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Haemophilus Infection in a Colony of Laboratory Rats

WERNER NICKLAS

Central Animal Laboratories, German Cancer Research Centre, D-6900 Heidelberg, Federal Republic of Germany

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During routine quality control of laboratory rodents, short gram-negative rods with satellite growth adjacent to a *Staphylococcus* strain were isolated from rats. They proved to be members of the family *Pasteurellaceae*. On the basis of their dependence on V factor they were classified as *Haemophilus* sp. Systematic investigations in our laboratory rat colony revealed a high prevalence of these bacteria. They were isolated from 75 of 446 rats (16.8%) which were monitored by culture during a 2-year investigation. Most strains were isolated from the lungs and the trachea; some were cultured from the nasal cavity and the female genital tract. Antibodies to these bacteria were detected in sera from 385 of 829 rats (46.5%) by using an indirect immunofluorescence test. The majority of culturally and serologically positive animals came from three separate holding areas; they all came from the same breeder. Investigation of rats immediately on receipt from the breeder showed that they were culturally and serologically positive for *Haemophilus* sp. Histological examination of rats which were monoinfected with *Haemophilus* sp. showed a mild inflammatory cell infiltration in the lungs and a light diffuse hyperemia. In the physiological and biochemical investigations of 53 isolates, all strains had an identical biochemical profile. On the basis of the 35 criteria examined, a definite classification is not possible. These *Haemophilus* bacteria are probably members of a hitherto unknown species.

Members of the family *Pasteurellaceae* are very common bacteria in the respiratory tracts of humans and animals. This family consists of the closely related genera *Actinobacillus*, *Haemophilus*, and *Pasteurella*. The classical criteria for differentiation between these genera are the production of certain enzymes and dependence on X and V factors. The validity of these growth factors as primary genetic criteria is now being questioned (7), since Pohl (11) demonstrated by DNA hybridization techniques that classical methods do not reflect the real relatedness. However, it is still common to classify growth factor-requiring members of the *Pasteurellaceae* as *Haemophilus* spp. (7).

In rodents, *Pasteurella pneumotropica* is the only species of the family which is very common in conventional laboratory animal colonies (1). The isolation of other members of this family from rodents is reported less frequently. Only a few reports exist about the presence of X- or V-factor-requiring members of the *Pasteurellaceae* in laboratory rodents. Csukas (3, 4) isolated *Haemophilus influenzae-murium* from mice. Harr et al. (5) and Kilian (6) described the isolation of *Haemophilus* spp. from rats. Another unknown *Haemophilus* species was recently cultured from a laboratory rabbit (12). However, Boot et al. (2) were not able to isolate *Haemophilus* spp. from feral rodents in a systematic search for members of the *Haemophilus-Pasteurella-Actinobacillus* group.

In this report I describe an endemic with V-factor-requiring members of the *Pasteurellaceae* in a colony of rats. These bacteria were first cultured during routine microbiological quality control in laboratory rodents. It was the purpose of this work to investigate the prevalence of these bacteria in our colony as well as their physiological properties and their significance as pathogens. On the basis of classical criteria for differentiation, they are here referred to as members of the genus *Haemophilus*, although it is not our intention to give a final systematic classification based on the criteria which are reported in this paper.

MATERIALS AND METHODS

Cultural examination. From January 1985 to December 1986, organs of 446 rats belonging to different strains were cultured for bacteria during routine health monitoring. The animals came from two different populations housed behind barriers and from three conventional holding areas. Each of these five units was run independently with no common contact via personnel, equipment, or exchange of animals. Two or three rats from each animal room were examined each month; animals from commercial suppliers were tested less frequently. Generally, blood samples were obtained by orbital bleeding under anesthesia with CO_2. The animals were subsequently killed with CO_2. Swabs from the nasal cavities, tracheas, and lungs were streaked on blood agar which was cross-inoculated with *Staphylococcus aureus* and on chocolate agar (blood agar base containing 7% defibrinated sheep blood; Oxoid, Wesel, Federal Republic of Germany). In 1986, swabs from the uteri and vaginas of 58 rats were cultured in a similar manner. The agar plates were incubated in an atmosphere supplemented with 5% CO_2 at 37°C and read after 24 and 48 h of incubation. The qualitative composition of the bacterial flora was determined on the basis of colonial morphology and primary diagnostic tests such as Gram staining, oxidase, oxidative and fermentative glucose breakdown, motility, and catalase. Here I report only on short gram-negative rods or coccobacilli with satellite growth adjacent to the *Staphylococcus* strain.

