J. Hyg., Camb. (1982), **89**, 243–252
Printed in Great Britain

Studies on the growth of *Vibrio cholerae* biotype eltor and biotype classical in foods

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(*Received* 7 *May* 1982; *accepted* 8 *June* 1982)

SUMMARY

The growth of *Vibrio cholerae* biotype eltor and biotype classical was studied in a range of cooked foods, shellfish and raw vegetables, incubated at 22°, 30° and 37 °C. Both biotypes grew in all cooked foods but growth was not demonstrated on raw shellfish. The organism multiplied on some vegetables to levels of the order of 10^6/g. The classical biotypes of *V. cholerae* showed a longer lag period than the eltor biotypes in some foods particularly when incubated at 22 °C. The eltor biotypes reached a higher level in the stationary phase than the classical biotypes.

INTRODUCTION

The cholera pandemic which began in Indonesia in 1961 has spread through parts of Asia and Africa and caused explosive outbreaks in Europe. *Vibrio cholerae* biotype eltor has superseded *V. cholerae* biotype classical as the major cause of the disease; the latter is now rarely isolated in clinical cases. Epidemics caused by the eltor biotype produce a lower morbidity and mortality rate and a higher incidence of asymptomatic infections than those caused by the classical biotype. Nevertheless, cholera still presents a tremendous public health problem. Access to clean water and hygienic sanitary and cooking conditions would reduce or eradicate the disease in many areas where it is endemic.

In recent years there has been some evidence to suggest that food plays a greater role in the transmission of cholera than was previously suspected. Outbreaks have occurred in several countries where the transmission of the disease has been associated with shellfish including raw or partially cooked cockles in Portugal (Blake *et al.* 1977), cooked crab in the U.S.A. (Blake *et al.* 1980) and raw mussels and other raw seafoods in Italy (Baine *et al.* 1974). Other foods implicated as the vehicle of infection include raw vegetables in Israel (Cohen *et al.* 1971) and a dish of cold cooked hors d'oeuvres on an aircraft to Australia (Sutton, 1974). There are other outbreaks where the actual vehicle of transmission of the organism was not identified, but food was suspected together with water in conveying the disease within communities and homes (Gunn *et al.* 1981). Outbreaks where transmission has occurred via food as well as water were reviewed in detail by Feacham (1981).

Initial studies with *V. cholerae* biotype eltor show that it can multiply in foods (Sutton, 1974). Presumably if the organism could multiply to the levels which constituted an infectious dose, foods could be involved in the transmission of the disease more frequently than is usually realised especially in areas where standards of hygiene are low and where direct contamination of food could occur. The infectious dose for the classical strain of cholera has been reported to be 10^8–10^9 organisms/g in water, but prior neutralization of gastric acid within the stomach reduces this to 10^4–10^6/g (Hornick *et al.* 1971). The eltor biotype of *V. cholerae* has been shown to have an infectious dose level as low as 10^3/g with prior neutralization of gastric acid (WHO Scientific Working Group, 1980). It is therefore feasible that only small numbers of the organism need be present on food to initiate infection.

This present study was undertaken to investigate the growth of eltor and classical biotypes of *V. cholerae* in a range of foods, particularly those that have been incriminated in outbreaks of cholera. Foods used as part of staple diets in cholera endemic areas were also used for growth experiments, although no attempt was made at this stage to prepare the foods according to local recipes. Foods were incubated at temperatures of 22°, 30° and 37 °C; 22 °C is the ambient temperature for Europe and 30 °C for Asia at the times of the year when outbreaks have occurred.

MATERIALS AND METHODS

Bacterial strains

Strains of *V. cholerae* were supplied by the Maidstone Public Health Laboratory. The four classical strains were all isolated in Bangladesh from clinical cases, F4289 (VL 9546/1980), F4290 (VL 9560/1980), F2618 (VL 9528/1971) and F2619 (VL 9545/1979): all these strains were serotype Ogawa. The eltor biotypes were all recent isolates, F4291 (VL 7484/1978) serotype Inaba isolated from shrimps in the U.S.A., F4292 (VL 8957/1979) serotype Ogawa isolated from clams in Italy, F4293 (VL 9757/1980) serotype Ogawa isolated from a patient in Tanzania and F4294 (VL 9931/1980) serotype Inaba isolated from a patient in Korea.

