THE CARBON DIOXIDE REQUIREMENT OF KLEBSIELLAE

G. BROOKES AND J. BARKER

*Regional Public Health Laboratory, East Birmingham Hospital,
Bordesley Green East, Birmingham, B9 5ST*

PLATE I

THE REQUIREMENT of some brucellae, pneumococci and gonococci for carbon dioxide is well established. A strain of *Staphylococcus aureus* dependent on CO₂ for characteristic growth was first recorded by Hale (1951) and subsequently there have been other reports of staphylococci with similar requirements (Thomas, 1977). This is a feature not usually associated with members of the Enterobacteriaceae; however, we reported strains of *Klebsiella* that needed CO₂ for growth (Barker, Brookes and Johnson, 1978) and a strain of *Escherichia coli* with this requirement was described by Eykyn and Phillips (1978).

This paper describes the growth of a variety of *Klebsiellae* in air with added CO₂ and its relevance to the isolation and recognition of these organisms in the laboratory.

MATERIALS AND METHODS

Culture media. The following were used: MacConkey Agar (Oxoid) (without NaCl); Columbia Agar (Oxoid) with defibrinated horse-blood added to a final concentration of 5% (v/v); Diagnostic Sensitivity Test Agar (DST) (Oxoid) with saponin-lysed defibrinated horse-blood, added to give a final concentration of 5% (v/v); and Nutrient Agar (Oxoid).

All the organisms were grown in 3 ml of Peptone Water (Oxoid) for 6 h at 37°C to produce turbid suspensions containing approximately 10⁸–10⁹ organisms/ml. These were then diluted in 100-fold steps from 1 in 10² to 1 in 10⁸ in quarter-strength Ringer's solution. One drop from a 50-dropper pipette of each dilution was seeded on the surfaces of the test plates which had been dried at 37°C for 45 min. Paint tins of 2.5-litre capacity with airtight lids and holding 10 test plates, were used for incubation. The lids had a hole cut for a small rubber stopper, so that 10% CO₂ could be injected by syringe and needle to give final concentrations of 0.07%, 0.1%, 0.18% and 3% (v/v), neglecting the volume of the petri dishes in each tin.

Organisms. Ninety-five strains of *Klebsiella* were studied. They included strains from the National Collection of Type Cultures: *K. ozaenae* (NCTC nos. 9601, 9659, 9662 and 10313), *K. pneumoniae* (NCTC nos. 8849, 9635, 5056, 9637 and 9633), *K. edwardsii* var. *edwardsii* (NCTC nos. 5054, 8839, 10209, 7761 and 9616), *K. edwardsii* var. *atlantae* (NCTC nos. 9496, 10213, 9495, 9498 and 9617), *K. rhinoscleromatis* (NCTC nos. 5046, 10273, 5048, 7799 and 5047). The remaining strains were clinical isolates: 23 strains of *K. ozaenae*, 7 of *K. edwardsii* var. *atlantae*, one of *K. pneumoniae* and 40 of *K. aerogenes*; these were identified according to the scheme of Cowan (1974). The clinical isolates of *K. ozaenae* were also identified with API 20E biochemical strips from API Laboratory Products.

RESULTS

Effect of CO₂ on growth

The results of preliminary investigations on the growth of the organisms after incubation for 18 h at 37°C in air with 3% carbon dioxide are shown in table I. More than half the *K. ozaenae* strains showed a 10⁴ increase in viable count compared with parallel cultures in air; only one strain grew in air without additional CO₂. There was no significant difference between the viable counts on blood or MacConkey agar except for three strains of *K. rhinoscleromatis* which failed to grow on the latter even after incubation in CO₂. Four of the six *K. pneumoniae*, two of the 12 *K. edwardsii* var. *atlantae* and one of the five *K. edwardsii* var. *edwardsii* strains showed enhanced growth in CO₂ but this was not seen in any of the *K. aerogenes* or *K. rhinoscleromatis* strains.