Examination of physiological properties. Altogether, 97 *Haemophilus* strains were isolated. The physiological and biochemical properties of 53 isolates (Table 1) were determined. These strains had been isolated from rats housed in three separate holding areas and from rats which were monitored directly on receipt from our supplier. The remaining 44 isolates had been cultured from rats of various origins. They had somewhat different physiological properties and were not included in this study. In detail, requirements for X and V factors were tested with filter paper disks containing 12.5 μg of NAD (Boehringer GmbH, Mannheim, Federal Republic of Germany) or hemin (Sigma Chemical Co., St. Louis, Mo.). *o*-Nitrophenyl-β-D-galactopyranoside, urease, indol, and porphyrin tests were performed as recommended by Kilian and Frederiksen (8). Amino acid decarboxylation and nitrate reduction were measured in standard bacterio-

TABLE 1. Physiological properties of 53 *Haemophilus* isolates from rats

Test	Reaction[a]
Symbiotic growth	+
V factor required	+
X factor required	−
Hemolysis	−
CAMP	−
CO_2 growth enhancement	d[b]
Porphyrin test	+
Fumarate effect	+
TMPD-oxidase	+
Nitrate reduction	+
Indole production	−
H_2S production	+
Ornithine decarboxylase	+
Arginine dihydrolase	−
Lysine decarboxylase	−
Catalase test	+
ONPG[c] test	−
Urease test	+
Gas from D+ Glucose	−
Acid from:	
D+ Glucose	+
D+ Sucrose	+
D+ Lactose	−
D+ Xylose	−
D− Ribose	d[d]
D+ Mannose	+
D− Mannitol	−
D+ Galactose	+
D− Sorbitol	−
Dulcitol	−
L+ Arabinose	−
Maltose	+
Myoinositol	−
Trehalose	−
Raffinose	−
D− Fructose	+

[a] +, Positive reaction; −, negative reaction.
[b] 29 positive strains.
[c] *o*-Nitrophenyl-β-D-galactopyranoside.
[d] 30 positive strains.

logical media (ornithine decarboxylase test broth, nitrate culture medium [E. Merck AG, Darmstadt, Federal Republic of Germany]) containing 10 μg of NAD per ml. Carbohydrate breakdown was tested in phenol red broth base (Difco Laboratories, Detroit, Mich.) supplemented with 1% of the respective carbohydrate and 10 μg of NAD per ml. Presence of catalase was determined by suspending bacteria from a 24- to 48-h agar culture in a drop of 30% H_2O_2 on a glass slide. Fumarate effect and the production of hydrogen sulfide (measured by the lead acetate method) were determined as described by Mannheim et al. (9). Differential media were inoculated with bacteria from a 24- to 48-h chocolate agar culture. Amino acid decarboxylation and carbohydrate breakdown were read after incubation for 5 days; all other reactions were read after incubation for 18 to 24 h or as recommended by the author cited.

Serological examination. Serum samples from 829 rats were tested for antibodies to *Haemophilus* sp. by an indirect immunofluorescence test with an isolate from a rat as antigen. A bacterial suspension of a 24-h agar culture was dropped onto Teflon-coated microscope slides (BioMerieux, Nürtingen, Federal Republic of Germany) and air dried. The sera were tested at a dilution of 1:20. Anti-rat immunoglobulin G (IgG) labeled with fluorescein isothiocyanate (Dianova, Hamburg, Federal Republic of Germany) was used as second antibody. In addition, each serum sample was monitored for the presence of antibodies to murine viruses and mycoplasmas by using hemagglutination inhibition tests (antibodies to pneumonia virus of mice, minute virus of mice, Theiler's GD VII, Kilham virus, and Toolan's H-1 virus), indirect immunofluorescence test (antibodies to rat corona virus/sialodacryoadenitis virus), and enzyme-linked immunosorbent assay (Sendai virus, *Mycoplasma pulmonis*, and *Mycoplasma arthritidis*). Furthermore, selected rat sera which reacted positively to *Haemophilus* sp. in our indirect immunofluorescence test were tested for cross-reactivity to *P. pneumotropica* strains isolated from our rodent colony. Sera from germ-free rats, from colonies which had been proven to be free of members of the *Pasteurellaceae*, or from *Haemophilus* sp.-negative but *P. pneumotropica*-positive rat populations were used as negative controls and tested accordingly.

