Characterization of a *Haemophilus paracuniculus* Isolated from Gastrointestinal Tracts of Rabbits with Mucoid Enteritis

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The isolation, characterization, and identification of a microorganism isolated from gastrointestinal tracts of rabbits with mucoid enteritis are described. The isolated organism did not grow on standard media. This organism grew around colonies of *Staphylococcus aureus* and *Lactobacillus desidiosus* and around disks saturated with diphosphopyridin nucleotide (factor V) on brain heart infusion agar. The growth of this organism was also observed on media supplemented with β -nicotinamide adenine dinucleotide. The organism appeared as gram-negative, pleomorphic rods or coccobacilli. It was positive for urease, oxidase, catalase, glycosidases, porphyrin, and indole, and it fermented glucose and sucrose. All of these characteristics suggest that the organism is a member of the genus *Haemophilus*. Because of its isolation from rabbits and differences in some characteristics from other species of this genus, the name *Haemophilus paracuniculus* is proposed for this organism.

It is known that members of the genus *Haemophilus* are strict parasites characteristically requiring accessory factors for growth in vitro, i.e., β -nicotinamide adenine dinucleotide (NAD) and/or hemin. Most species of this genus are indigenous to the upper respiratory tract of healthy human subjects and animals, where they may either reside in small numbers or colonize without evidence of overt infection (1, 6). Occasionally, these microorganisms become primary or secondary invaders and, as a result, etiological agents of disease. Infections include, but are not limited to, meningitis, pharyngitis, sinusitis, pneumonia, otitis media, and bacteremia; less frequently encountered or reported are conjunctival episodes. In the present study, characterization and identification to species of a *Haemophilus*-like organism isolated from gastrointestinal tracts of rabbits with mucoid enteritis are described.

Mucoid enteritis is a disease of young rabbits with a mortality of about 50% in some commercial rabbitries. The etiology of this disease is unknown. The most important clinical symptoms include dehydration, diarrhea of varying intensity, and normal or subnormal temperature. Pathological changes are confined to the gastrointestinal tract. The small intestine may show increased mucus production in acute cases. In chronic or subacute cases, the cecum and large intestine may be packed with a clear, thick

gelatinous substance. Histopathologically, hyperplasia of goblet cells in the duodenum, jejunum, and ileum is a characteristic finding in mucoid enteritis (3).

MATERIALS AND METHODS

Specimens. Specimens were collected from different portions of the gastrointestinal tracts of 9 New Zealand White rabbits with mucoid enteritis and 20 normal New Zealand White rabbits during postmortem examination. The content of the duodenum, jejunum, cecum, large colon, or small colon was inoculated onto sheep blood plates. Strips of a "feeder culture" (see below), which promoted growth of *Haemophilus*-like organisms, were streaked across the line of the original inoculum.

Media. Tests for growth of the isolated organism were conducted on the following media: beef, horse, or sheep blood agar, chocolate agar (sheep blood heated to 70°C for 30 min), blood agar base, brain heart infusion (BHI) agar and broth, nutrient agar, mycoplasma agar, and purple broth base, all from Difco Laboratories, Detroit, Mich.; phenol red broth base from Baltimore Biological Laboratory, Cockeysville, Md.; and phenol red sorbitol broth from BBL Microbiology Systems, Cockeysville, Md. Purple broth base was used to determine fermentation of various carbohydrates. The following carbohydrates were tested: D-glucose, D-galactose, D-sucrose, D-lactose, inulin, D-mannitol, and D-xylose from Difco Laboratories; and 2-deoxy-D-ribose from Sigma Chemical Co., St. Louis, Mo.

