ISOLATION AND CHARACTERIZATION OF *VIBRIO PARAHAEMOLYTICUS* AND *AEROMONAS HYDROPHILA* FROM FISH IN KENYAN WATERS AND THEIR POSSIBLE ROLE IN CAUSING INFECTION TO MAN

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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This thesis has been submitted for examination with our approval as university supervisors.

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To my beloved parents, brothers and sisters

ABSTRACT

With the increase in utilization of fish resources by man, there has been a concomitant demand for quality control of fish and fish products. An important aspect of this has been the need for the study of microbiological contribution to spoilage changes. Both the inherent bacterial flora of fish and those acquired through contamination after catch, together with other factors like the enzymatic activities, are important in causing changes which lead to lowering of quality. Of major public health importance, however, is the fact that some of these microorganisms cause infection in man. Amongst this group are Vibrio parahaemolyticus and motile Aeromonas species.

Vibrio parahaemolyticus has long been known to be one of the aetiologic agents in food-poisoning causing acute gastroenteritis, particularly common in areas where marine fish is eaten raw in large quantities. Aeromonas species, together with Campylobacter and Yersinia species, on the other hand, are considered to be in the group of emerging enteropathogens, which cause acute diarrhoeal diseases particularly in infants. This study was aimed at assessing the hygienic standards of fish from some of the fishing areas in Kenya by determining the total bacterial counts, isolation and characterization of V. parahaemolyticus and motile Aeromonas species.

Total bacterial counts were performed on fish and water samples obtained directly from landing sites of Lake Naivasha, River Sombeli (Magadi), and Masinga Dam. A total of 30 samples were collected.

For isolation of the two bacteria, a total of 763 samples were collected from all the major fishing sites in Kenya. Of these, 723 were fish and 40 were water samples. Primary isolation was done by the use of selective media and the isolates were confirmed by means of biochemical tests. In the case of Aeromonas isolates, two methods, that is, biochemical and CAMP-like factor detection, were used to further identify to species level as A. caviae, A.hydrophila and A. sobria.

The ability of these isolates to cause infection was determined by assessing their pathogenic potential in terms of toxin production. For V.parahaemolyticus, this was done by detection of Kanagawa haemolysis. In case of Aeromonas species, toxigenicity was tested for by detection of enterotoxin and cytotoxin production using suckling mice and vero cell lines respectively.

The antibiograms of the isolates of the two genera were studied by determining the zones of inhibition of bacterial growth on Mueller-Hinton agar by various antimicrobial agents. Analysis of the plasmid contents was performed by lysis of the cell-walls of the isolates and then separating them by means of electrophoresis.

Slide coagglutination technique using antisera raised against the somatic antigens of the two genera coated on Staphylococcus aureus strain Cowan I, was used to identify isolates having homologous antigens.

Results obtained for total bacterial counts on fish sampled from the three areas ranged from 10^5 to 10^8 and 10^1 to 10^5 for water samples.

Out of 666 fish and water samples from both freshwater and marine environs screened for V. parahemolyticus, 29 isolates were made, representing an isolation rate of only 4%. However, a high isolation rate of 46% (27/62) was found amongst fish samples obtained from the coastal waters. The prevalence in fish from this area was 44% (25/57) and in water was 40% (2/5). Only 1% (1/99) and 0.3% (1/325) of the samples from Lake Naivasha and Lake Victoria respectively yielded the organism. The two isolates in both cases were from fish. The organism was not isolated from lake Turkana, River Sombeli (Magadi) and Masinga Dam samples.

All the 763 fish and water samples collected were screened for motile Aeromonas species.
An isolation rate of 47% (356/763) was made. The two methods used for species differentiation were observed to give a slight variation of different rates of occurrence of the three species. The rates obtained by using biochemical tests were A. caviae 42%

(150/356); A. hydrophila 37% (132/356) and A.sobria 21% (74/356) while that of CAMP-like factor detection were A.caviae 44% (155/356), A. hydrophila 35% (123/356) and A. sobria 22% (78/356).

The 29 V.parahaemolyticus isolates were all Kanagawa negative. The general optimum salt requirement for growth was 3%, although one isolate from Lake Naivasha had a low salt requirement because it grew very well in a medium containing 1% salt concentration. Of the typable twenty isolates serotyped, only five were typable, representing 20% typability. All the typable isolates were from the coastal waters and were serotypes 02:K28; 03:K29 and 04:K42.