Foods

The foods studied included cooked rice, lentils, chicken, kidney beans, potatoes, hard boiled eggs, pâté, prawns, mussels and cockles, raw mushrooms, fennel, courgettes, beanshoots, oysters and mussels. The oysters and cooked prawns were taken from samples received for routine microbiological testing in this laboratory. Other foods were purchased locally and those requiring cooking, prepared in the laboratory.

Media

Alkaline peptone water (APW) pH 8·6 and 0·1% peptone water (PW) pH 7·0 were used as diluents for the foods and counting techniques respectively, and quarter-strength Ringer solution (Ringers) was used to dilute the inoculum. Counts were made on Thiosulphate Citrate Bile Salts Agar, Oxoid (TCBS), and Columbia Base Agar (CBA) with 5% whole horse blood.

Methods

Portions (10 g) of most foods were distributed into a series of sterile 450 g screw-capped jars. Oysters and mushrooms were used whole and the weight in each jar noted. A second batch of oysters were homogenized before dividing into 10 g portions. Mussels were removed from their shells and distributed raw. In subsequent experiments they were first cooked for two minutes in their shells in boiling water.

The inoculum was prepared by subculturing one colony of the appropriate strain from overnight growth at 37 °C on CBA into 10 ml of nutrient broth. This was incubated for 16 h at 37 °C and then diluted in Ringers to give *c.* 10^3–10^4 organisms/ml. Each jar of food was inoculated with 5 drops from a 50 drop/ml Pasteur pipette; i.e. a total of 0·1 ml of inoculum per jar, to give a final level of *c.* 10^2–10^3 organisms/g food. The volume of inoculum added to the oysters and mushrooms was adjusted according to the amount of sample in each jar to ensure that each jar received *c.* 10^2–10^3 organisms/g food. Courgettes and fennel were inoculated on the outer skin of the vegetable. One set of mushrooms was inoculated on the gills and another set on the cap.

Two strains of each biotype were individually grown in each food except for prawns, pâté, mushrooms, eggs and chicken where only one strain of each biotype was used. Sets of jars were held at 22°, 30° and 37 °C and removed for counts after 0, 3, 6, 8, 10, 15, 18 and 24 h incubation with some counts being carried out at 32 and 48 h. Food showing evidence of complete spoilage was discarded without sampling. Each food was diluted 1 in 10 with APW and counts of *V. cholerae* made in 0·1 % PW on CBA and TCBS using a rapid microdilution technique (Kramer, 1977). The plates were incubated at 37 °C for 24 h.

RESULTS

Both eltor and classical biotypes of *V. cholerae* grew well in all the cooked foods tested. The rate of growth was fastest on cooked mussels, prawns and eggs. At 37 °C, levels of 10^{10}/g were reached within 12 h on mussels (Fig. 1), 12 h on prawns and 14 h on eggs. In other cooked foods all strains tested grew to levels of at least 10^7/g within 24 h at 37 °C.

At 22 °C both biotypes grew in all cooked foods except in pâté, where there was no growth after 48 h by which time the food had spoiled. Maximum numbers reached by the two biotypes were generally similar although the eltor biotype usually reached a higher level than the classical biotype. In some foods the variation between the final levels of the two biotypes was $< 10^1$/g as in mussels at 30 °C (Fig. 1), but in other foods the difference was $> 10^2$/g as in rice at 30 °C (Fig. 2).

When held in rice at 22 °C, the classical biotype had a lag phase of 15 h and the eltor biotype of 8–10 h before entering the log phase (Fig. 2). Similarly, in mussels at the same temperature the classical biotype had a lag phase of 8 h compared with only 4 h for the eltor biotype (Fig. 1). In pâté at 30 °C the classical biotype entered the growth phase after 15 h of incubation compared with only 6 h for the eltor biotype. At 30° and 37 °C in both rice and mussels and at 37 °C in pâté the lag

