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A TAXONOMIC STUDY OF THE GENUS *PASTEURELLA* USING A NUMERICAL TECHNIQUE

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Received 10.xi.64

The genus *Pasteurella*, at present included in Bergey's Manual (Breed, Murray & Smith 1957) as a member of the family *Brucellaceae*, is listed as containing nine species, *multocida*, *septicaemiae*, *haemolytica*, *anatipestifer*, *pestis*, *pfaffi*, *pseudotuberculosis*, *tularensis* and *novicida*. Since publication of the current edition of the Manual the last two species on this list have on account of their special cultural requirements been re-classified as *Francisella tularensis* (Olsufiev, Emelyanova & Danayura 1959, Philips & Owen 1961) and *F. novicida* (Owen *et al.* 1963), using the generic name originally suggested by Dorofeev (1947). Three others, *Past. pfaffi*¹, *septicaemiae* and *anatipestifer* are of dubious status: the first is almost indistinguishable from *Past. pseudotuberculosis* while the latter two, both gelatine-liquefiers, are excluded if only by the Manual's own definition of the genus which unequivocally states that gelatine is not liquefied.

The grouping of the remainder, *Past. multocida*, *haemolytica*, *pestis* and *pseudotuberculosis*, and of certain more recently described forms, presents further difficulties. *Past. multocida* (syn. *Past. septica*) which includes the organisms of bovine haemorrhagic septicaemia, a common form of pneumonia in pigs, fowl cholera and a variety of other disease conditions in mammals and birds, though biochemically (Smith 1958) and serologically (Roberts 1947, Carter 1955, Namioka & Murata 1961) heterogeneous, is usually regarded as a single species. Its relationship with *Past. haemolytica* (Newsom & Cross 1932), a group of bacteria associated with some forms of pneumonia in cattle and sheep, is however not clear, although there are some resemblances between the two. The question of the systematic position and species rank of *Past. haemolytica* has been further complicated by the isolation from sheep of two distinct types, A and T (Smith 1961) and, from the human respiratory tract, of another group of superficially similar strains

¹ The American Type Culture Collection has no *Past. pfaffi* on its lists. The National Collection of Type Culture of Great Britain has one culture, labelled 1102, isolated by Dr. F. Pfaff from an unstated source; it was received in 1921 and is a *Past. pseudotuberculosis* group I.

termed *Past. haemolytica* var. *ureae* (Henriksen & Jyssum 1960, 1961). *Past. pneumotropica*, first obtained from the lungs of mice (Jawetz 1950) and later from humans (Henriksen 1962) is also of uncertain status but resembles in many respects *Past. multocida*.

The close relationship of the plaque bacillus, *Past. pestis*, with *Past. pseudotuberculosis*, causal agent of pseudotuberculosis in rodents, birds and other animals, has been well authenticated (Girard 1953, Pollitzer 1954, Thal 1956, Parnas 1961) but many properties of these two species tend to set them apart from the other members of the genus. A further type isolated from various sources including pseudotuberculosis in chinchillas and lymphadenitis mesenterica in man and temporarily named "*Pasteurella X*" (Daniels & Goudzwaard 1963) appears to be related to but distinct from *Past. pseudotuberculosis* (Knapp & Thal 1963, Mollaret & Chevalier 1964).

The classification techniques reviewed by Sneath (1962) and variously termed numerical, arithmetical, computer or Adansonian taxonomy have been widely applied in bacterial systematics and are of particular value in the study of heterogeneous and closely related groups. On one occasion a collection of *Past. multocida* strains was analysed (Talbot & Sneath 1960) but the genus *Pasteurella* has otherwise been neglected. The present communication describes an attempt to elucidate the relationships between *Past. multocida*, *haemolytica*, *haemolytica* var. *ureae*, *pneumotropica*, *pseudotuberculosis*, *pestis* and "*X*" by the use of a numerical technique.

MATERIALS AND METHODS

Cultures.

