonstration of "*Pasteurella X*" in a chinchilla

has been made in the meantime by Thal also in Sweden) and Switzerland related or identical on the basis of their cultural, biochemical, serological, animal-experimental and immunological characteristics and can they be assigned to a species?

3. What cultural, biochemical, serological, animal-experimental and immunological differences exist between these strains and *P. pseudotuberculosis* type I-V?

4. What method of examination will provide positive differential diagnosis of these strains from *P. pseudotuberculosis*?

5. What position should be assigned to the strains in the system of bacteria according to Bergey's Manual (1957)?

I - Investigation Material

a) Strains investigated: The strains and their sources are listed below:

1 strain No. 2533 (human; "Hässig strain") from Dr. Grumbach, Institute for Hygiene of Zurich University;

1 strain No. 897 (human; "Strain Winblad") from Dr. Winblad, Allmänna-Sjukhuset, Malmö;

14 strains No. 13, 18, 49, 51, 57, 59, 71, 85, 115, 122, 134, 151, 157 (chinchilla) and 200 (dog; number assigned by Institute of Hygiene of Tübingen University) from Dr. Becht, Veterinary-Bacteriological Institute of Zurich University;

10 strains No. 905, 924, 931, 975, 1046, 1054, 1055, 1078, 1165 (chinchilla) and 1028 (hare) from Dr. Daniels, St. Francis Hospital, Rotterdam;

3 strains No. P-71, P-76, P-77 (chinchilla) from Dr. Frederiksen, National Serum Institute, Copenhagen.

11 strains No. 266, 267, 268, 271, 612, 709, 988, 1100, 1154, 1524, and 2737 from Dr. Siegmann, Federal Research Institute for Small-animal Breeding, Celle;

93 strains of *P. pseudotuberculosis* (both human and animal) from the stock cultures of the Institute of Hygiene of Tübingen University and the National Veterinary-Medicine Institute at Stockholm.

b) Rabbit Immune Sera Investigated: For the production of the OH-sera designated as "H-sera" in the following, we utilized elutions from soft

agar (0.75-1.0%) plates incubated at 22° C; for the sera designated as "OH", elutions of slant agar (2.5%) cultures also incubated at 22° C; and for the sera designated as "O", elutions of slant agar cultures incubated at 37° and subsequently boiled for 150 minutes. Antigens not boiled were killed with 0.3% phenol or 0.37 formalin. The difference between the sera designated as "H" and/or "OH" consists in the fact that the "H" sera have a higher H and an appreciably lower O titer than the OH-sera. The animals were immunized in the customary manner without adjuvants. The following rabbit sera were available for our initial investigations:

<u>Serum Number</u>	<u>Immunized with Strain</u>
H-85)	No. 2533 (Hässig)
OH-722)	
O-725)	
OH-2900)	No. 897 (Winblad)
O-2899)	
H-83)	No. 18 (Becht)
OH-2177)	
OH-3199)	No. 151 (Becht)
H-86)	No. 975 (Daniels)
OH-809 & 1434)	
OH-800 & 4575)	
H-81)	No. 268 (Siegmann)
OH-1500)	
O-705 & 307)	

II - Investigation Data

1. Investigations on Identity of "Human" Strains No. 2533 and 897.

Introductory Note: Parallel investigations of all strains was made in both institutes. Differences in findings extensively due to differences in the length of incubation periods gave rise to control examinations. A part of the cultural and biochemical investigations reported (Table 1) were made by Miss Struve for her dissertation to be published in 1964.

a) Cultural and Biochemical Characteristics: Incubation in culture media of the strains of both human and animal origin presented no difficulty and was successful on customary nutrient media without special growth-favoring components. The growth of the gram-negative bacteria

Table 1
Cultural and Biochemical Examination

Cultural media and tests	Number and origin of strains investigated						
	1 (Hässig)	1 (Winblad)	14 (Becht)	10 (Daniels)	3 (Fredericksen)	11 (Siegmann)	93 (P. pseudo-TB Type I-V)
Glucose	+	+	+	+	+	+	+
Lactose	+	+	+	+	(+)	+	+
Saccharose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Salicin	-	-	-	-	+	-	+
Inositol	-	-	-	-	-	-	+
Adonitol	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-
Sorbitol	+	+	(-)	(-)	+	+	-
Raffinose	-	-	-	-	-	-	(+)
Trehalose	+	+	+	(-)	+	+	+
Arabinose	+	+	+	+	+	+	+
Inulin	-	-	-	-	+	+	+
Dextrin	+	+	(-)	(-)	+	+	+
Rhamnose	-	-	-	-	+	+	+
Katalase	+	+	+	+	+	+	+
Oxydase	-	-	-	-	+	+	+
β -Galactosidase	+	+	+	+	+	+	+
Gelatine	-	-	-	-	-	-	-
Litmus milk	-	-	-	-	-	-	-
Aesculin	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	+
Voges-Proskaver	-	-	-	-	-	-	-
Ammonium citrate	-	-	-	-	-	-	-