Supplements. The isolated organism from the gastrointestinal tracts of the affected rabbits did not grow on standard media. For growth of this organism, media had to be supplemented with NAD. In the present study, the majority of the media were supplemented

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with NAD (Sigma Chemical Co.) at a concentration of 10 $\mu\text{g}/\text{ml}$ or with disks saturated with diphosphopyridin nucleotide (factor V), and other media were supplemented with iron protoporphyrin (factor X) (differentiation disks BV or BX [Difco Laboratories]). In some experiments, media were supplemented with the supernatant fluid from the feeder culture after 48 h of incubation in BHI broth. The supernatant fluid, after filtration by a membrane filter (0.22- μm pore size; Millipore Corp., Bedford, Mass.), was added to autoclaved and cooled (50°C) 10 $\times$ concentrated BHI broth or agar to restore a standard concentration.

Biochemical and physiological tests. The requirement for factor V was tested by observing the satellitism phenomenon on beef, horse, and sheep blood agar, BHI agar, nutrient agar, and mycoplasma agar. Satellitic growth of the organism isolated from affected rabbits was studied around colonies of *Staphylococcus aureus*, certain strains of *Staphylococcus epidermidis*, *Bacillus brevis*, *Bacillus cereus*, and *Bacillus subtilis* (American Type Culture Collection, Rockville, Md.). As a confirmatory test, factor V disks were used on BHI agar.

The requirement for factor V was also tested by observing growth in broth media supplemented with the supernatant fluid from the feeder culture. As a confirmatory test, factor V disks or NAD at a concentration of 10 $\mu\text{g}/\text{ml}$ was used in BHI broth.

The requirement for increased CO_2 tension in the incubation environment was determined by comparing growth on a lightly inoculated triplicate set of BHI agar plates supplemented with NAD in aerobic and anaerobic conditions with growth in a candle jar.

Hemolysis of horse blood around single colonies was examined after growth for 24 to 48 h on 5% (vol/vol) defibrinated horse blood in blood agar base supplemented with NAD.

A hemagglutination test was performed by mixing equal quantities (50 μl) of a 1% (vol/vol) suspension of human group O erythrocytes in saline (0.85% [vol/vol] NaCl) and a suspension of the bacteria in saline at a concentration of 10^6 cells per ml in a U-type microtiter plate (Cooke Engineering Co., Alexandria, Va.). The results were read from the pattern of sedimented cells on the bottom after incubation at room temperature for 8 h.

Reduction of nitrate was examined after 2 days of incubation in indole-nitrate broth (Baltimore Biological Laboratory) supplemented with NAD.

Indole production was demonstrated after 48 h of incubation in indole-nitrate broth by using Kovac reagent.

Oxidase activity was tested by using a 1% solution of *N,N*,dimethyl-*p*-phenylenediamine oxalate (Eastman Kodak Co., Rochester, N.Y.).

Catalase activity was determined by effervescence of a suspension of bacteria in 3% (vol/vol) H_2O_2 on a glass microscope slide.

Hydrogen sulfide production was determined by placing a lead acetate strip (Fisher Scientific Co., Rochester, N.Y.) in the lid of an inoculated BHI agar plate supplemented with NAD, which was read after 5 days of incubation.

Production of acid carbohydrates was determined in purple broth base. The respective carbohydrates

were added to the autoclaved and cooled broth as sterile solutions to give final concentrations of 1% (vol/vol). NAD was added in filter-sterilized solution to a final concentration of 10 $\mu\text{g}/\text{ml}$. A Durham tube was inserted in the glucose broth for the detection of gas. Tubes containing the same medium with various carbohydrates were inoculated with a loopful of bacteria and incubated for 5 days. A control medium without carbohydrates was also inoculated.

Enzymatic activity determinations were performed by suspending a loopful of bacteria from a 24-h BHI agar culture into 1 ml of the respective enzyme substrates. Urease activity was tested in urea broth (Difco Laboratories). Decarboxylation of ornithine and lysine and dihydrolation of arginine were determined in purple broth base. The glycosidases β -galactosidase and α -glucosidase were determined in 0.1% (wt/vol) solutions of 2-nitrophenyl- β -D-galactopyranoside and 4-nitrophenol- α -D-glucopyranoside (Sigma Chemical Co.), respectively. The organism isolated from the gastrointestinal tracts of rabbits was also tested for β -lactamase production. The method used routinely was that described by Thornsberry and Kirven (11).