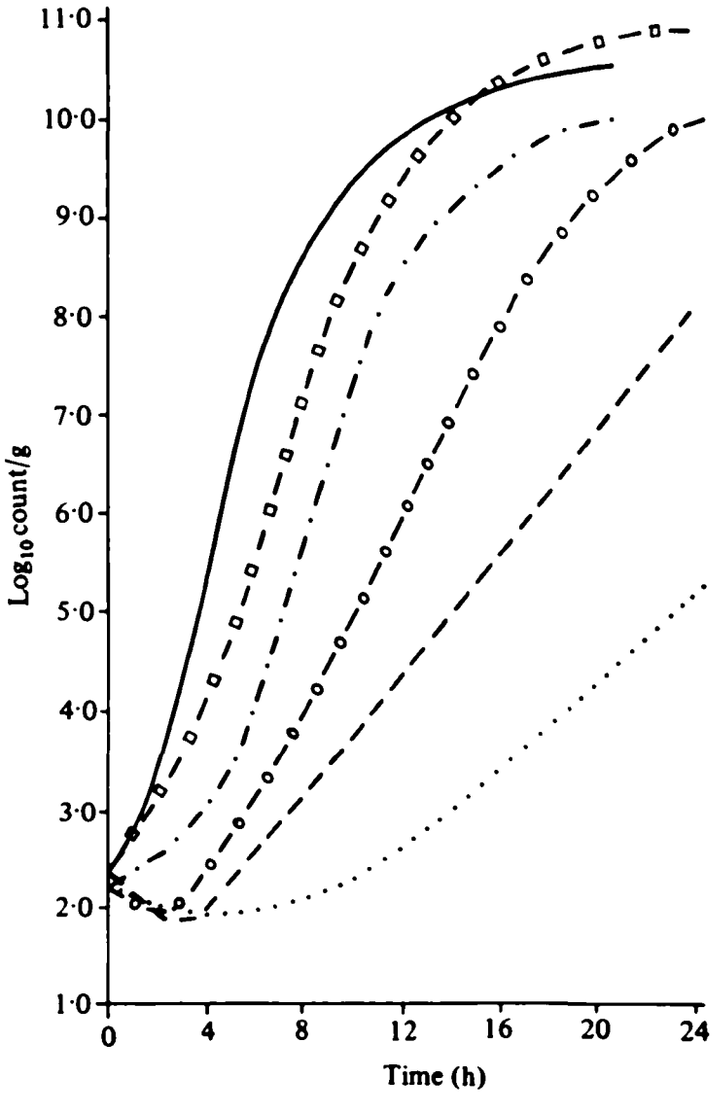

Fig. 1. Growth of *V. cholerae* in cooked mussels. F2618 (classical) at 22 °C (·····), at 30 °C (o——o) and 37 °C (—·—); F4292 (eltor) at 22 °C (----), at 30 °C (□——□) and 37 °C (——).

phase was considerably reduced. On prawns, kidney beans and cockles there was also a shorter lag phase for the eltor biotype than for the classical biotype at 22 °C. In eggs and chicken the lag phase at 22 °C was less apparent; this is exemplified in the growth curve for chicken (Fig. 3).

With some foods the number of *V. cholerae* recovered at 0 h was < 100/g. Such foods included lentils, potatoes, fennel and courgettes and with these the lag phase could not be studied because these levels are not detectable by the counting method employed. It was noticeable, however, that strains of the classical biotype of *V. cholerae* began their growth phase later than strains of the eltor biotype of *V. cholerae*, particularly at lower incubation temperatures: in lentils (Fig. 4) there was at least 5 h difference between the classical and eltor biotypes in the commencement of the growth phase at 22 °C, the lag phase of the classical biotype being much longer.

Due to the more extended lag phase of the classical biotypes compared to the eltor biotypes in courgettes, fennel and all the cooked foods except eggs and chicken, there were often great differences between the levels of the two biotypes.

