The hygienic and pathogenic consequences of the isolation of *Vibrio cholerae NAG* from the conjunctiva of two ducklings, from brackish water samples in their surroundings, and from the intestinal contents of a duckling, are discussed in relation to present knowledge of the occurrence of *Vibrio cholerae NAG* in man and animals.

**INTRODUCTION**

In recent years, certain species of vibrio pathogenic to man have attracted increasing attention. The reasons are several, one of them being that increased travel has caused a wider spread of cholera, and at the same time other enteropathogenic vibrios, which have previously been largely disregarded, are becoming more frequently incriminated as the cause of cholera-like diseases or gastroenteritis.

The taxonomy of the so-called NAG vibrios is controversial; but they are now commonly classified as *Vibrio cholerae NAG* (Non Agglutinable) (Sakazaki, 1970), i.e. they are not agglutinated in cholera antiserum 0 1. Apart from that difference, they show great similarity to the classic *Vibrio cholera* type with respect to morphology, biochemical properties, H antigens, and G-C percentages (Sakazaki et al., 1967; Colwell et al., 1968; Sakazaki, 1970). These similarities may consequently cause differential diagnostic difficulties, as certain mucoid forms of *V. cholerae* may occur which are not agglutinated in 0 1 serum (Sakazaki, 1970).

*V. cholerae NAG* may cause a cholera-like disease, giving the same symptom of rice-watery faeces as the classic vibrio type or the EL-TOR type. But often diarrhoea will be mild (Mc Intyre et al., 1965).

Chashi et al. (1972) state that antiserum against crude NAG toxin may completely
neutralize both purified and crude cholera enterotoxin. Zinnaka and Carpenter (1972) mention that NAG vibrio isolated from patients causes accumulation of fluid in ligated loops of ileum in rabbits, whereas similar vibrios isolated from water do not.

Since 1954 (Yajnik and Prasad, 1954) *V. cholerae NAG* has been known to cause disease in man. The bacterium has been isolated from cases of diarrhoea in the Philippines, Thailand, East Pakistan, India, Iran, Sudan, Egypt, Czechoslovakia, West Germany and Sweden (Gaines *et al.*, 1964; Mc Intyre and Feeley, 1965; Mc Intyre *et al.*, 1965; Aldova *et al.*, 1968; Chatterjee and Neogy, 1971; Ohashi *et al.*, 1972; Ko *et al.*, 1973; Zafari *et al.*, 1973; Bäck *et al.*, 1974).

Apparently, the extent and occurrence of *V. cholerae NAG* in waste water, surface water, and carriers, is not precisely known. An extensive investigation made in Iran of approximately 22,000 samples of faeces showed that 160 (0.72%) were positive (Zafari *et al.*, 1973). However, the carrier percentage was much higher (about 16%) in returned pilgrims.

Examinations in Thailand showed that 10% of patients with diarrhoea excreted *V. cholerae NAG*, whereas it was only found in 0.6% of clinically healthy persons (Gaines *et al.*, 1964).

Examinations in Germany of waste water from a big city showed the surprising result that all samples examined were positive. The bacterium was also isolated from 9 out of 11 samples of river water (Ko *et al.*, 1973).

**MATERIAL AND METHODS**

The institute received 13 live ducklings for examination from a farm, and 4 from a hatchery which had no connection with the former.

Conjunctival scrapings from 13 birds were seeded on BA (Tryptose blood agar base, Difco, with 5% calf's blood added) and TCBS-agar (Merck).

Intestinal contents from 10 of the ducklings were investigated for the presence of *V. cholerae NAG* by mixing 20g of faeces and 300ml of *V. cholerae* enrichment broth (Merck) containing 0.5% soluble starch and 0.3% alkylbenzene-sulphonate (Lutensit A-BA). The broth was incubated for 18 hours at 37°C, and subcultured on TCBS-agar. Suspect sucrose positive colonies were subcultured on BA.

The following environmental samples were collected at the farm: 7 water samples, each of 250ml, were collected in sterile bottles (5 of the samples were taken from a pool and 2 from drinking water troughs); 5 faeces-contaminated soil samples were collected in sterile petri dishes.

A fortnight later the sampling procedure was repeated and 11 water samples and 10 faeces/soil samples were collected.

Both water and faeces/soil samples were examined for vibrios according to the technique described for the intestinal contents. For practical reasons, two samples were usually bulked for testing. In all cases, 20g faeces-contaminated soil or 30ml water per 300ml broth were used.