The ability to carry out the biosynthesis of porphyrine from α -aminolevulinic acid was determined by two tests of Kilian (5). One test detects the production of porphyrine. A heavy suspension (milky) of organisms from BHI agar was made in 0.5 ml of α -aminolevulinic acid at a concentration of 2 mM (Sigma Chemical Co.). The tubes were incubated in a water bath at 37°C for 2 to 4 h. Subsequently, the tubes were examined for red fluorescence (positive test) against a white background under a Wood lamp. The second test for porphobilinogen was prepared in the same manner as the porphyrin test. After the appropriate incubation period, a few drops of Kovac reagent was added to the tubes. Development of a red aqueous layer indicated a positive test.

Determination of DNA base composition. Extraction of DNA from the organism and equilibrium density gradient centrifugation in CsCl of its DNA were performed by H. Kubinski. The method was previously described by Kubinski et al. (7) and Szybalski et al. (9). Briefly, after a 48-h incubation bacteria were harvested from BHI agar supplemented with NAD by washing in cold saline. Subsequently, bacteria were lysed, and nucleic acids were extracted with chloroform-butanol and phenol. The fraction containing DNA was centrifuged in CsCl, and base composition of DNA was determined.

Serotyping. Serological typing by Quellung reaction and slide agglutination were performed with specific antisera. Antisera against *Haemophilus influenzae* types a through f were obtained from a commercial source (Hyland Laboratories, Inc., Costa Mesa, Calif.).

Antibiotic susceptibility test. Various antibiotics were tested for inhibition of the growth of the organism isolated from the gastrointestinal tracts of rabbits on Mueller-Hinton agar supplemented with NAD under aerobic conditions. The test was performed with commercially prepared disks saturated with ampicillin, bacitracin, chloramphenicol, chlortetracycline, furadantin and macrodantin, gentamicin, lincomycin, neomycin, penicillin, streptomycin, tetracycline, or tri-

ple sulfa (Baltimore Biological Laboratory). The diameter of inhibition of growth was measured after 24 h of incubation, and susceptibility of the microorganism to various antibiotics was determined by Bauer-Kirby criteria (C. Thornsberry, personal communication).

RESULTS

Isolation of a satellitic-growing organism. A satellitic-growing organism was isolated on sheep blood agar plates inoculated with specimens from the duodenum, jejunum, and cecum of six of nine rabbits with mucoid enteritis. This organism grew in close proximity to heterologous large microbial colonies. It appears that the large colonies of feeder cultures excreted nutrient(s) into the medium that promoted satellitic growth. The satellitic-growing organism was not isolated from gastrointestinal tracts of normal rabbits. Unfortunately, five of six satellitic-growing isolates which were able to convert δ -aminolevulinic acid to porphyrin were lost during storage at -40°C . The specific properties of the satellitic-growing microorganism were defined for the remaining isolate. This isolate was deposited in the American Type Culture Collection (ATCC 29986).

Isolation and identification of the feeder culture. The feeder culture was isolated from various specimens from the gastrointestinal tracts of rabbits affected with mucoid enteritis or from the large colons of normal rabbits. This culture was identified as *Lactobacillus desidiosus*. The nutrient(s) excreted by this culture promoted satellitic growth of *H. influenzae* on sheep blood agar and of the organism isolated from affected rabbits on BHI agar and sheep blood agar. With *L. desidiosus* as a feeder, a pure culture of our satellitic-growing organism was isolated from some of the affected rabbits without other bacteria.

Satellite phenomenon. The satellitic

growth of the isolated organism was observed around colonies of *L. desidiosus*, appearing on beef, horse, and sheep blood agar, BHI agar, mycoplasma agar, and nutrient agar. Satellitic growth of the organism isolated from rabbits with mucoid enteritis was also promoted by *S. aureus*, certain strains of *S. epidermidis*, *B. brevis*, *B. cereus*, and *B. subtilis* on BHI agar or blood agar.

Nutritional requirements. The nutritional requirements for growth in vitro of the *Haemophilus*-like organism from the gastrointestinal tracts of affected rabbits are presented in Table 1. Growth of the isolated organism was observed only on media supplemented with either factor V or supernatant fluid from a *L. desidiosus* culture. In contrast, growth was not observed around the disk saturated with factor X. The basic media before supplementation were relatively simple and included nutrient agar, purple broth base, and phenol red sorbitol broth. However, growth was not observed in the phenol red broth base. A major compositional difference between the phenol red broth base and phenol red sorbitol broth that affected growth is the configuration of the Trypticase peptone employed as a source of nitrogen: Trypticase from Baltimore Biological Laboratory in the former and Trypticase from BBL Microbiology Systems in the latter (Baltimore Biological Laboratory, personal communication). The presence or absence of sorbitol in the medium did not enhance growth of the *Haemophilus*-like organism. Growth in purple broth base indicated that this organism could utilize proteose peptone no. 3 as a source of nitrogen.

Biochemical and physiological characteristics. The isolated organisms were gram-negative, nonmotile, coccobacillary or pleomorphic rods, occasionally with filaments. In the broth medium supplemented with factor V, the

TABLE 1. Nutritional requirements for growth of *Haemophilus*-like organism

Medium supplement	Growth on:							
	Blood agar ^a	Chocolate agar ^b	BHI agar	Nutrient agar	Mycoplasma agar ^c	BHI broth	Purple broth base	Phenol red broth base
None	—	+	—	—	—	—	—	—
Factor V	+	+	+	+	+	+	+	—
NAD (10 $\mu\text{g}/\text{ml}$)	+	+	+	+	+	+	+	—
NAD (0.2 $\mu\text{g}/\text{ml}$)	+	+	—	—	—	—	—	—
Factor X	—	+	—	—	—	—	—	—
Factors V + X	+	+	+	+	+	+	+	—
Culture fluid ^d	+	+	+	+	+	+	+	—

^a The same pattern of growth was observed on beef, horse, and sheep blood agar.

^b Chocolate agar was prepared from sheep blood.

^c PPLO agar (Difco) was supplemented with 20% horse serum.

^d Supernatant fluid collected from *L. desidiosus* culture after 48 h of incubation.

organism formed chains of short rods. Biochemical and physiological characteristics of the organism are summarized in Table 2. Optimal growth of this organism was observed in a candle jar; less growth occurred under aerobic or anaerobic conditions, regardless of humidity. With this organism, tests for conversion of δ -aminolevulinic acid to porphyrin and for the presence of porphobilinogen were positive, and nitrates were reduced. The guanine plus cytosine content of its DNA was 40.0 ± 0.5 mol%. On the basis of the Bauer-Kirby criteria, the *Haemophilus*-like organism was resistant to bacitracin and lincomycin and susceptible to ampicillin, chloramphenicol, chlortetracycline, furadantin and macrodantin, gentamicin, penicillin, streptomycin, tetracycline, and triple sulfa. This organism did not produce β -lactamase. As expected, this organism was not agglutinated by type-specific sera for *H. influenzae*.

DISCUSSION

Satellitic growth of the organism isolated from the gastrointestinal tracts of rabbits with mucoid

TABLE 2. Biochemical and physiological characteristics of *Haemophilus*-like organism^a

Characteristic	Occurrence
Indole	+
Urease	+
Ornithine decarboxylase	+
Lysine decarboxylase	—
Arginine dihydrolase	+
H ₂ S (lead acetate)	—
Hemolysis	—
Glucose acid ^b	+
Glucose gas	—
Galactose acid	—
Sucrose acid	+
Lactose acid	—
Xylose acid	—
Deoxyribose acid	—
Inulin acid	—
Mannitol acid	—
β -Galactosidase	+
α -Glucosidase	—
Catalase	+
Oxidase	+
CO ₂ improves growth	+
Capsule	—
Hemagglutination	—

^a All media were supplemented with 10 μ g of NAD per ml. Proper controls were introduced for each of the tests.

^b Carbohydrates were added to give final concentrations of 0.5% in purple base broth.

enteritis around colonies of various cultures and disks saturated with factor V indicates that this organism is a member of the genus *Haemophilus*. The requirement of factor V for growth on BHI agar, the ability to convert δ -aminolevulinic acid to porphyrin, and the guanine plus cytosine content of its DNA are attributes characteristic of *Haemophilus parainfluenzae*. Production of indole by this organism indicates the similarity to the unclassified strains of *H. parainfluenzae* or the members of taxon B of Kilian (6). However, this organism differs from the members of the two aforementioned groups by the lack of production of hydrogen sulfide, by improved growth under CO₂, and by the production of arginine dihydrolase. Additional differences are in the fermentation of xylose and deoxyribose, production of gas from glucose, hemagglutination, and the guanine plus cytosine content of its DNA. These characteristics indicate that the satellitic-growing organism isolated from the rabbits with mucoid enteritis substantially differs from the members of taxon B and unclassified strains of *H. parainfluenzae*. Therefore, this organism could be considered to be a new species of the genus *Haemophilus*. Because of its isolation from rabbits and its characteristics, the name *Haemophilus paracuniculus* is proposed for this organism.

Results of the antibiotic susceptibility test indicate that the majority of the tested antibiotics inhibited growth of *H. paracuniculus* on Mueller-Hinton agar supplemented with NAD. The possibility of interference of NAD with the inhibitory activity of some antibiotics was considered. However, similar results of the antibiotic sensitivity test with *S. aureus* obtained from supplemented Mueller-Hinton agar and Mueller-Hinton agar did not indicate such interference. Similarly, supplementation of Mueller-Hinton agar with 1% IsoVitaleX (Baltimore Biological Laboratory) and 1% hemoglobin was recommended by Thornsberry et al. (10) for identifying ampicillin-resistant strains of *H. influenzae*. Unfortunately, Mueller-Hinton agar supplemented with 1% IsoVitaleX has too low a concentration of NAD to support growth of *H. paracuniculus*. In this study, susceptibility of *H. paracuniculus* to various antibiotics was determined by criteria for rapidly growing pathogens. Therefore, the interpretation of the antibiotic susceptibility results for *H. paracuniculus* should be considered to be tentative.

In the present study, *L. desidiosus* was isolated from the gastrointestinal tracts of sick and normal rabbits. This culture promoted the satellitic growth of *H. paracuniculus* and *H. influenzae*. It seems that *L. desidiosus* supplied members of the genus *Haemophilus* with those

essential nutrients presumably also provided by *S. aureus* or a *Pseudomonas* species (8).

Previously described members of the genus *Haemophilus* were isolated from various sites of the bodies of humans and animals but not from the gastrointestinal tract. The high incidence of isolation of *H. paracuniculus* from affected rabbits may indicate some correlation between appearance of this organism in the gastrointestinal tract and mucoid enteritis in rabbits. Evidence of other investigators (12) suggests that mucoid enteritis is an infectious disease and can be transmitted to young rabbits. Isolation of *H. paracuniculus* from the gastrointestinal tracts of affected rabbits is quite difficult because of its slow growth. Frequently, the colonies of this microorganism are overgrown by colonies of fast-growing microorganisms.

The results of a preliminary study on the pathogenicity of this organism indicate reproducibility of mucoid enteritis in rabbits administered *H. paracuniculus* per os. However, our data are insufficient to implicate unequivocally *H. paracuniculus* either as a tacit pathogen or as an etiological agent of epizootic episodes. The presence of homologous serum antibody in high concentrations (ca. 10%) in healthy rabbits supports the tenet of its pathogenicity. Several bacterial species have been isolated from affected rabbits, but their importance has been difficult to assess. Coliform bacilli and anaerobic bacteria have been isolated, but these are normally present in intestines, and their pathogenicity has not been proven (2, 4).

The biological significance of *H. paracuniculus* in rabbits remains to be determined by fulfilling the requirements of the Henle-Koch postulates and by assessing normal and infected rabbits for humoral antibody responses. These studies currently are in progress.

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