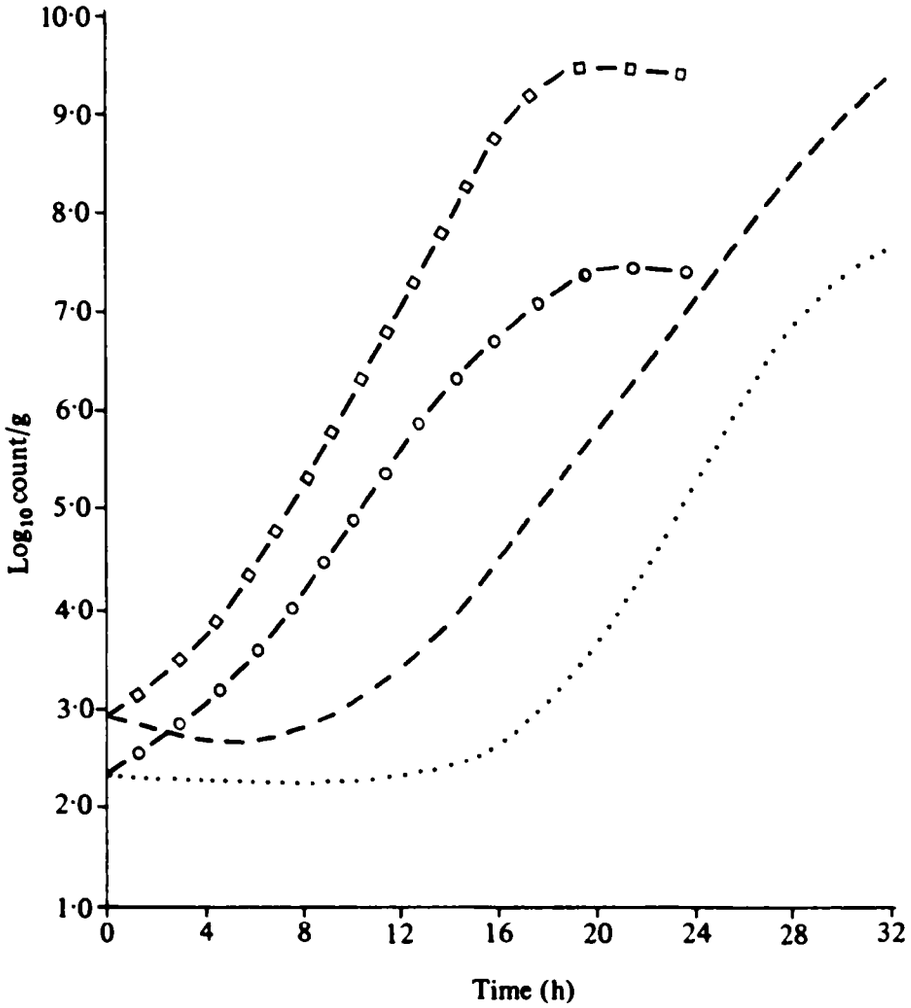

Fig. 2. Growth of *V. cholerae* in cooked rice. F4290 (classical) at 22 °C (·····) and 30 °C (○——○); F4292 (eltor) at 22 °C (----) and 30 °C (□——□).

For instance in mussels at 30 °C after 12 h, the eltor biotype was ahead of the classical biotype by 10^3 organisms/g (Fig. 1). The rate of growth however was generally similar for each food at each temperature (Figs. 1–5).

Growth of the cholera organism was not demonstrated on any raw shellfish. The pH value of whole oysters was 6·4, of homogenized oysters 5·95 and of mussels 6·7. Adjustment of the pH values of the homogenized oysters using Oxoid phosphate buffered saline to 6·7, 7·2 and 7·9 did not facilitate the growth of *V. cholerae* at any of the temperatures tested.

V. cholerae failed to grow on either bean shoots or mushrooms at the temperatures tested. The eltor biotypes grew at 22°, 30° and 37 °C on both courgettes and fennel but the classical biotypes grew only at 30° and 37 °C on courgettes and only at 37 °C on fennel. Growth on courgettes is demonstrated in Fig. 5. The pattern of growth was irregular on both vegetables, probably due to the interfering background flora; total viable counts at 0 h were 10^6–10^7/g. The trend however, was for an overall increase in levels of the *V. cholerae* strains from < 100/g to $10^5 - 10^6$/g.

https://doi.org/10.1017/S0022172400070777 Published online by Cambridge University Press