Results similar to those shown in table I were obtained when cultures were incubated in air at 30°C—the concentration of CO₂ in air is approximately 0.03%. Although the viable count was the same, the colonies were only about 1 mm in diameter whereas with added CO₂ at 37°C a diameter of 3 mm would be expected.

Some of the clinical strains exacting towards CO₂, together with the NCTC organisms and a selection of *K. aerogenes*, were retested in air containing varying amounts of CO₂ and incubated for 18 h at 30°C, 37°C and 41°C. For each temperature two controls were set up; one to test for growth in air and the other for growth in the complete absence of CO₂, achieved by passing air through a saturated solution of KOH. The minimum concentration of CO₂ required to give mucoid colonies of 3 mm diameter after incubation for 18 h is shown in table II. *K. aerogenes* was the only organism capable of growth at 30°C in the complete absence of CO₂; all the remaining species grew at this temperature with the atmospheric level of CO₂. At 37°C one NCTC strain of *K. ozaenae* grew in the absence of CO₂ but the other 19 strains required a

TABLE I

Ability of 3% CO₂ to enhance the viable count of klebsiellae during incubation for 18 h at 37°C on horse-blood agar

<i>Klebsiella</i> species	Number of strains showing the indicated enhancements of viable count in 3% CO ₂ compared with air		
	10000-fold	100-fold	None
<i>K. ozaenae</i>	12	10	1
<i>K. pneumoniae</i>	2	2	2
<i>K. rhinoscleromatis</i>	0	0	4
<i>K. edwardsii</i> var. <i>atlantae</i>	0	2	10
<i>K. edwardsii</i> var. <i>edwardsii</i>	0	1	4
<i>K. aerogenes</i>	0	0	40

TABLE II

Effect of incubation temperature and CO₂ concentration on the production of normal-sized mucoid colonies by various klebsiellae after incubation for 18 h

<i>Klebsiella</i> species (total number of strains)	Number of strains giving normal-sized (3–4 mm diameter) mucoid colonies at*							
	30°C in		37°C in			41°C in		
	CO ₂ -free air	air	CO ₂ -free air	air	air with 0.07% CO ₂	CO ₂ -free air	air	air with 0.07% CO ₂
<i>K. ozaenae</i> (20)	...	20	1	...	19	...	1	...
<i>K. pneumoniae</i> (4)	...	4	...	1	3	...	...	4
<i>K. edwardsii</i> var. <i>atlantae</i> (5)	...	5	1	2	2	...	...	5†
<i>K. aerogenes</i> (5)	5	...	5	...	...	5	...	...

* Organisms that grew at the lowest CO₂ concentration also grew at the higher concentrations; air contains approximately 0.03% CO₂.

† Poor growth: colony size *c* 1 mm diameter.

‡ In addition, four strains failed to grow at 41°C.

concentration above that of the atmosphere. When the incubation temperature was increased to 41°C the requirement for CO₂ increased to 0.1%. However, some organisms produced minute colonies, particularly the *K. edwardsii* var. *atlantae* strains and this growth limitation may have been influenced by the temperature. At 41°C, only the *K. aerogenes* and *K. pneumoniae* strains grew well.

Growth after deprivation of CO₂

Twenty strains of *K. ozaenae* and four strains of *K. pneumoniae* were inoculated on blood agar to give 10³ colony-forming units and incubated for 48 h at 30°C and 41°C in the absence of CO₂. The plates were then transferred to an atmosphere containing 3% CO₂ and incubated for a further 24 h at 37°C. Of the *K. ozaenae* strains deprived of CO₂ at 30°C, 15 grew normally, the other five and the four *K. pneumoniae* strains failed to grow. After deprivation of CO₂ at 41°C only one organism grew; this was a *K. ozaenae*.

