The use of the API 20 NE bacteria classification procedure to identify *Pasteurellaceae* strains in rodents and rabbits.

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Summary

Forty growth-factor independent *Pasteurellaceae* strains representing most known taxa from rodents and rabbits were API 20 NE profiled by four laboratory animal diagnostic laboratories using their routine methodology. Significant differences were found in the number of bacterial strains classified with the *Pasteurellaceae*. The results of the four laboratories taken together showed that 136 (85 %) of the 160 tests carried out with the 40 strains led to classification with the family. The 23 *Pasteurellaceae* strains from species *(Pasteurella aerogenes, P. multocida, P. pneumotropica)* present in the API 20 NE data base (API taxa strains) and the 17 strains from taxa not included (non - API taxa strains) were classified with the family with similar frequency.

Pasteurella species designation differed significantly between the laboratories and full agreement in speciation was found with only 9 (22.5 %) of the 40 strains. Of the tests carried out with *P. multocida and P. pneumotropica,* 42 and 52 % respectively led to misclassification. Conversely 38 % of the profiles obtained with non- API taxa strains led to identification as *P. multocida or P. pneumotropica.*

We conclude that identification of *Pasteurellaceae* to the species level by the API 20 NE system is unreliable, but the system is useful to classify various *Pasteurellaceae* taxa from rodents and rabbits with the bacterial family.

Introduction

Pasteurellaceae belong to the most frequently occurring pathogenic bacteria in laboratory rodents and rabbits *(Boot 1997)*. The Federation of Laboratory Animal Science Associations (FELASA) recommends monitoring rodents and rabbits for all *Pasteurellaceae* taxa *(Nicklas et al. 2002)*. One reason for this recommendation is that various *Pasteurellaceae* other than *P. multocida* and *P. pneumotropica* have been cultured from both healthy and diseased rodents and rabbits *(Boot 1997)*. Further, many *Pasteurellaceae* have through a variety of pathogenicity attributes, the capacity to influence the immune system and to interfere with respiratory research *(Boot 1997; Boot et al 1999)*. Finally, until the taxonomy of the *Pasteurellaceae* is fully elucidated *(Bisgaard 1995)* species identification of members of this bacterial family will remain difficult, as the key characteristics separating the species are unclear.

Due to their ease of use commercial biochemical identification systems have been widely used by laboratory animal diagnostic laboratories *(Hansen 2000)*. Such systems have been developed for the classification of bacteria from human and some

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important veterinary sources. The API 20 NE test kit (BioMerieux) is indicated as suitable for the diagnosis of *Pasteurella* spp from laboratory animals *(Hansen 2000)*, but surprisingly data substantiating this have to our knowledge not been published. In this study the first author profiled 40 growth-factor independent *Pasteurellaceae* strains from rodents and rabbits in the API 20 NE system, and requested three other laboratory animal diagnostic laboratories to profile the strains by their 'standard methodologies' to get an insight into the suitability of the system under the (predictable) variations in the way tests are carried out. We first evaluated the classification of *Pasteurellaceae* strains with the family and then possible differences in their species designation between the laboratories.

Materials and Methods

Bacterial strains: the 40 *Pasteurellaceae* investigated were well documented type and other reference strains (Table 1) along with our own strains that were characterized and classified as described

Table 1. *Pasteurellaceae* taxa studied and their host origin

Bacterial species	host	n =	collection numbers *
A. muris	mouse	3	SSI P719; DKFZ J384012 & L032091
H. influenzaemurium	"	1	DKFZ R025011 T
P. aerogenes	rabbit	2	ATCC 27883 T, HIM 612.4
P. multocida	guineapig	2	SSI P397 & P403
"	hamster	2	MHH 911/83, LAM Pm29
"	rabbit	2	CDI Pm02642 & Pm47459
P. pneumotropica Heyl	mouse	2	ATCC 12555, LAM Ppn4
P. pneumotropica Jawetz	"	3	NCTC 8141; SSI P185 & P440
"	rat	2	LAM Ppn135 & Ppn330
P. pneumotropica	gerbil	2	LAM Ppn176 & Ppn183
"	hamster	2	MHH 522/82 & 995/83
"	mastomys	2	LAM Ppn325 & Ppn326
"	rat	2	HIM 599/5 & 651/1
P. ureae #	mouse	2	SSI P687 & P688
SP group pasteurella	guineapig	2	SSI P603 & P605
"	hamster	2	LAM Pg22 & Pg23
Bisgaard Taxon 6	guineapig	2	LAM Ac46 & Ac47 (IAD)**
" Taxon 7	"	2	CCUG 15569 & 24852
" Taxon 8	"	1	CCUG 16494
" Taxon 25	"	2	LAM Pg19 & Pg 20 (IAD)**

disputed species designation (*Mutters, Frederiksen and Mannheim* 1984).