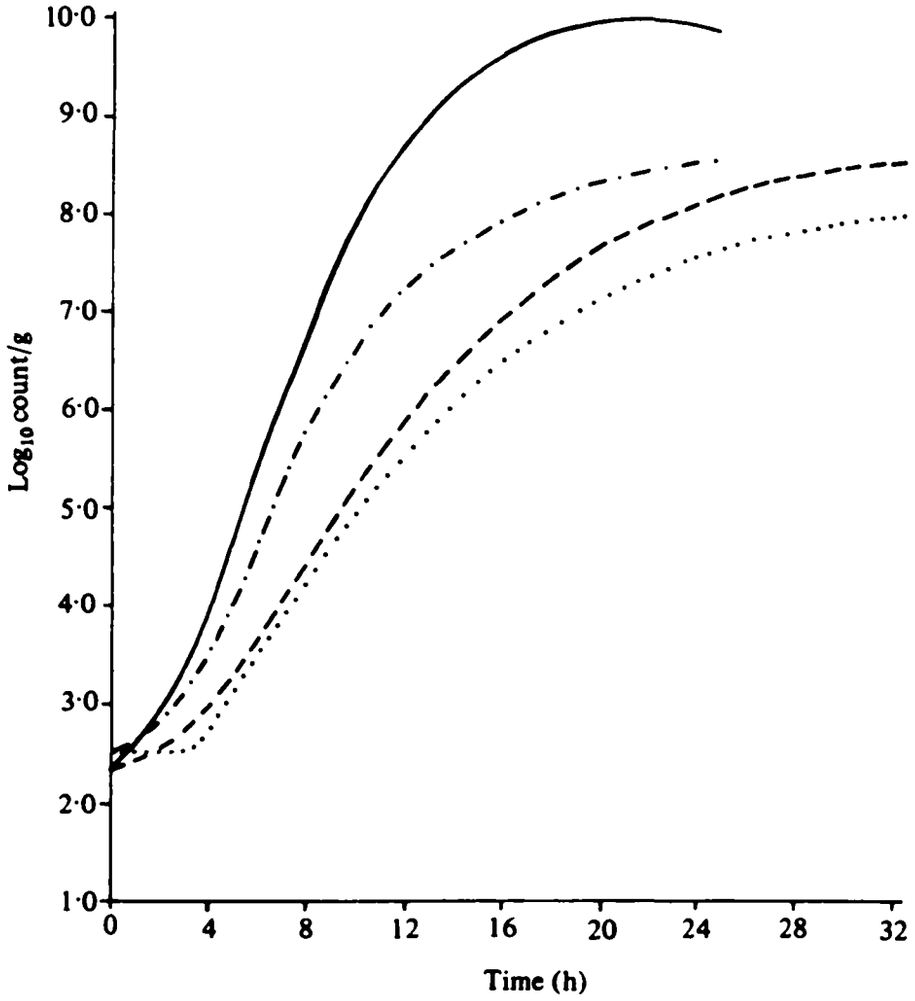

Fig. 3. Growth of *V. cholerae* in cooked chicken. F4290 (classical) at 22 °C (·····) and 37 °C (—·—); F4291 (eltor) at 22 °C (----) and 37 °C (——).

DISCUSSION

The concept that cholera may be food-borne as well as water-borne has been widely debated but research studies on the ability of *V. cholerae* to multiply in foods are limited. In 1926 Takano, Ohtsubo & Inouye reported multiplication of *V. cholerae* biotype classical on raw fish, beef and shelled oysters at 20 °C: an increase in numbers occurred overnight on raw fish and beef and within 68 h on shelled oysters. A Cholera Research Laboratory Technical Consultant Report (1965) described growth studies with a classical strain of *V. cholerae* on cooked rice incubated at 25 °C. An increase in numbers of up to 10^5 organisms/g overnight was obtained. Following the outbreak of cholera on the airline Sutton (1974) attempted to grow the strain which was isolated from patients, on foods similar to those served on the flight: growth occurred on stuffed egg, milk–cream mix, duck and pâté. A study on the growth of non 0–1 cholera vibrios in foods showed that these organisms could multiply in a range of cooked foods to levels of up to 10^{10}/g food (Roberts & Gilbert, 1979).

In this study growth of *V. cholerae* was most rapid in foods with an alkaline pH such as prawns, eggs and cooked mussels with pH values of 7·9, 7·85 and 7·05 respectively; these values are close to the optimum for growth of vibrios. Other