25 strains were used and for the purpose of the study were temporarily labelled a to y; details of their sources are given below:

Past. pseudotuberculosis

a.	Turkey, pseudotuberculosis.	E. Thal, Stockholm,	(14/I)
b.	Hare	" "	(30/II)
c.	Mink,	" "	(43/III)
d.	Hare,	" "	(32/IV)
e.	Hare,	" "	(25/V)

Past. haemolytica

f.	Sheep, pneumonia.	G. Smith, Edinburgh,	97, type A)
g.	Sheep, pneumonia.	J. Smith, London,	(RVC. 1057, type A)
h.	Lamb, septicaemia.	G. Smith, Edinburgh,	(1190/1, type T)
i.	" "	" "	(121, type T)
j.	" "	" "	(158, type A)

"*Past. X*"

k.	Human, mesenteric lymphadenitis.	S. Winblad, Malmö,	(897)
l.	Dog, abdominal cyst.	H. Becht, Zürich,	(200)
m.	Chinchilla, pseudotuberculosis.	H. Becht, Zürich,	(18)
n.	Human hepatic abscesses.	A. Hässig, Zürich,	(2533)
o.	Chinchilla, pseudotuberculosis.	O. Siegmann, Celle,	(268)

Past. multocida

- p. Buffalo, haemorrhagic septicaemia. *E. Wijewanta*, Peradiniya, (561)
- q. Buffalo, haemorrhagic septicaemia. *G. Sharma*, Mukteswar, (S. 138).
- r. Pig, pneumonia. *J. Smith*, London, (RVC. 518)
- s. Cat, condition unknown. *N. Stamatin*, Bucarest, (4118)
- t. Sheep, pneumonia. *L. Badiati*, Perugia, (5)

Past. pneumotropica

- u. Human, nose. *S. Henriksen*, Oslo, (953/60)
- v. Human, throat. *S. Henriksen*, Oslo, (4225/60)
- w. Human, dog-bite. *J. Talbot*, London, (12/Venn)

Past. pestis

- x. Human, plague vaccine strain. *G. Girard*, Paris, (EV. 76)

Past. haemolytica var. *ureae*

- y. Human, ozaena. *S. Henriksen*, Oslo, (320/59)

No cultures of *Past. pfaffi*, *septicaemiae* or *anatipestifer* were available for study.

Tests.

All cultures were examined by the following tests, the methods employed being those described by *Kauffmann* (1954), *Ewing* (1960) and *Edwards & Ewing* (1962) unless otherwise stated:

Motility (Gard swarm-plates incubated at 22° C); diameter of giant colonies on five per cent ox blood agar (Oxoid, CM 55) plates, 3 mm thick, after 24 hours at 37° C (character-states: 1.5 mm or less, 1.6–2.0 mm, 2.1–2.5 mm, 2.6 mm or more); increase in giant colony size after a further 24 hours incubation (character states: × 1.80 or less, × 1.81–2.10, × 2.11 or more); haemolysis on the same medium; growth on MacConkey's agar, in ammonium citrate medium (fluid) and in the presence of KCN; action on litmus milk; M.R. test; production of indole, acetoin, catalase, oxidase (using 0.5 per cent aqueous tetramethyl-p-phenylene-diaminehydrochloride), urease, gelatinase and arginine, lysine and ornithine decarboxylase; production of acid within 10 days from aesculin, adonitol, amygdalin, l-arabinose, cellobiose, dextrin, dulcitol, fructose, galactose, glucose, glycerol, glycogen, inositol, inulin, lactose, maltose, mannitol, mannose, melezitose, raffinose, rhamnose, salicin, sorbitol, starch, sucrose, trehalose and xylose; width of inhibition zones around Oxoid Multodisks containing bacitracin 5 u, chlortetracycline 10 µg, erythromycin 10 µg, furazolidone 50 µg, neomycin 10 µg, nitrofurantoin 10 µg, novobiocin 5 µg, oleandomycin 5 µg, oxytetracycline 10 µg, penicillin 1.5 u, polymyxin B 100 u, streptomycin 10 µg, sulphafurazole 100 µg and tetracycline 10 µg (character states: 0.5 mm less, 0.6–3.0 mm, 3.1 mm or more).

Numerical Analysis.

The reactions of the strains were scored in columns on strips of paper, using scoring method III of *Beers et al.* (1962); for each test a + sign was entered to indicate the result, any preceding character-states being scored—and any subsequent ones 0. Percentage similarity coefficients (S) were calculated mentally for each possible pair of strains by laying the record strips side by side, counting n, the number of times + signs coincided and n', the number of times + and — signs coincided, and substituting in the formula

$$S = \frac{100 n}{n + n'}$$

0 signs were ignored.

The strains were sorted manually by accumulative pairing at falling similarity levels, the simplest of the methods of cluster analysis described by *Sneath* (1962), and the resultant groupings depicted diagrammatically. Electronic or other computing devices were not used at any stage in processing.

Fig. 1.