Cultural media and tests	1 (Hässig)	1 (Winblad)	14 (Becht)	10 (DanteIs)	3 (Fredericksen)	11 (Siegmann)	93 (P. pseudo-TB Type I-V)
Liquid medium	-	-	-	-	-	-	-
Solid medium	-	-	-	-	-	-	-
Urea							
Harnstoff Bouillon	-	+	+ +	+ +	+ +	+ +	+
Christensen Bouillon	+	+	+ +	+ +	+ +	+ +	+
Nitrate reduction	+	+	(-)	(-)	+	+	+
H ₂ S (Kligler)	-	-	+	+	-	+	-
KCN	-	-	+	+	-	+	-
Malonat	-	-	-	-	-	-	**
Phenylalanine Desaminase	-	-	-	-	-	-	-
Decarboxylase							
Lysin	-	-	-	-	-	-	-
Arginine	-	-	-	-	-	-	-
Ornithine	+	+	+	+	+	+	+
Motility 22° C	+	+	+	+	+	+	+
37° C	-	-	-	-	-	-	-
PST-phagolysis	-	-	-	-	-	-	+

- * = Alkaline
- ** = Except type IV + at 22° C
- + = positive reaction
- = negative reaction
- + = More frequently positive reaction
- = More frequently negative reaction
- (+)(-) = 1 or 2 strains negative or positive
- (+) = slightly positive delayed reaction
- g = growth-inhibited, i.e. medium is clear with sediment and bacterial growth takes place in smear preparation.

showing predominantly ovoid or rod form was facultatively anaerobic and optimum at temperatures between 30-37° C. In contrast to observations on *P. pseudotuberculosis*, the intensity of growth of these strains was greater at 37° than at 22° C. Demonstration of motility was successful (as with *P. pseudotuberculosis*) only between about 22-28° C and not at 37° C. Further details on the morphological and cultural characteristics of the strains of animal origin will be found in the paper of Struve (1963).

Table 1 shows the manner and results of our cultural-biochemical investigations. In order to check on the fermentation of the sugars and higher alcohols, the cultures were incubated at 37° C for three weeks. Most of the biochemical investigations and/or reactions were carried out on the principles for the identification of enterobacteriaceae indicated by Ewing as well as Edwards and Ewing.

As shown in Table 1, the strains of human origin isolated by Hässig and Winblad are uniform in their cultural-biochemical behavior. They differ from *P. pseudotuberculosis* primarily through the fermentation of saccharose and the absence of fermentation of rhamnose, adonitol and aesculin. In contrast to *P. pseudotuberculosis*, ornithine-decarboxylase reaction was positive for both strains. Moreover, they were not subject to lysis by the PST-phage of Girard (Knapp, 1962).

b) Serological Characteristics: Experiments for agglutination in non-saturated and in crossover-saturated sera demonstrated the serological identity of both strains. A tabulation of the experiments and results was therefore superfluous. Antigen relations to *P. pseudotuberculosis* type I-V were not demonstrated.

c) Animal Pathogenicity: 10 mice and guinea pigs as well as one rabbit survived, without manifest disease symptoms, intraperitoneal or intramuscular infection with as much as 5.0 ml of 24-hr bouillon cultures of both strains. Two additional rabbits showed no indications of disease although they were infected simultaneously intraperitoneally and intramuscularly with 1.0 ml of a 24-hr bouillon culture of strain 897. Dissection of the animals showed only sometimes an appreciable amount of clear serous peritoneal exudate, an inflamed reddening of the peritoneum and an enlargement of mesenterial lymph nodes but no changes in spleen, liver or other organs. The findings from histological examinations are not yet available. Hässig et al had already pointed out the absence of virulence of the strain in regard to the customary small laboratory animals. The same finding was also made in the case of strain 897 (Winblad).

d) Immunological Investigations: Three guinea pigs immunized with strain 897 intramuscularly and/or intraperitoneally showed no protection through vaccination when infected with *P. pseudotuberculosis*, strain 10⁷ (cf. 2-d).

Moreover, 3 weeks after infection with the non-virulent strain 897, 1 rabbit and 2 guinea pigs did not show any dermal indications of any cross reactions after an intracutaneous test with various antigen preparations of *P. pseudotuberculosis* type I-V. Where slight reactions occurred in the sensitized rabbits, these did not differ in kind and intensity from the manifestations observed in the control animals. The animals were tested with 8 different antigen preparations of *P. pseudotuberculosis* type I-V and one antigen preparation each of *Salmonella dublin* and *S. schottmuelleri*.

2. Identity of Strains of Animal Origin

a) Cultural-biochemical Characteristics: The result of cultural-biochemical investigations of the 38 strains of animal origin is also listed in Table 1.