Prolonged incubation in CO₂ and air

Five strains of *Klebsiella* which grew well in air were subcultured daily in an atmosphere containing 3% CO₂ in an attempt to induce a cultural requirement for the gas. This attempt failed; there was no detectable change in the recovery rates after this treatment. However, two strains of *K. pneumoniae* which had shown a preference for CO₂ became aerotolerant after daily subculture in air for one month.

Effect of pH on growth

Nutrient-agar plates buffered to pH 5.7, 7.2, 8.5 and 10.0 were inoculated with strains of *K. ozaenae*. Only a few colonies were observed after incubation

in air at 37°C for 18 h on the plates at pH 5.7, 7.2 and 8.5. No growth was obtained at pH 10.0. Parallel cultures incubated in 3% CO₂ produced large mucoid colonies at all the pH values.

Influence of antibiotics and other substances on growth

Antimicrobial-sensitivity testing was done on *K. ozaenae* with Oxoid DST Agar incubated at 37°C in air for 18 h. When a Mastring (Diamed Diagnostics Ltd) containing ampicillin, tetracycline, co-trimoxazole, cephalixin, colistin and gentamicin was used, a characteristic pattern of growth developed; the centre of the plate was free from organisms and what appeared to be a ring of stimulated growth was seen around the co-trimoxazole and tetracycline discs. The colonies were 1–2 mm in diameter and non-mucoid. Sensitivity-test plates incubated in CO₂ produced mucoid growth with the following susceptibility pattern: moderately sensitive to ampicillin, tetracycline, co-trimoxazole; sensitive to cephalixin, colistin, gentamicin. Growth in air was stimulated by other unrelated substances including the antibacterial agents trimethoprim and clindamycin. The nature of the bacterial growth is shown in the figure. The growth appeared after 18 h as a ring of minute colonies which became larger and mucoid after 24 h. Barium nitrate, 5% (w/v), magnesium chloride, 5% (w/v) and dipotassium ethylene diamine tetra-acetate 0.1% (w/v) produced a similar effect. The growth around a well of sodium barbitone 0.8% (w/v), was almost the same as that obtained in CO₂. None of the substances could replace CO₂ because no growth was evident when the tests were made without CO₂. A 3% (w/v) solution of sodium bicarbonate enhanced the growth of most of the strains tested but did not replace atmospheric CO₂.

DISCUSSION

We believe that CO₂ dependence is a characteristic of *K. ozaenae* because all but one of the strains tested required a final concentration of CO₂ of around 0.07% to produce typical mucoid "klebsiella colonies" after growth at 37°C for 18 h. The CO₂ produced two observable changes: it increased the viable count compared with parallel aerobic cultures and stimulated the formation of mucoid colonies. *K. ozaenae* was not the only species with a preference for CO₂; a few strains of *K. pneumoniae* had similar requirements and so did two strains of *K. edwardsii* var. *atlantae*. However, none of the *K. aerogenes* strains demonstrated a need for CO₂.

The organisms requiring added CO₂ for growth at 37°C grew in air at 30°C at the concentration of CO₂ in the atmosphere and in this respect appeared similar to the staphylococci reported by Thomas (1977). As the temperature of incubation increased, the *K. ozaenae* strains required more CO₂; this was greatest at a temperature of 41°C. Unlike *K. aerogenes*, the *K. pneumoniae* and most of the *K. ozaenae* strains did not survive after deprivation of CO₂. The lowest survival rate was found at the higher temperatures of incubation.

Cultures of *K. ozaenae* grown at 37°C in air for 18 h often produced minute

CARBON DIOXIDE REQUIREMENT OF *KLEBSIELLAE*

FIGURE.—*K. ozaenae* on Oxoid DST Agar with added lysed horse-blood 5% (v/v), growing around a well containing tetracycline 100 mg/litre after incubation for 24 h at 37°C in air. The growth contains mucoid and non-mucoid colonies.

colonies which could easily have been missed without close examination. After incubation for a further 18 h a mixture of small non-mucoid and large (3–4 mm diameter), mucoid convex colonies, typical of klebsiellae were produced. With the *K. ozaenae* strains subculture never yielded a pure growth containing only one type of colony.