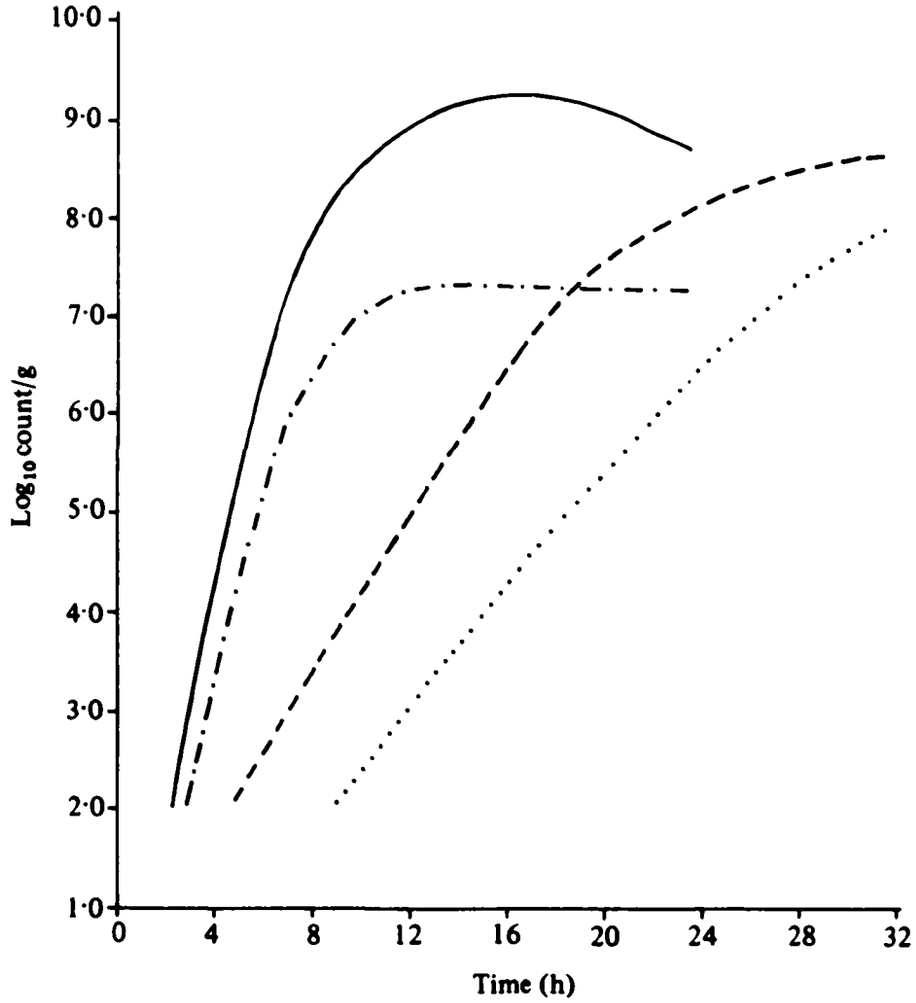

Fig. 4. Growth of *V. cholerae* in cooked lentils. F4289 (classical) at 22 °C (·····) and 37 °C (—·—); F4294 (eltor) at 22 °C (----) and at 37 °C (——).

cooked foods supported the growth of *V. cholerae* well, but where the fat content of the food was high such as in pâté, growth was slower. The reason for the ability to grow on some vegetables but not on others is not completely understood. Vegetables usually support a high background flora which arises from contamination from the soil and environment and multiplication of some species of bacteria on the vegetable. The low nutrient and high moisture content probably makes them unable to support multiplication of all types of bacteria.

The ability of *V. cholerae* to multiply on raw shellfish was not demonstrated in this study. Although Takano, Ohtsubo & Inouye (1926) reported growth of the organism on raw oysters, there is little other evidence to suggest *V. cholerae* will multiply on raw shellfish. In an outbreak on Guam raw fish had been salted, refrigerated and subsequently warmed in the sun for 5 h before eating during which time any surviving organisms were able to multiply (Merson *et al.* 1977). However, other outbreaks may have resulted from high level contamination of the fish in the sea (McIntyre *et al.* 1979), as well as concentration of the organisms during filter feeding. The present study has shown that the organism will multiply on cooked shellfish. Thus inadequate cooking of highly contaminated shellfish or contamination with *V. cholerae* following cooking could lead to multiplication of the organism on the fish, as occurred in an outbreak in the U.S.A. (Blake *et al.* 1980).