The comparison of the cultural-biochemical characteristics of the strains -- divided in groups corresponding to their origin only for technical reasons -- shows an extensively uniform behavior. Special mention should be made of minor cleavage of lactose which can be observed very late in the strains P-71, P-76 and P-77 as well as of variable biochemical characteristics in the various investigations of strain 1046 and strain 200. Individual differences in our biochemical findings and those of Becht, Daniels and Goudzwaard as well as Daniels alone can very probably be ascribed to the different length of incubation and observation periods of these cultures. Becht observed his cultures only 7 days and Daniels does not furnish the respective time indications. Moreover, diverging findings raise the question of the utilization of chemically impure substances such as we noticed for inositol in our investigations carried out in separate locations (cf. Struve).

A comparison of the cultural-biochemical behavior of the two strains of human origin with that of the strains of animal origin shows their identical behavior. The animal strains were also not subject to lysis by PST-phages.

b) Serological Behavior: The initial serological examinations communicated in this report were intended only to answer the question whether there exist, between the strains of animal and of human origin, antigen relations and these eventually between the H- and O-antigens and/or only between the H- or O-antigens? Clarification of the second question whether eventual differences in the partial antigen structure of the individual strain make possible an arrangement into different serological subgroups and/or types was left to further investigations on which we shall report elsewhere.

Repeated investigations with the OH-sera produced by us with strain 2533, 897, 18 and 151, 975, and 268 demonstrated that all strains

of human and animal origin -- although with different intensities -- were agglutinated as live antigen. Publication of the individual experimental findings attesting the antigen relations of the strains to each other is superfluous.

Further investigations were to answer the above question whether the antigen relations of the strains to each other are based on common O- and H-antigens and/or only on common O- or H-antigens. Arbitrarily selected strains were therefore tested for their characteristics of agglutination in our H- and/or O-sera (Table 2).

The H-antigens were obtained from elutions of cultures incubated on soft agar (0.75-1.0%) plates at 22° C for 48 hours and had abundantly propagated ("Schwarmen"). The suspensions were mixed with 0.3% formalin. O-antigens came from elutions of agar cultures incubated at 37° C for 48 hours and subsequently boiled for 150 minutes. Antigen density was adjusted to Wellcome II and the H- and/or O-agglutination noted after 4 hours at 52° C (water bath) and/or 37° C for 18 hours (thermostat). The result of the various serological examinations is summarized in table 2 which shows the agglutination of the H-antigens in different H-sera and that of the O-antigens in different O-sera.

The evaluation of these and other agglutinations not listed in detail shows that, with the exception of the strains P-71, P-76 and P-77, antigen relations, although of differing intensity, exist between all strains in the O- and H-antigen complex. In transmitting the P-strains, Fredericksen had already pointed out the different O-antigen structure of the latter from each other as well as from various other chinchilla strains. The serological differences between the strains of human origin and some chinchilla strains will be discussed elsewhere. Antigen relations between P. pseudotuberculosis type I-V and the strains of animal origin could not be demonstrated.

c) Animal Pathogenicity: The animal experiments which were also carried out with very large doses of infection as for the strains 2533 and 897, again did not produce any clinical disease manifestations in any of the animals. We infected 40 guinea pigs and 40 mice with 10 different chinchilla strains (Daniels) and also 16 mice with 8 chinchilla strains of different sources. About half of the animals was dissected but only a few of the animals showed the same autopsy findings as after infection with strains of human origin.

In one each of the rabbits and guinea pigs considered clinically as healthy after infection, Becht (1962) found especially in the spleen and less so in the liver slightly brilliant glassy foci whereas Daniels (1963) was not able to make the same observation in mice and guinea pigs with his strains.

Table 2. Serological Examination

Serum number	H- and O-aggglutination of individual strains in different H- and/or O-sera											
	2533 (Hassig)	897 (Winblad)	18 (Hoch)	151 (Recht)	200	975 (Daniels)	1046 (Daniels)	268 (Sigmund)	1154 (Sigmund)	71 (Frederiksen)	76 (Frederiksen)	77
	H	O	H	O	H	O	H	O	H	O	H	O
H - 85	1000	400	400	1000	800	3200	3200	400	800	1600	1600	1600
H - 83	800	200	200	3200	200	6100	6100	1000	1600	200	1000	1000
H - 84	800	400	400	800	800	800	800	200	800	200	400	400
H - 86	800	400	400	3200	800	6100	3200	1000	200	1000	6100	3200
H - 81	3200	1000	1000	6100	500	6100	6100	3200	800	1600	6100	3200
Antigene												
O - 725	100	160	320	320	80	640	640	40	640	20	160	160
O - 2174	40	80	640	640	160	640	640	80	320	40	320	80
O - 2899	640	320	640	640	320	640	640	80	320	40	160	160
O - 4575	160	320	160	160	160	320	320	160	160	40	160	160
O - 705	320	320	160	160	320	1280	160	160	160	80	—	—