Although we were unable to produce CO₂ dependence *in vitro*, it seems significant that all our CO₂-requiring organisms were isolated from the respiratory tract. Only a few were regarded as being clinically significant but many of the strains were found in bronchitic patients who are known to suffer from CO₂ retention. This may account for the frequent isolation of these organisms from this site and their requirement for relatively small amounts of CO₂. In substantiation of this hypothesis, we obtained typical mucoid growth by incubating plates at 37°C for 18 h in a plastic bag, filled by mouth with expired air and sealed.

An unusual characteristic of many of the *K. ozaenae* strains and of a recently isolated *S. aureus* requiring CO₂ for growth at 37°C, is the ability of the organism to grow in air at 37°C without added CO₂ but in the presence of antibiotics and other unrelated substances. This phenomenon appeared similar to that described by McGhie, Hutchison and Finch (1972) in which the growth of a strain of *K. aerogenes* was stimulated by sulphonamides and other substances. Our organisms did not grow with the added substances except at the atmospheric concentration of CO₂. The *S. aureus* demonstrated a similar growth enhancement with antibiotics, notably chloramphenicol and tetracycline and grew well at 30°C in air without added CO₂. It may be that the gram-positive and gram-negative organisms exhibiting this type of dependence use similar metabolic pathways. It seems unlikely that the effect of CO₂ is due simply to pH change because alteration in the H⁺ ion concentration did not allow growth unless CO₂ was present.

Dependence on CO₂ for growth is a characteristic not usually associated with the Enterobacteriaceae and may present the laboratory with difficulties in isolation, identification and antimicrobial sensitivity testing. We consider that any organism that grows poorly in air should always be tested for CO₂ requirement because this may be more widespread than is generally recognised. Perhaps air containing extra CO₂ should be regarded as "normal" for human pathogens and growth in ordinary air abnormal and fortuitous rather than the reverse (Stokes, 1975).

SUMMARY

The growth of 95 strains of *Klebsiella* cultured in air with added CO₂ is described. Twenty-two strains of *K. ozaenae* required CO₂ for characteristic growth when cultured at 37°C and growth was promoted by co-trimoxazole, tetracycline and sodium barbitone. Four strains of *K. pneumoniae*, two of *K. edwardsii* var. *atlantae* and one of *K. edwardsii* var. *edwardsii* gave increased viable counts after incubation in CO₂. None of the 40 strains of *K. aerogenes* tested required CO₂ for growth.

We are grateful to Dr J. G. P. Hutchison for his advice in preparing the manuscript and to Dr P. R. Mortimer of Coventry Public Health Laboratory for supplying strains of klebsiellae.

REFERENCES

- BARKER, J., BROOKES, G. AND JOHNSON, T. 1978. Carbon dioxide dependent Klebsiellae. *Br. med. J.*, **1**, 300.
- COWAN, S. T. 1974. Cowan and Steel's Manual for the identification of medical bacteria. Cambridge University Press: London, p. 110.
- EYKYN, S. AND PHILLIPS, I. 1978. Carbon dioxide-dependent *Escherichia coli*. *Br. med. J.*, **1**, 576.
- HALE, J. H. 1951. Studies on staphylococcus mutation: a naturally occurring "G" gonidial variant and its carbon dioxide requirements. *Br. J. exp. Path.*, **32**, 307.
- MCGHIE, D., HUTCHISON, J. G. P. AND FINCH, R. G. 1972. Stimulation of growth on a deficient medium of a *Klebsiella aerogenes* species by sulphonamide, para-amino benzoic acid, nitrofurantoin, and some other substance. *J. clin. Path.*, **25**, 976.
- STOKES, E. J. 1975. Clinical bacteriology, 4th ed. Arnold: London, p. 13.
- THOMAS, M., 1977. Carbon dioxide dependent dwarf staphylococcus. *Br. med. J.*, **2**, 830.