TABLE 2. Isolation of V-factor-dependent members of the *Pasteurellaceae* (*Haemophilus* spp.) from rats

Yr of expt	No. of animals		No. of *Haemophilus* isolates from:				
	Cultured	Positive (%)[a]	Nasal cavity	Trachea	Lungs	Uterus	Vagina
1985	195	24 (12.3%)	6	12	10	NT[b]	NT
1986	251	51 (20.3%)	9	25	33	1[c]	1[c]

[a] Average, 16.8%.
[b] NT, Not tested.
[c] Genital tracts of only 58 rats were cultured.

Histopathological examination. Only organs from rats derived from a closed colony were used. Animals from this colony were proven to be free of antibodies to murine viruses and mycoplasmas and were culturally negative for *P. pneumotropica* and other respiratory bacterial pathogens, as determined by regular microbiological monitoring for several years. Each rat used for histopathology was serologically positive for *Haemophilus* sp., and in some rats these bacteria were cultured. After necropsy, organs were fixed in 4% formaldehyde solution. Paraffin sections (5 to 7 μm) of tracheas, bronchi, and lungs were stained with hematoxylin and eosin and examined under a light microscope.

RESULTS

From 446 rats which were culturally monitored during the 2-year investigation, 97 strains of V-factor-dependent members of the *Pasteurellaceae* were isolated from different organs of 75 rats. Most of the *Haemophilus* bacteria were cultured from trachea and lungs and less frequently from the upper respiratory tract (Table 2). The female genital tract was cultured from only 58 animals; one isolate was obtained from uterus, and one was obtained from a vaginal smear.

The majority of *Haemophilus* strains were isolated in three separate holding areas. Of 180 rats which were cultured, 37 (20.6%) were positive. Rats in these areas came from the same breeder. Examination of rats immediately on receipt from the breeder showed that they were culturally and serologically free of known pathogens but nonetheless positive for *Haemophilus* sp. (Table 3).

Antibodies to the *Haemophilus* strain used were detected in 385 (46.5%) of 829 rats (194 [47.1%] of 412 rats in 1985 and 191 [45.8%] of 417 rats in 1986). There was no cross-reaction to *P. pneumotropica*, which was the only known member of *Pasteurellaceae* occurring in our colony, nor did sera from

TABLE 3. Results of cultural and serological monitoring for *Haemophilus* spp. obtained from rats

Source of rats and yr of expt	No. of rats with results by:			
	Culture		Serology	
	Monitored	Positive (%)[a]	Monitored	Positive (%)[b]
Exptl colony				
1985	93	22 (23.7)	122	117 (95.9)
1986	87	15 (17.2)	156	145 (92.9)
Commercial breeder				
1985	15	3 (20.0)	68	44 (64.7)
1986	17	12 (70.6)	22	21 (95.5)

[a] Averages for laboratory and commercial rats were 20.6 and 46.9% positive, respectively.

[b] Averages for laboratory and commercial rats were 94.2 and 72.2% positive, respectively.

Haemophilus sp.-negative but *P. pneumotropica*-positive rats or from germ-free rats show a positive serological reaction. As in the cultural investigation, the majority of serologically positive rats had been housed in holding areas where all the rats came from one breeder. Within these areas, 94.2% of the rats had antibody titers to *Haemophilus* sp. and 65 (72.2%) of 90 serologically monitored rats from the corresponding breeder were positive (Table 3).

The histopathological examination revealed mild but typical lesions in the lungs. In contrast to normal lungs (i.e., those from germ-free rats or from rats which were determined by serology and by culture to be free of known pathogens, including *Haemophilus* spp.), a light, diffuse hyperemia combined with modest thickening of the alveolar walls and slight inflammatory cell infiltration was found. Additionally, a mild peribronchiolar hyperplasia was observed.