H = H-Antigen
O = O-Antigen

Table 3. Differential Diagnosis of P. pseudotuberculosis and "Pasteurella X"

Past. pseudotuberculosis	Type I-V	"Pasteurella X"
Saccharose	—	+
Rhamnose	+	—
Adonitol	+	—
Asculin	+	—
Ornithine	—	+
Arginine	+	—
PST-Phago	+	—
Agglutination in R. pseudo-TB sera	+	—

d) Immunological Examinations: As a result of his experiments of immunization, Becht considers an immunological relation between his chinchilla strains and *P. pseudotuberculosis* as possible. In 4 of 6 animals, he found a difference of length of survival of 2 and/or 6 more days in comparison with the control animals.

As already mentioned, since we could not demonstrate, with the agglutination method, any antigen relations between the strains of human or animal origin and *P. pseudotuberculosis* type I-V and the low number of experiments of Becht appeared to make further investigation with a larger number of animals necessary, we carried out our own experiments of immunization with 10 and/or 9 strains of Becht and/or Daniels. All of the guinea pigs which survived a high dose of antigen (2.0-5.0 ml of an 18-hour bouillon culture) without disease manifestations during the 4-week control period, succumbed to an infection with *P. pseudotuberculosis*, strain 285¹, within 3-5 days, as did 6 control animals.

These investigative findings concord with observations of Struve (-). After intraperitoneal and intramuscular infection of guinea pigs and mice with 10 strains (Daniels), Struve also did not find any crossover immunity against *P. pseudotuberculosis*, strain 2¹.

The result of our cultural, biochemical, serological and immunological investigations on the question of identity of strains of animals origin made so far therefore permits both the human and animal strains to be classified as a species.

3. Cultural, biochemical, serological and animal-experimental differences between "Pasteurella X" and *P. pseudotuberculosis*:

The cultural and biochemical differences existing between both bacterial varieties are shown by the findings in table 1 and 3 which need not here be listed and discussed. Serological examination disclosed a different antigen structure and no antigen relations between the two bacterial varieties. Experiments with infection on guinea pigs and rabbits showed definitely differences in their pathogenicity. The "pasteurella X"-strains were either only slightly or not at all pathogenic for these animals, in contrast to *P. pseudotuberculosis*.

4. Differential Diagnosis of "Pasteurella X" and *P. pseudotuberculosis*

The repeated erroneous diagnosis of "pasteurella X" strains as *P. pseudotuberculosis* made it necessary to identify the most important differential-diagnostic characteristics and to summarize them. Table 3 shows the cultural and biochemical characteristics, the differing reaction to lysis by the PST-phage and the difference of antigen structure which made possible reliable differential diagnosis.

In addition to the characteristics compiled by us in table 3, Daniels (1963) suggests testing for the cleavage of sorbitol, salicin and xylose. In contrast to *P. pseudotuberculosis*, his *pasteurella-X* strains did cleave sorbitol but not salicin and xylose. We found cleavage of xylose in all *pasteurella-X* strains as a rule only between the 7th and 14th day and that of salicin between the 10th and 20th day for some of the strains. We also noted a different fermentative behavior of the *pasteurella-X* strains in regard to sorbitol. Becht found a delayed cleavage of inositol and xylose for the strains used by him; maltose and sorbitol were attacked only by some of the strains and salicin by none of the strains during the control period of one week.

Diagnostic differentiation of *pasteurella-X* strains from strains of the species *Proteus* is easily possible through the absence of phenylalanine-deaminase in the former.

5. Position of "Pasteurella X" in the System of Bacteria

If we were to follow the suggestion of Daniels (1963) and classify these strains under the genus *pasteurella*, they would have to be classified in accordance with Bergey's manual (1957) under I-B in the genus *pasteurella* because they are not motile at 37° but are motile and flagellate at 18-28° C.

However, we believe that the eventual designation of a new species in the genus *pasteurella* must be preceded by a change in the position of *P. pestis* and *P. pseudotuberculosis* in the System of Bacteria and their designation as genus. For a number of years, various authors have justifiably pointed out that the two closely related species *P. pseudotuberculosis* and *P. pestis* differ so greatly in their cultural, biochemical, serological, immunological and pathogenic characteristics from other species of this genus such as *P. multocida* as "type species" and *P. tularensis* that their classification in this genus and in the family *parvobacteriaceae* and/or *brucellaceae* is not justified (cf. literature in Knapp, 1959). This statement is true also for the new species tentatively designated as "*pasteurella X*" by Daniels.

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- i - Letter Addressed to the First International Conference on Diseases of Fur-Bearing Animals, Celle, 1963.
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