Similarity matrix: 25 *Pasteurella* strains.

RESULTS

The calculated values of *S* ranged from 51 per cent (strains *d* and *i*) to 97 per cent (*b* and *d*). In accordance with the usual practice in publications of this nature the cumbersome table of similarity values has been omitted but, using these values, the strains were re-arranged into final order *v u w y t q r s p j f g h i l m k n o x e b d a c*. Fig. 1, based on the rearranged list, is a similarity matrix giving in symbol form approximations of all *S* values and thus showing cross relationships between strains and clusters of strains. Fig. 2, a dendrogram, is also based on this list.

Two main groups may be discerned, Group 1 containing all strains of *Past. multocida*, *haemolytica*, *haemolytica* var. *ureae* and *pneumotropica*, and Group 2, composed of *Past. pseudotuberculosis*, "X" and *pestis*. This is best seen in Fig. 2 which shows the two groups linking at 65 per cent similarity. Fig. 1 gives the additional information that there was little cross relationship between the two groups: the rectangular area *lv, li, ci, cv*, contains only three entries indicating similarities greater than 75 per cent, and most are less than 70 per cent.

Within the two main groups, the clusters representing different spe-

Fig. 2.

Dendrogram: 25 *Pasteurella* strains.

cies linked at 75 to 85 per cent similarity (Fig. 2). All strains of a given species appeared in the same cluster in the re-arranged list and, except for those of *Past. haemolytica*, were linked by S values ranging from 78 per cent (*multocida*) to as high as 93–97 per cent (*pseudotuberculosis*). In general the Group 2 species were the more homogeneous, as shown in Fig. 1 by the denser shading of the triangular areas delineating clusters, and were the more sharply defined. Within Group 1, the two strains of *Past. haemolytica* type T formed a small, homogeneous subgroup (S = 92 per cent) but were linked to type A only at the relatively low level of 76 per cent.

Although the principal object of the study was not to examine the differential reactions of the various *Pasteurella* species, a summary of the properties of the strains may be of value and is given in Tables 1, 2 and 3.

Table 1 shows the results of a variety of biochemical and other tests. *Past. pseudotuberculosis* and "*Past. X*" reacted to these in a similar

TABLE 1
Biochemical and other Reactions.

Character	Pneumo- tropica	Haemo- lytica var. ureae	Multocida	Haemo- lytica type A	Haemo- lytica type T	»X«	Pestis	Pseudo- tuber- culosis
Motility, 22° C	—	—	—	—	—	+	—	+
Haemolysis	—	+	—	+	+	—	—	—
MacConkey, growth	—	—	—	+	+	+	—	+
Indole	+	—	+	—	—	—	—	—
V.P.	—	+	—	—	—	—	—	—
M.R.	—	+	—	—	—	+	+	+
Urea	+	+	—	—	—	+	—	+
KCN	— ²	—	+ ⁴	—	—	— ³	—	—
Oxidase	+	+	+	+	+	—	—	—
Litmus milk	—	Alk.	—	—	—	—	Clot	Alk.
Lysine decarboxylase	—	—	—	—	—	—	—	—
Arginine decarboxylase ...	—	—	—	—	—	—	—	+ ³
Ornithine decarboxylase ...	—	—	+	—	—	+	—	—
Strains examined	3	1	5	3	2	5	1	5

Catalase: all strains + except *Past. haemolytica* var. *ureae* (trace +); gelatine, citrate: all strains —. Where results varied, the majority reaction is given, followed by the number of strains which reacted thus, Alk.: litmus milk alkaline.

TABLE 2
Fermentative Reactions.

Substrate	Pneumo- tropica	Haemo- lytica var. ureae	Multocida	Haemo- lytica type A	Haemo- lytica type T	»X«	Pestis	Pseudo- tuber- culosis
Aesculin	—	—	—	— ²	—	—	—	+
Adonitol	—	—	—	— ²	+	— ³	—	+
Amygdalin	— ²	—	—	—	+	+	+	+
Arabinose	—	—	—	+	+	+	+	+
Cellobiose	—	—	—	—	+	+	—	+
Dextrin	+	+	— ⁴	+ ²	—	+	+	+
Galactose	+	+	+	+	—	+	+	+
Glycerol	— ²	—	+ ³	+	+	+	—	+
Glycogen	—	—	—	+	+	—	—	—
Inositol	—	—	—	+ ²	+ ¹	—	—	—
Maltose	+	+	— ⁴	+	+	+	+	+
Mannitol	—	+	+	+	+	+	+	+
Mannose	+	+	+	+ ²	—	+	+	+
Raffinose	+ ²	—	—	—	—	—	—	—
Rhamnose	—	—	—	—	—	—	—	+
Salicin	—	—	—	—	+	—	+	+
Sorbitol	—	+	— ³	+	+	+ ³	—	—
Starch	+	—	—	— ²	—	—	—	+ ³
Sucrose	+	+	+	+	+	+	+	—
Trehalose	+	—	+ ³	—	+	+	+	+
Xylose	—	—	+ ⁴	+	—	+	+	+
Strains examined	1	1	5	3	2	5	1	5