* ATCC: American Collection of Type Cultures; CDI: Institute for Animal Science and Health, Lelystad NL (Dr A Kamp), CCUG: Culture Collection University of Goteburg S; DKFZ: Deutsches Krebsforschungs Zentrum, Heidelberg FRG (Dr W Nicklas); HIM, Hygiene Institut der Philipps Universität, Marburg FRG (Dr W Mannheim) MHH: Medizinische Hochschule Hannover FRG (Dr I Kunstyr), LAM: own collection, NCTC: National Collection of Type Cultures, Central Public Health Laboratory, London UK; SSI: Statens Serum Institute, Copenhagen DK (Dr W Frederiksen); T: type strain

** Diagnosis by Dr M Bisgaard, Royal Veterinary and Agricultural University, Copenhagen Denmark

(Boot et al 1993). The strains represent the wide variety of growth-factor independent *Pasteurellaceae* taxa isolated from rodents and rabbits. They comprised 23 strains belonging to species included in the API 20 NE data base *(P. aerogenes, P. multocida, P. pneumotropica,* further referred to as API taxa strains) and 17 strains belonging to taxa not included (non - API taxa strains). The laboratory technicians carrying out the tests were informed only that the bacteria under study were members of the *Pasteurellaceae* family.

API 20 NE testing

Tests were carried out by the four laboratories using their respective daily employed 'standard methodology' based on the manufacturer's (BioMerieux) instructions (Table 2). Kits were inoculated with bacterial suspensions standardized to MacFarland 2 (laboratories A and B) or 0.5 (laboratories C and D) and incubated 48 hrs at 37 °C (laboratories A, B and C) or 30 °C (laboratory D). Test results were read after 48 hrs and the numerical profiles (API codes) reported were compared by the first author to the profiles of species listed in the computerized API 20 NE database version 6. In laboratory D, two strains were not tested as efforts to subculture them failed by repeat.

Scoring / evaluation of profiles

'No identification' was scored if the API code did not compute (was not listed) or if the identification was considered 'not valid' or 'unacceptable' by the API system. The identification was considered to be valid if the API score did compute and yielded a family / species designation with one of each of the following qualifications: low discrimination (ld), doubtful (d), acceptable (a), good (g), very good (vg) and excellent (e). Where 'very good identification to the (*Pasteurella*) genus level' was obtained the *Pasteurella* sp indicated with the highest percentage of identification was considered the right species designation.

Table 2. Identifications derived from API 20 NE profiles reported by 4 laboratories (A – D) on 40 *Pasteurellaceae*

		A	B	C	D
API test					
	Macfarland density	2	2	0.5	0.5
	incubation hrs	48	48	48	48
	incubation temp °C	37	37	37	30
Identification	no identification	5	2	2	2* + 1
	identification	35	38	38	37
identified as	non *Pasteurella*	6	2	0	4
	Pasteurella	29	36	38	33
P. species	*aerogenes*	4	1	4	4
	haemolytica	7	22	5	4
	multocida	9	8	4	6
	pneumotropica	9	2	24	19
	not indicated	0	3	1	0

* 2 strains not alive after shipment

Identification of a strain was considered to agree between the laboratories if all four profiles excluded a strain from the *Pasteurellaceae* or if all profiles led to the same *Pasteurella* species designation. An identification was considered to be aberrant when a profile led to 'member of the *Pasteurellaceae'* where three other laboratories agreed that the strain did not belong to the family and if for instance *'P. multocida'* was obtained and the three other laboratories diagnosed *'P. pneumotropica'* etc. The two strains that could not be evaluated by laboratory D were considered not to be identified.

Statistical evaluation

The x^2 test was used to evaluate possible differences in the number of identifications and other scores between laboratories. Similarly we evaluated presumed differences in the number of identifications obtained with API taxa and non-API taxa strains and the quality of the diagnoses.

Results

The number of identifications within the *Pasteurellaceae* family was different (x^2 = 9.01; p = 0.03; df = 3) as was the *Pasteurella* species designation (x^2 = 50,9; p < 0.001; df = 12). Laboratory B reported profiles leading to *P. haemolytica* more often than the other laboratories (x^2 = 28.1; p < 0.001; df = 3) whereas laboratories C and D found *P. pneumotropica* more often than both other laboratories did (x^2 = 31.6; p < 0.001; df = 3). The number of strains classified as *P. multocida* or *P. pneumotropica* differed significantly (x^2 = 19.3; p = 0.002; df = 3) between the laboratories.