Preliminary tests, colonial and bacterial morphology, haemolytic activity, citrate, D-tartrate, mucate and malonate utilisation, gelatin liquefaction, nitrate reduction, KCN sensitivity, lysine and ornithine decarboxylation, arginine and esculine hydrolyzation, formation of acid and gas from carbohydrates, hydrogen sulhide, urease,
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phenylalanine deaminase, indole, acetoin and phosphatase production were performed as earlier described by Bisgaard (1975). ONPG and ability to reduce tellurite in concentrations of 0.001, 0.01 and 0.04 took place according to Cowan and Steel (1970). Deoxyribonuclease, lipolytic and lecithinase activity was studied on bacto DNA's test agar (Difco), tributylin agar (Merck) and egg yolk medium (Nordic Committee on Food Analysis, 1968), respectively. Haemagglutination of chicken red blood cells was carried out according to Finkelstein and Mukerjee (1963), "string-test" and sensitivity to 2,4-diamino-6,7-di-isopropyl pteridine according to Smith (1970) and Caselitz et al. (1961) respectively.

Examination for growth on EBM, McConkey and BLSF agar was made on the respective Difco media.

Growth at different pH values was tested in peptone water. After adjustment of pH to 5, 6, 7, 8, 9, 10, 11, and 12, respectively, the broth was seitz-filtered before inoculation.

The temperature limits for growth were determined in veal infusion broth (Difco). The same medium was used to examine sodium chloride tolerance.

Hydrolysis of starch at different sodium chloride concentrations was examined on agar plates. To obtain a sodium chloride concentration of 2, 4 and 6% respectively, sufficient sodium chloride was dissolved in tryptose blood agar base (Difco) containing 1% starch.

Pigment formation at 22°C was examined on Pseudomonas agar F and P (Difco).

Finally, the strains were inoculated into the yolk sack and allantoic cavity of 7 and 11 days old chick embryos, respectively, to determine their lethality. The inoculum used was 0.1 ml of 2 hours veal infusion broth culture per egg (5 x 10⁷ cells/ml).

Unless mentioned to the contrary, incubation took place at 37°C and the final result was taken after a week.

RESULTS

After having spent the first 3 weeks of their lives in a heated house, all ducks were moved to an open field in hilly grounds, without natural pools or streams. They had free access to a commercial feed mixture and to running water from a drinking water source. The running water formed artificial pools in the low parts of the area. Normally, however, the ducks had no access to these pools.

*V. cholerae NAG* was isolated from the conjunctival scrapings of 2 ducklings suffering from serous conjunctivitis, although not in pure culture, but mixed with Micrococcus — and Bacillus species, from 4 bulked water samples and from the intestines of 1 out of 4 ducklings originating from the hatchery.

**Description of the isolated *V. cholerae NAG* strains**

**Colonial morphology.** Colonies were circular, 2-3mm in diameter, low convex or slightly conical, with glistening surface, and entire edge; they were butyrous in consistency and easily emulsifiable. The colonies were surrounded by a zone of partial or complete haemolysis, 4-6mm in diameter. The zone of haemolysis was surrounded by a dark, 1mm wide, band. Where the growth was confluent, the zone of partial haemolysis showed a tendency to clear. Single colonies were centrally greyish, but transparent at the periphery. After further incubation, the colonies became larger and assumed a brown/greenish colour, especially where the growth was confluent. At
the same time the agar showed a dark greenish discoloration.

Three of the strains isolated dissociated into R- and S forms.

**Bacterial morphology.** Gram-stained smears revealed slightly curved Gram-negative rods, arranged singly and often resembling a comma. Size was variable, the axis generally being curved with parallel sides and rounded ends.

**Biochemical reactions.** All strains were positive in the following tests: catalase, citrate, DNA'se, gelatin, haemolysis, indole, lipase, lysine, motility, nitrate, ornithine, oxidase, pellicle formation, pteridine, Voges-Proskauer (22°C), fructose, galactose, glucose, glycerol, maltose, mannitol, sucrose, starch, trehalose, hydrolysis of starch with 2 and 4% NaCl added, growth on McConkey, at pH 6, 7, 8, 9, 10 and 11, at 15, 40, 42 and 44°C and in 2, 4, 6 and 7% NaCl.

Negative reaction was obtained in the other tests, as follows: arginine, D-tartrate, hydrogen sulphide, KCN, lecithinase, malonate, methyl red, mucate, phenylalanine, phosphatase, pigment, urease, adonitol, amygdalin, arabino, cellulose, dulcitol, gas from glucose, inositol, inulin, melezitose, melibiose, raffinose, rhamnose, salicin, sorbitol, sorbose, xylene, esculin, hydrolysis of starch with 6% NaCl added, growth at pH 5 and 12, at 10 and 45°C, in 8% NaCl and on EMB agar.