Glucose, fructose: all strains +; melezitose, inulin, dulcitol, lactose: all strains —. Where results varied, the majority reaction is given, followed by the number of strains which reacted thus.

manner except for the decarboxylase tests and litmus milk. *Past. multocida* was distinguished from *Past. pneumotropica* only by the ornithine decarboxylase and urea tests and the two types of *Past. haemolytica* gave identical reactions. The oxidase test was uniformly positive with Group 1 strains and negative with Group 2.

TABLE 3
Antibiotic Sensitivity Tests.

Antibiotic	Pneumotropica	Haemolytica var. ureae	Multocida	Haemolytica type A	Haemolytica type T	X _α	Pestis	Pseudotuberculosis
Bacitracin	— ²	±	± ³	± ²	V	—	—	— ⁴
Chlortetracycline	+	+	+	+	+	+	+	+
Erythromycin	+	+	+	+	+	— ⁴	—	— ⁴
Furazolidone	+	+	+	+	+	± ³	+	+
Neomycin	+	—	± ³	± ²	±	± ⁴	+	± ⁴
Nitrofurantoin	+	+	+	+	+	+	+	+
Novobiocin	± ²	±	± ⁴	—	— ¹	—	—	—
Oleandomycin	± ²	—	— ³	—	±	—	—	—
Oxytetracycline	+	+	+	+	+	+	+	+
Penicillin	+	+	± ⁴	+	+	—	—	± ⁴
Polymyxin B	+	+	± ⁴	± ²	±	+	—	V
Streptomycin	+	+	+	+	+	+	+	+
Sulphafurazole	—	+	± ⁴	± ²	+	—	—	—
Tetracycline	+	+	+	+	+	+	+	+
Strains examined	3	1	5	3	2	5	1	5

Where results varied, the majority result is given, followed by the number of strains which reacted thus. V: results too variable for summary.

—: Inhibition zone 0.5 mm wide, or less.

±: Inhibition zone 0.6 to 3.0 mm wide.

+: Inhibition zone 3.1 mm wide, or more.

No comment on the reactions of *Past. pestis* and *Past. haemolytica* var. *ureae* is made since only one strain of each was examined.

Fermentation reactions are summarized in Table 2. *Past. pseudotuberculosis* and "*Past. X*" differed consistently in their action on aesculin, adonitol, cellobiose, rhamnose, salicin and sucrose and less consistently with sorbitol and starch, while *Past. multocida* and *pneumotropica* were distinguishable by their reactions with dextrin, maltose, mannitol, starch and xylose. Cellobiose, galactose, glycogen, salicin and trehalose fermentation tests were among those for which types A and T of *Past. haemolytica* gave different results.

Certain of the antibiotic sensitivity tests (Table 3) are also of interest. *Past. pseudotuberculosis* and "*X*" for example tended to be erythromycin and novobiocin resistant, in contrast to *multocida* and *pneumotropica*, while *Past. multocida* and *pneumotropica* showed differences in their sensitivity to sulphafurazole, oleandomycin and bacitracin.

DISCUSSION

The major finding of interest was the clear division into Group 1 (oxidase positive) and Group 2 (oxidase negative). The low overall similarity between the two groups gives justification for *van Loghem's* (1945, 1946) proposal of a separate *Yersinia*, to accommodate the causal organisms of plague and pseudotuberculosis, and in subsequent discussion these two species will be referred to as *Yersinia pestis* and *Y. pseudotuberculosis* respectively. *Thal* (1954) considered that *Yersinia* might well be classified within the family *Enterobacteriaceae* and though the present work offers no help on this point it is noteworthy that *Sneath & Cowan* (1958), in a more general computer study of bacteria, placed *Y. pestis* between *Escherichia* and *Klebsiella*.