All laboratories excluded one *Actinobacillus muris* strain from the *Pasteurellaceae*. They reached the same *Pasteurella* species designation with 8 strains, comprising four API taxa strains (one *P. aerogenes,* two *P. multocida,* one *P. pneumotropica*) and four non-API taxa strains (one *P. ureae,* one SP group pasteurella, and two Bisgaards taxon 25). So full agreement was found with only 9 (22.5 %) of the 40 strains examined. Bilateral diagnostic agreement varied considerably (Table 3): it was lowest between laboratory B and the others and highest between laboratories C and D. The number of aberrant (unique) identifications ranged from 1 to 10 and laboratories A and B reported significantly more profiles leading to unique diagnoses than both other laboratories (x^2 = 12.9; p = 0.005; df = 3).

Table 3. Bilateral diagnostic agreement on 40 *Pasteurellaceae* strains studied by 4 laboratories (A - D)

	B	C	D
A	20 *	10	20
B		13	31
C			15

* number of strains

Taken together the 68 profiles produced by the four laboratories on the 17 non-API taxa strains more often lead to 'no identification' than the 92 profiles obtained with the 23 API taxa strains (x^2 = 4.3; p = 0.04; df =1). There was no difference in the classification as a member of the *Pasteurellaceae* between both groups of strains (x^2 = 1.06; p = 0.30; df =1), but the quality (probability) of the identifications obtained with API taxa strains (Table 5) was significantly better than the quality of the identifications obtained with the non-API strains (x^2 = 22.4; p < 0.001 ; df = 1). With the 23 API taxa strains, 62 of 92 (67 %) of the profiles led to the correct species identification. Of the tests carried out with *P. multocida* and *P. pneumotropica* 42 and 52 % respectivily led to misclassification. Conversely with the 17 non-API taxa strains 28 of 68 (38 %) of the profiles led to identification as *P. multocida* or *P. pneumotropica.*

The profiles (Table 4) leading to 'no classification' (n = 12) or classification outside the *Pasteurellaceae* (n = 12) indicated one or more positive readings in assimilation tests (which should be negative). When we considered all assimilation tests to be negative the number of profiles leading to 'no classification' was reduced to 5 and involved the three *A. muris* strains only. The number of pro-

Table 4. Identification of API taxa and non-API taxa *Pasteurellaceae* strains (totalized results of 4 laboratories)

	strains	tests	no identification	non *Pasteurella*	*Pasteurella* diagnosis	Species* *aerogenes*	*haemolytica*	*multocida*	*pneumotropica*	not indicated
API taxa										
P. aerogenes	2	8	0	1	7	7				
P. multocida	6	24	1	6	17		1	10	3	3
P. pneumotropica	15	60	2	1	57	15	11	31		
Subtotal	*23*	*92*	*3*	*8*	*81*	*7*	*16*	*21*	*34*	*3*
non-API taxa										
A. muris	3	12	6	2	4		1		3	
H. influenzae-murium	1	4	1		3		1		2	
SP group pasteurella	4	16		2	14	6	8			
P. ureae	2	8			8		1		6	1
taxon 25	2	8			8		8			
taxon 6	2	8	2		6		2		4	
taxon 7	2	8			8			6	2	
taxon 8	1	4			4		1		3	
Subtotal	*17*	*68*	*9*	*4*	*55*	*6*	*22*	*6*	*20*	*1*
Total	40	160	12	12	136	13	38	27	54	4
%		100	7.5	7.5	85	8	24	17	34	2.5

files leading to classification outside the *Pasteurellaceae* was reduced to zero by assuming negative assimilation test results. Positive assimilation test results were also read in 19 / 136 (14 %) of the profiles that yielded a classification within the *Pasteurellaceae*, notably with *P. aerogenes* and the SP group pasteurella strains. When we assumed assimilation test results to be negative the species designation did not change (n = 4), changed to another *Pasteurellaceae* species (n =14) and one classification was now outside the family. Laboratories inoculating the kits with bacterial suspensions standardized to MacFarland density 2 did not report more positive assimilation tests (56 of 960 [2 laboratories x 12 tests on 40 strains] = 6 %) than did laboratories (57 of 960 tests) that standardized to density 0.5