Variations between strains were seen in the following tests: haemagglutination, where 2 of the strains became strongly positive within 10 seconds, while the rest showed weaker and delayed positive reactions within 1-2 minutes, the control still being negative; ONPG (4 strains positive); "string-test" (3 strains positive); Voges-Proskauer at 37°C (5 strains positive), lactose (4 strains positive) and mannose (3 strains positive).

A pronounced alkaline reaction was observed in meat extract peptone broth after a few days incubation. With some strains the same reaction could be seen as well on TCBS as on BLSF agar.

Tellurite was reduced at a concentration of 0.01% potassium tellurite even though growth was greatly retarded. A concentration of 0.04% potassium tellurite completely inhibited growth.

**Serology.** None of the strains isolated were agglutinated by *V. cholera* O-group I serum. Serological investigation of 4 strains revealed that 3 of the strains belonged to O-group 39 and the fourth to O-group 41.

**Inoculation of chick embryos.** All strains killed chick embryos after 18 hours.

**DISCUSSION**

Apart from negative fermentation reactions in d-tartrate, lactose and ONPG, the biochemical reactions were in accordance with those reported by Sakazaki (1970).

Because of the alkaline properties of these Vibrio, sugar fermentation results should be taken daily, as the later readings may be affected by the concomitant production of alkaline substances.

Recent investigations have shown that *V. cholerae NAG* is widespread not only in South East Asia but also in Europe. The realization that this species may cause severe cases of diarrhoea has given rise to concern as to whether there may be a non-human reservoir besides the human one. It has been reported that fish and molluscs form a reservoir of importance for the spread and persistence of these vibrios in a given region (Dutt et al., 1971).
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Investigations in India of cattle, buffaloes, sheep and goats in the Ganges estuary showed that *V. cholerae NAG* could be demonstrated in 5-38% of samples of rumen liquor (Abou-Gareeb, 1960). Examination in India of dogs revealed the presence of NAG Vibrio in 28 of 203 (14%) faeces samples. It was isolated from crows also (Sack, 1973).

Unlike *V. cholerae NAG*, the normal type of *Vibrio cholera* seems exclusively to occur in human populations (Sack, 1973).

The isolation, without any aetiological connection to pathological changes of *V. cholerae NAG* from the conjunctiva and gut contents of ducks in Denmark indicates that the bacterium also can be associated with poultry, the living habits of which bring them into close contact with soil and water.

Our present knowledge about the occurrence and significance of *V. cholerae NAG* in Europe is based on very few publications. But the present observations in conjunction with Swedish and German reports (Bäck et al., 1974; Ko et al., 1973) give reasons for greater interest in this microorganism from the point of view of water and food hygiene. It should be pointed out that the bacterium was isolated from water samples as late as the 21st of September, after a period of low temperatures.

The source of the initial contamination of the surroundings is not known. No large stream to which the ducks had access passes by the farm. Hence the contamination was unlikely to have originated from human sources through contaminated waste water effluent. As waste water and contaminated surface water might be contaminated with *V. cholerae NAG* (Dutt et al., 1971; Ko et al., 1973) migratory birds foraging in these areas could be excreters of the organism, thus carrying the infection over long distances. The results indicate that *V. cholerae NAG* can survive in the soil/water environment. More knowledge is therefore considered to be necessary on the role played by the animal reservoir in the dissemination and ecology of these vibrios potentially pathogenic to humans.

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RESUME

Isollement et caractérisation de Vibrio cholerae NAG provenant d’un élevage danois de canards. Aspects hygiéniques de l’infection.

Dans cet article, les conséquences hygiéniques et pathogéniques de l’isolement de Vibrio cholerae NAG de la muqueuse conjunctive de deux canetons, de quatre spécimens d’eau boueuse constituant l’environnement normal de ces canetons et enfin du contenu intestinal d’un caneton sont discutées par rapport aux connaissances actuelles sur Vibrio cholerae NAG chez les animaux et l’homme.

ZUSAMMENFASSUNG

Vibrio cholerae NAG in einer dänischen Entenfarm – Isolierung, Charakterisierung und hygienische Aspekte

Es werden im Anschluß an eine Isolierung von Vibrio cholerae NAG aus den Konjunktiven zweier Enten, aus Brackwasserproben in ihrer Umwelt und aus dem Darminhalt eines Entchens die hygienischen und pathogenetischen Konsequenzen unter Bezug auf unsere derzeitigen Kenntnisse über Vibrio cholerae NAG bei Mensch und Tier diskutiert.