In our physiological and biochemical investigations, the 53 *Haemophilus* isolates, whether derived from rats of our colony or from newly purchased animals, had very similar biochemical profiles. Among the isolates there were differences only in the ability to produce acid from ribose and the effect of CO_2 on growth. The physiological properties of the *Haemophilus* bacteria are listed in Table 1. On the basis of the 35 criteria examined, a definite classification is not possible. A few strains were selected for genotypic investigations. By using DNA:DNA hybridization methods, they were found to be closely related to *P. pneumotropica* (over 50% DNA homology; W. Mannheim, personal communication).

It seems very likely that the *Haemophilus* bacteria were introduced into our laboratory rodent colony by commercially obtained specific-pathogen-free rats.

DISCUSSION

There are only a few reports in the literature that V-factor-requiring members of the *Pasteurellaceae* are to be expected in laboratory rodents. On the basis of standard criteria for differentiation, such bacteria were considered *Haemophilus* spp. These criteria are no longer decisive, although we use the term *Haemophilus* sp. for our isolates until a final classification is possible. This requires further investigation such as DNA hybridization. Nonetheless, the biochemical results lead us to assume that this is the first detailed report of an infection in rats with an unknown bacterial species belonging to members of the *Pasteurellaceae*. The only report of the occurrence of similar bacteria in a colony of laboratory animals was published by Harr et al. (5), who described a respiratory epidemic and a high mortality in laboratory rats. Similar isolates and clinical symptoms were found in humans who were in contact with the diseased rats. Unfortunately, the bacteriological investigations by Harr et al. were incomplete, so their bacteria cannot be compared with our isolates. A more detailed description of *Haemophilus*-like bacteria from rats is given by Kilian (6). Since he tested only a small number of biochemical criteria in a few strains, I am not certain if our bacteria are identical with his strains.

Haemophilus bacteria are usually species specific (10). By many criteria, our bacteria resemble *Haemophilus parainfluenzae*, but it is very unlikely that a species occurring in humans can cause an infection in rats. Indeed, the first results of genotypic investigations performed by Mannheim (personal communication) indicate that our bacteria are closely related to *P. pneumotropica* and are not *Haemophilus* spp. Most of our strains were isolated from the lower respiratory tract. In contrast to *P. pneumotropica*, they were less frequently isolated from the nasal cavity. Mild but typical morphological alterations were found in the bronchi of rats which were proven to be monoinfected with these bacteria. As we never observed obvious clinical symptoms, the bacteria are considered to be of low pathogenicity. Nevertheless, they can colonize the lungs of rats which are free of other bacterial, mycoplasmal, and viral pathogens or parasites which might cause immunosuppression.

The question of how these bacteria were introduced into our rodent colony was answered by routine health monitoring of purchased animals. *Haemophilus* bacteria with an identical profile were repeatedly isolated from different shipments of rats from a commercial breeder. This supports the view that they were introduced into our animal house by commercially obtained rats. Since these *Haemophilus* sp.-positive rats were purchased from a commercial breeder, I expect that *Haemophilus* spp. are present in many laboratory rat colonies. Meanwhile, we have cultured similar bacteria from rats of different origins and confirmed my expectation.

I assumed that the *Haemophilus* isolates were introduced into the barrier-reared breeding colony of specific-pathogen-free rats by hysterectomized animals. This led me to culture the female genital tract of rats. During the period of investigation reported here, two isolates were obtained from vagina and uterus, but further investigations resulted in the isolation of several isolates from the female genital tract. This confirms my assumption that an intrauterine transmission to fetuses might be possible during pregnancy. In contrast to bacteria which are easily detectable by routine sterility controls in hysterectomized germ-free animals, *Haemophilus* spp. may remain undetected because of their special nutritional requirements.

Knowledge about *Haemophilus* spp. in rats is very limited. We found slight but typical pathological changes in the respiratory system. Thus, this organism may have importance as a hitherto undiscovered pathogen in rats. Finally, I cannot exclude the possibility that these bacteria modify the results of animal experiments.

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