Discussion

The API 20 NE kit has been developed for Gram negative rod shaped bacteria other than *Enterobacteriaceae* from human and some veterinary sources. The system contains only part of the key characteristics of *Pasteurellaceae*, namely (pos) glucose fermentation, (neg) arginine dihydrolase, (pos) NO3 reduction. This implies that bacterial strains belonging to the family may not be recognized as such. This seems to be substantiated by the fact that up to 11 (27. 5 %) of the strains were classified not at all or outside the family (Table 2). As any relationship with the way the tests were carried out by the laboratories is not obvious, difficulties in reading test results might be the cause of classifying strains outside the *Pasteurellaceae*. Nevertheless the majority of the strains were

classified within and although the probability of the identifications obtained with the non-API taxa strains was much lower than that obtained with the API taxa strains (Table 5), the likelihood of identification as a *Pasteurellaceae* did not differ between both groups of strains. This suggests that the API 20 NE system is suitable to classify *Pasteurellaceae* strains with the family irrespective of their taxonomic position. Our study included strains belonging to *Pasteurella* sensu stricto *(P. multocida)*, strains affiliated to the genus *Actinobacillus (A. muris,* mouse and rat *P. pneumotropica,* and strains originally described as *P. ureae*), and strains whose taxonomic position is not yet clear (growth factor independent *H. influenzae-murium*, hamster *P. pneumotropica*, and the various taxa from guinea pigs) *(De Ley et al 1990; Dewhirst et al 1993)*.

The species designations derived from the profiles reported by the four laboratories revealed significant differences (Table 2), in that laboratories C and D over-diagnosed *P. multocida* and *P. pneumotropica* and laboratory B frequently came up with *P. haemolytica* (not in our study being an exclusive ruminant pathogen). Despite the API 20 NE database allowing five different *Pasteurella* spp identifications, full diagnostic agreement between the laboratories existed only for 20 % of the strains. That API taxa strains were misidentified in 37 % of the tests indicates that identification to the species level by this test system is not very reliable. Of course all *Pasteurella* species identifications obtained with the non-API taxa strains were inevitably wrong. Pitfalls in the identification of growth-factor independent *Pasteurellaceae* by the API 20 NE system have been reported *(Hamilton-Miller 1993; Frederiksen and Tonning 2001)*. In addition, growth-factor requiring *Pasteurellaceae (Haemophilus* spp) type and reference strains were not properly classified by commercial rapid-test kits *(Brands and Mannheim 1996)*.

The reasons for the discrepancies of results found by the laboratories must lie in differences in the way the tests were performed and read. API tests are said to be sensitive to variation in the density of the inoculum, incubation temperature, age (and hence quality) of the reagents to be added prior to reading. Reading is subjective by definition and in assimilation tubes turbidity may be due to a heavy inoculum and not to growth. As a consequence different test results may be obtained even if tests are repeatedly carried out and read by the same person. We deliberately did not attempt to standardize methodologies in the laboratories as the aim of the study was to explore differences in test outcome in different laboratories using their daily applied 'standard methodology'.

Traditionally *P. multocida* and *P. pneumotropica* are considered of greater importance than other *Pasteurellaceae*, although this seems to be unjustified *(Boot 1997; Boot et al. 1999)*. The fact that 42 and 52 % respectively of the tests carried out with *P. multocida* and *P. pneumotropica* strains led to misclassification suggests that rodents and rabbits might be easily and falsely considered uninfected by these 2 species. This is partly counterbalanced by the misidentification of non-API taxa strains as *P. multocida* or *P. pneumotropica*. Animal colonies

Table 5: quality of identification of API taxa and non-API taxa *Pasteurellaceae* strains (totalized results of 4 laboratories)

	no diagnosis	non *Pasteurella* diagnoses						*Pasteurella* diagnoses					
		ld *	d	a	g	vg	e	ld	d	a	g	vg	e
API taxa strains	3	2	3	1	2			6	1	2	34	10	28
Non-API taxa strains	9	2	1	1				14	13	4	15	9	

ld: low discrimination; d: doubtful; a: acceptable; g: good; vg: very good; e: excellent

considered to be infected by *P. multocida* or *P. pneumotropica* will more likely be repopulated than when other *Pasteurellaceae* species were diagnosed.

We conclude that identification of *Pasteurellaceae* to the species level by the API 20 NE system is unreliable due to the existence of taxa that are not included in the API database and due to the limited diagnostic agreement between laboratories studying the same strains. Despite variations in the way the system is used, the kit is useful to classify *Pasteurellaceae* strains of a wide variety of presently known taxa from rodents and rabbits with the bacterial family, and so remains a valuable tool for the laboratory animal microbiologist *(Hansen 2000)*. However species identification of *Pasteurellaceae* should be carried out by reference laboratories that will use comprehensive conventional phenotypic and genetic methods such as 16S rRNA gene sequencing.

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