A total of 166 Aeromonas were tested for their enterotoxigenicities. Fourty seven (28%) isolates were found to produce enterotoxins. The occurrence of enterotoxin producing strains amongst the isolates varied considerably with A. sobria having the highest rate of 51% (20/39), followed by A. hydrophila 35% (23/65) and then A. caviae 6% (4/62). In the cell culture model, 53% (42/80) of the isolates were found to be cytotoxic to vero cells.

The V. parahaemolyticus strains were generally sensitive to chloramphenicol, sulphonamides, while being resistant to penicillin and fucidin. Aeromonas isolates ,on the other hand, showed resistance to more

agents like ampicillin, bacitracin, neomycin, penicillin, oxacillin and nitrofuran. Most of them, however, responded to chloramphenicol, tetracyclines, nalidixic acid, fucidin and sulphonamides. Among the Aeromonas isolates, 32 of them were found to carry plasmids of varying sizes and numbers. Most of them were light, although some isolates carried heavy plasmids as well.

Coagglutination test using V. parahaemolyticus isolates showed that only four of the 29 isolates were serologically identical to the somatic antigen of the reference strains to which the antiserum was raised. Among the Aeromonas isolates, only 29 out of 366 gave positive reaction. Two of these were A.sobria while the rest were A. hydrophila.

The total bacterial counts for fish were found to be generally higher than those for water, because apart from serving as a substrate for attachment of microorganisms, some fish can actively concentrate bacteria during feeding as they filter water through their gills.

The prevalence of V. parahaemolyticus in Kenyan marine fish was higher than that of fresh water fish. A high percentage of motile Aeromonas species was isolated from both freshwater and marine environs. Although the results of differentiation into the three species using the two methods differed slightly the

general trend was that A. caviae had the highest frequency of occurrence followed by A. hydrophila and A. sobria

Toxigenicity assay for both enterotoxins and cytotoxins revealed that higher toxigenic strains occur amongst A. sobria followed by A. hydrophila and then A. caviae. Since pathogenicity is associated with toxin production, it can be deduced that although A. sobria has low occurrence among environmental isolates, they are more pathogenic than the other two species.

Antimicrobial susceptibilities of V. parahaemolyticus showed that the isolates were susceptible to most broad spectrum antibiotics. The plasmid carriage amongst the isolates was low which might imply that plasmid-induced resistance is not present in these isolates. The few light plasmids detected were assumed to be cryptic as no specific functions could be assigned to them.

Aeromonas species, on the other hand, showed multiple resistance to a number of agents. One isolate was found to be resistant to all the tested agents. It was found that Aeromonas species showed greater variation in antibiotic susceptibility patterns than V. parahaemolyticus.

Although coagglutination technique enhanced sensitivity of slide agglutination test, its use for identification of the two organisms was limited because

of the heterogenicity of the antigens of the organisms.

In conclusion, this study reports a high prevalence of V. parahaemolyticus amongst marine fish but a low prevalence in fresh water fish in Kenya. The high prevalence of the organism amongst marine fish might be a potential health hazard. There is also a high occurrence of Aeromonas species in Kenyan waters and fish, and the isolation of toxigenic strains of A. sobria and A. hydrophila among them indicates that they may present a potential health problem.

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INTRODUCTION

1.1 TOTAL BACTERIAL COUNT

Fish is assuming great importance in human diet as an alternative source of animal protein in many parts of the world; and it has been estimated that it contributes about 10% of the world's total animal protein food supply (Anderson, 1977). This is reflected in the ever increasing production of fish industry the world over. According to FAO statistics, world total fish landings have increased from about 20 million tons in 1948 to 60 million tons in 1968 and the average annual rate of increase is estimated to be 7% (Roy, 1971). In Kenya, total fish production has also been rapidly increasing, especially in the past decade. In 1985, the production was almost four times that of 1974 (Table 1). Total production increased from 28,581 metric tons in 1974 to 105,815 metric tons in 1985.

Fish being a highly perishable food requires the establishment of quality control measures to ensure an extended retention of organoleptic properties. Spoilage due to microbial organisms is a major factor which affects the quality of fish; therefore assessment of microbial load is necessary to reflect on the shelf-life (Hobbs,1983). