A HAEMOLYTIC V-DEPENDENT CO₂-PREFERRING *HAEMOPHILUS* SPECIES (*HAEMOPHILUS PARAPHRO-* *HAEMOLYTICUS* NOV. SPEC.)

K. ZINNEMANN, K. B. ROGERS, JOYCE FRAZER AND S. K. DEVARAJ

*Bacteriology Department, School of Medicine, Leeds, and Department of Microbiology,
The Children's Hospital, Birmingham*

PLATES X AND XI

AFTER the existence of *Haemophilus paraphrophilus* had been recognised in 1959 (Zinnemann *et al.*, 1968), a haemolytic strain of a V-dependent, increased-CO₂-tension-preferring haemophilus was isolated by K. B. R. in March 1960 and sent to K. Z. for classification. Subsequently, seven more such strains were isolated and all were freeze-dried for further investigation. However, about 10 yr later these strains could not be recovered from the ampoules after suitable reconstitution of the freeze-dried material. Amongst 20 strains of haemolytic haemophili isolated by K. B. R. in 1969 from various sites in children during a 4-wk period there was one strain, Corinthian, that had the characters seen 10 yr previously. The strain was isolated from the throat of a 12-yr-old asthmatic child, where it was present as the predominant organism together with a few colonies of a coagulase-positive staphylococcus. Subsequently two similar strains were isolated by S. K. D. on two different occasions from the sputum of a 45-yr-old male patient with pyrexia who had received a skin graft after an accident. On both occasions the strains, Lawton 1 and Lawton 2, were the predominant organism in a mixed, upper respiratory tract flora. They were haemolytic and V-dependent and preferred an increased tension of CO₂.

MATERIALS AND METHODS

Strains. Cultures of the strains Corinthian, Lawton 1 and Lawton 2 were kept at 37°C on plates of heated blood ("chocolate") agar, 25 ml per 8.5 cm diameter plastic petri dish, in a jar with air plus 5–10 per cent. CO₂, and were subcultured at least thrice weekly. The control strains used in demonstrations of growth-factor requirements were: (1) a type-b strain, Hind, of *H. influenzae*, (2) strain MB of *H. parainfluenzae*, and (3) strain Funagli of *H. paraphrophilus* (Zinnemann *et al.*). The Heatley (Oxford) strain of *Staphylococcus aureus* was used as feeder strain for the supply of V factor in rough screening tests for V-factor requirements.

Culture methods were as described by Zinnemann *et al.* except that nutrient agar (Oxoid Columbia Agar, CM331) containing 5 per cent. oxalated horse blood added at 45°C was used for tests of haemolysis.

Growth-factor requirements. These were tested as described by Zinnemann *et al.* One quadrant of a culture plate was spread with an inoculum of one of the three strains under investigation and the other three quadrants were spread with inocula of the three control strains Hind, MB and Funagli.

Biochemical and sensitivity tests were done by the methods of Zinnemann *et al.*

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RESULTS

Cultural and microscopical characters

When incubated at 37°C for 48 hr in air without added CO₂ the Lawton strains produced faint haemolysis without visible colonies on fresh blood plates, and did not give visible growth on heated blood ("chocolate") agar plates. Strain Corinthian grew feebly with the formation of uniformly tiny colonies on both fresh-blood and chocolate agar, and produced faint haemolytic zones on the fresh blood plates (fig. 1).

In air with c. 10 per cent. (v/v) CO₂ all three strains grew well on both fresh-blood and chocolate agar, and produced large zones of clear haemolysis similar to those produced by β -haemolytic streptococci on fresh blood plates (fig. 2).

Occasionally, when subcultures from the two Lawton strains were incubated in air, slight growth was obtained on chocolate agar as well as on fresh blood agar, but growth did not continue after the second subculture in air on fresh blood plates or after the fifth subculture in air on chocolate agar plates. On other occasions strains maintained in CO₂ failed to grow in air without added CO₂ on either fresh blood agar or chocolate agar. When growing in air on either fresh blood agar or chocolate agar, the colonies were smooth, round and dome-shaped, and never showed the wrinkled, bread-crumble-like appearance described for *H. paraphrophilus*. Chocolate agar promoted larger colonies than fresh blood agar, irrespective of the presence or absence of CO₂. The strains grew in Levinthal broth producing a uniform turbidity but no sediment.

Requirements of accessory growth factors were found to be the same as those previously demonstrated in strains of *Haemophilus paraphrophilus*. The strains were V-dependent both in air and in air with added CO₂, provided 0.6 per cent. NaCl was present in the medium incubated in air. No growth was obtained on inspissated serum or on MacConkey's agar. We succeeded in adapting strain Corinthian to grow in air without added CO₂ by repeated subcultures on chocolate agar. The adapted strain retained its colonial size and morphology, its haemolytic property and its V-dependence in the presence of 0.6 per cent. NaCl.

When growing on chocolate agar incubated in air with c. 10 per cent. CO₂, all three strains were Gram-negative and consisted of short to medium-length rods measuring 0.75–2.5 μ m and 0.4–0.5 μ m in width, with an occasional short filament (figs. 3, 5 and 7). When incubated in air without added CO₂, strain Corinthian showed mainly short rods, many of which resembled elongated cocci, and a proportion of globular involution forms (fig. 4); strain Lawton 1 formed coarser rods and filaments, some of which were twisted (fig. 6), and strain Lawton 2 formed even coarser and longer rods and filaments than strain Lawton 1. Some of the filaments produced by Lawton 2 showed extraordinary twisting (fig. 8) such as would suggest the term "sea serpent" used by Fleming and Maclean (1930) when investigating haemolytic haemophili isolated from the gums. The three strains were shown to be non-motile and non-spore-forming.

HAEMOPHILUS PARAPHROHAEMOLYTICUS

FIG. 1.—Poor growth and faint haemolysis on fresh horse blood agar plate spread with inocula of strain Lawton 1 (top left), Lawton 2 (top right) and Corinthian (bottom) and incubated in air without added CO₂. $\times 0.7$.

FIG. 2.—Good growth and strong haemolysis on plate with similar inocula to those in fig. 1, but incubated in air with c. 10 per cent. added CO₂. $\times 0.7$.

HAEMOPHILUS PARAPHROHAEMOLYTICUS

FIG. 3.—Bacillary morphology of strain Corinthian incubated in air with c. 10 per cent. CO₂.

FIG. 4.—Strain Corinthian incubated in air without added CO₂, showing swollen involution forms and globules.

FIG. 5.—Strain Lawton 1 incubated in air with c. 10 per cent. CO₂.

FIG. 6.—Strain Lawton 1 incubated in air without added CO₂, showing involution forms and twisted short filaments.

FIG. 7.—Strain Lawton 2 incubated in air with c. 10 per cent. CO₂.

FIG. 8.—Strain Lawton 2 incubated in air without added CO₂, showing coarse bacilli and long, twisted "sea serpent" forms.

FIGS. 3-8.—Cultures grown on heated blood (chocolate) agar for 24 hr at 37°C. Gram's stain. $\times 1200$.

Viability

An overnight growth of the strains in peptone water with added X and V factors, incubated at 37°C in air with added CO₂, was used for viability tests. Subcultures on chocolate agar incubated in air with added CO₂ were used to confirm growth in peptone water before heating, and afterwards for viability. When heated at 50°C strain Corinthian remained viable for 40–60 min. and the two Lawton strains did so for 10 min.; at 55°C strain Corinthian survived for 16–18 min. and the Lawton strains survived for 2–4 min.; at 60°C all three strains were killed in less than 2 min.

After initial incubation for 24 hr at 37°C in air with added CO₂, strain Corinthian, when left in air at 4°C, survived for 7–10 days, and the Lawton strains survived for 6, but not for 7 days. On chocolate and fresh-blood agar under the same condition all strains survived for up to 10 days. When cultures on chocolate and fresh-blood agar were first incubated overnight at 37°C in air with added CO₂ and then left at room temperature (20–22°C) in air, all three strains survived for 3 days but not for 6 days on fresh blood agar, and for 7 but not for 8 days on chocolate agar. When the plates were wrapped up in a Polythene bag as a protection against drying out, the survival times at room temperature were the same as when they were unwrapped. The strains survived for 6, but not for 7 days on fresh blood plates at room temperature in a jar containing air with added CO₂, and for 7 but not for 8 days on chocolate agar plates under the same conditions.

When first incubated overnight at 37°C in air with added CO₂ and then kept at 37°C in air on the incubator shelf they survived on both fresh blood and chocolate agar plates for 10 days but not for 13 days. When incubated in the same way but kept at 37°C in air with added CO₂ in a jar they survived for 19–21 days on fresh blood agar. On chocolate agar under these conditions the Lawton strains survived for 13 but not for 16 days, and strain Corinthian survived for 21 but not for 23 days.

Other characters

All three strains reduced nitrate to nitrite and gave a positive catalase reaction on chocolate agar. Each gave a positive urease reaction, though strain Corinthian did so rather slowly. The oxidase reaction was negative and the indole reaction variable. Gelatin was not liquefied.

Acid was produced regularly by all three strains from fructose, glucose and maltose. Acid production was variable, though consistent for each strain, from dextrin, galactose, lactose, mannitol, sucrose and xylose. The strains were insensitive to disks containing 1 unit of penicillin and variably sensitive to disks containing 10 units of penicillin when tested on either fresh blood or chocolate agar. The different results for 10 units were given by the same strain grown on different media. All three strains were completely inhibited by 10 µg of ampicillin, chloramphenicol, erythromycin and streptomycin. They were not inhibited by optochin.

None of the three strains caused signs of illness, lesions or death when 0.5 ml

of a 24-hr Levinthal broth culture was injected subcutaneously, intraperitoneally or intravenously into a white mouse weighing *c.* 15 g, or 1 ml was injected subcutaneously into a guinea-pig weighing *c.* 250 g. The experiments were repeated once in guinea-pigs and twice in mice.

TABLE
Sites of isolation of the V-dependent, CO₂-preferring haemolytic strains of Haemophilus

Number of strains	Place of origin	Site of lesion or specimen from which strain was isolated
2	Birmingham	Throat swabs from a nurse and child with acute sore throats
1	Birmingham	Thumb print on a blood agar plate from a student nurse
1	Birmingham	Swabs from pharynx and ulcer of mouth of a 10-yr-old child
1	Birmingham	Throat swab from a child with laryngo-tracheo-bronchitis
1	Birmingham	Throat swab from a child
1 (Corinthian)	Birmingham	Throat swab from an asthmatic child
2 (Lawton 1 and 2)	Leeds	Sputum from an adult male with pyrexia after skin grafting of foot
2	Leeds	Urethral discharge from adult males suspected of having gonorrhoea

Sources of strains

Six of the previously isolated strains came from the Children's Hospital in Birmingham, and two were isolated from chocolate agar plates inoculated with urethral secretions from males suspected of suffering from gonorrhoea.

All strains grew in pure, or almost pure cultures and on account of this fact were regarded by the bacteriologist as presumptive pathogens. The table gives details of the sources of the total of 11 strains recognised by us.

DISCUSSION

The sources from which strains of this V-dependent, CO₂-preferring haemolytic species of *Haemophilus* have been isolated suggest that its normal habitat is the oral cavity. Probably Fleming and Maclean (1930) handled strains of this organism as well as some strains of *Haemophilus paraphrophilus* when they investigated the mouth flora of normal adults for the presence of haemophili. This is suggested by data provided in tables V, VI and VII of their paper, although they omitted to establish a link between colonial and microscopical appearance on the one hand, and haemolysis on the other. Moreover, they

do not appear to have used incubation in air with added CO₂ as a differential method, although Fleming was well aware that certain bacteria isolated from the upper respiratory tract grow only, or better, in an atmosphere containing c. 10 per cent. CO₂. Only since Khairat (1940) and Zinnemann *et al.* (1968) used preference for increased CO₂ tension as a species-specific character has the significance of this property come to be recognised. Fleming and Maclean found "haemolytic types" of haemophili on the gums of four out of six laboratory workers, and it is possible that the gums are their normal habitat, where they are a commensal and from where they can spread in large numbers to the pharynx and enter the sputum. Sims (1970), using incubation under increased CO₂ tension for the isolation of haemophili from the normal oral cavity, found that c. 13 per cent. of all isolates consisted of V-dependent haemolytic strains.

Strains of this kind are distinct from *Haemophilus parahaemolyticus* (Pittman, 1953) and similar to *H. aphrophilus* (Khairat) and *H. paraphrophilus* (Zinnemann *et al.*) in their marked requirement of CO₂. We propose, therefore, to name the new species *H. paraphrohaemolyticus* in view of the fact that, apart from producing haemolysis on solid blood media, it prefers increased CO₂ tension for growth. Strain Lawton 1 is designated as the holotype.

SUMMARY

The repeated isolation of a haemolytic species of *Haemophilus* requiring V factor and preferring increased CO₂ tension for growth is described. Other characters helpful in establishing the identity of such strains are given and the name *Haemophilus paraphrohaemolyticus* is proposed.

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The three strains on which this report is based have been deposited with the National Collection of Type Cultures, Central Public Health Laboratory, London; they have been allotted the following numbers: strain Corinthian, NCTC no. 10672; strain Lawton 1, NCTC no. 10670; strain Lawton 2, NCTC no. 10671.

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