The results confirm *Frederiksen's* (1964) assignment of the so-called "*Pasteurella X*" to the same genus, *Yersinia*; they also afford valuable supportive evidence for its species status since the strains examined formed a group whose homogeneity equalled that of *Y. pseudotuberculosis* but which was as clearly differentiated from *Y. pestis* and *Y. pseudotuberculosis* as these two species were from each other (Figs. 1 and 2). On grounds of prior description (*Schleifstein & Coleman* 1943) *Frederiksen* favoured the specific epithet *enterocoliticum* for the "*X*" group but as the two *enterocoliticum* strains available to him were atypical in producing indole, a character of some weight in conventional taxonomy, this question might perhaps be left open for further study; meanwhile "*Yersinia X*" would serve as a convenient label.

The residue, Group 1 (oxidase positive), may be regarded as constituting the genus *Pasteurella*. Here the differentiation of the subgroups is less sharp than in Group 2 (*Yersinia*), peripheral strains tending to possess intermediate properties and thus causing the subgroups to blend (See Fig. 1). *Past. multocida* appears as a somewhat heterogeneous cluster of strains all showing about 85 per cent similarity with each other and probably therefore acceptable as a species. *Talbot & Sneath* (1960) in a computer study of *Past. multocida* found no reason to subdivide the group but their collection of strains was by no means representative and several authors have noted biochemical and serological variations between strains isolated from the different host species (*Roberts* 1947, *Carter* 1955, *Smith* 1958, *Namioka & Murata* 1961). Further work in the form of a detailed, comparative study of strains from a wide variety of mammalian and avian hosts is still needed to investigate the validity of the *formae speciales* recognized by earlier authors under such specific epithets as *bovisepitica*, *suisepitica*, *avisepitica* and others.

The similarity matrix, Fig. 1, gives the better indication of the affinities of *Past. multocida* within Group 1 and shows its main cross relationships to be with *Past. pneumotropica*. (The impression given by Fig. 2 may be misleading here, since the dendrogram depends on similarities

of adjacent strains and clusters only.) The single strain of *Past. haemolytica* var. *ureae* appeared between *pneumotropica* and *multocida*, not with the *Past. haemolytica* group; Jones (1962) who examined 18 strains of this bacterium also found its affinities with *Past. haemolytica* to be slight and chose to call it *Past. ureae*, a decision justified by the present results.

The other finding of interest concerns *Past. haemolytica*. Smith (1961) recognized two types, A (arabinose positive) and T (trehalose positive) distinguished by colonial, fermentative and other properties. In this study, although the two types appeared side by side in the final classification, they differed markedly from each other, linking at as low as 75 per cent similarity (Fig. 2). The differences were largely fermentative (Table 2) but a more detailed taxonomic investigation of this group might well furnish grounds for regarding A and T as separate species rather than types of *Past. haemolytica*.

The study reported, like others of its kind, illustrates the value of the numerical technique in bacterial systematics as an adjunct to more conventional taxonomic procedures. Though time-consuming, the method had several valuable features: it is objective (at least to the extent that selection of material and characters for examination can be rendered objective), it enables the systematist to utilize, if he wishes, all available data concerning his material rather than a few empirically chosen properties, and the affinities of the microorganisms studied can be expressed in a quantitative form. Provided the number of strains is kept reasonably low, much useful work can be accomplished without recourse to calculating devices. The maximum number of strains which might conveniently be handled in this way would seem to be about 25, involving calculation of some 300 values for S; for larger problems electronic computation is essential. Most work in this field has so far been confined to cultural and biochemical characteristics but there is no fundamental reason why serological, phage-typing and pathogenicity tests should not also be used; although there are difficulties in presenting the results of such tests in a form suitable for analysis, their inclusion would greatly increase the usefulness of the technique.

SUMMARY

25 cultures, originally identified as *Pasteurella* species, were classified by a numerical (Adansonian) method, and fell into two main groups. Group 1 (oxidase positive) comprised *Past. multocida* and *pneumotropica*, which were related, the two types of *Past. haemolytica*, A and T, which differed markedly from each other, and *Past. haemolytica* var. *ureae*; the latter resembled *Past. pneumotropica* rather than *Past. haemolytica* and the name *Past. ureae* seems more appropriate. It is suggested that Group 1 should be regarded as constituting the genus *Pasteurella* and that Group 2 (oxidase negative), containing

species formerly designated *Past. pseudotuberculosis*, *pestis* and "X", should form the genus *Yersinia*.

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