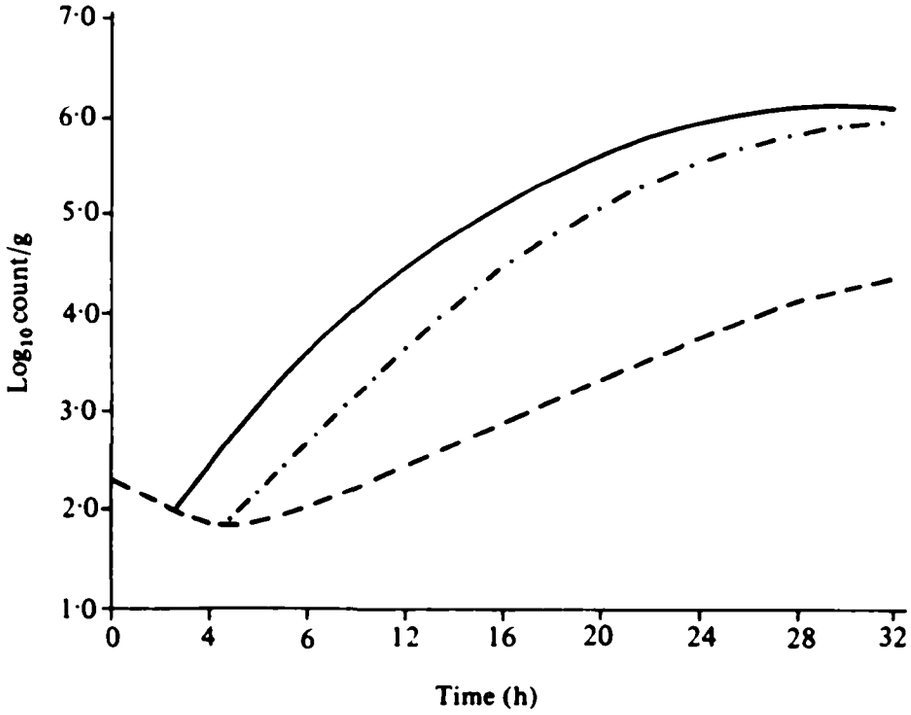

Fig. 5. Growth of *V. cholerae* in courgettes. F2618 (classical) at 37 °C (—·—); F4291 (eltor) at 22 °C (----) and 37 °C (——).

In some endemic areas it is common practice to cook sufficient rice for a whole day and store it at ambient temperatures. Results of this study show that when rice was contaminated with *V. cholerae* counts of 10^8/g were reached within 16 h at 30 °C; this would be sufficient to cause infection if consumed. Many workers have examined food and water used within homes during outbreaks of cholera. Felsenfeld (1972) in Thailand found that 7·5% of cooked rice samples were contaminated with *V. cholerae* but they were not isolated from other foods in the home or were present in small numbers only. More recently Spira (1980) found the organism in stored drinking water and in less than 1% of foods in affected households. These results would seem to indicate that food could only play a minor role in transmitting cholera. Success at isolating *V. cholerae* from foods sold locally during outbreaks is also limited (Prescott & Bhattacharjee, 1969). However, many factors, including the nature of the samples examined and the time of sampling the food, influence the degree of success in isolating the organism. Water is often suspected as being the initial source of the cholera organism and only subsequently are food sources investigated; this often leads to inconclusive results. For example, during one of a series of outbreaks of cholera in Israel, Gerichter *et al.* (1971) isolated *V. cholerae* from only three samples of vegetables from a total of 333 examined, although vegetables were suspected early in the outbreak as being the primary vehicle of infection.

Results of this study have shown that especially at low temperatures the lag phase of the eltor strains growing in some foods was significantly shorter than that of the classical strains. Classical strains also failed to grow at lower temperatures on the pâté, fennel and courgettes. In survival studies it has been found that eltor strains generally survive longer than classical strains of *V. cholerae* (Neogy, 1965;

Felsenfeld, 1965). It is possible that these strains have physiological and/or biochemical characteristics which enable them to compete and survive better than strains of the classical biotype of *V. cholerae*; this may also account for the predominance of the eltor biotype in the environment. Further investigation into these differences as well as competition studies and controlled survival studies would hopefully lead to an understanding of this predominance of eltor over the classical biotype.

It is evident that *V. cholerae* can multiply sufficiently on cooked foods and some raw foods to produce levels constituting an infectious dose. In endemic areas particularly this could be a mode of transmission within homes and small communities where hygienic food practices are difficult to adopt and follow. However, to verify this supposition, further work involving *in vitro* and *in situ* growth and survival studies needs to be carried out to clarify the role of food in transmission of the disease.

We wish to thank Dr A. L. Furniss and Dr J. V. Lee both of the Public Health Laboratory, Maidstone for providing the *V. cholerae* strains and for helpful comments. We are grateful to Dr R. J. Gilbert of the Food Hygiene Laboratory for his guidance and encouragement during this project.

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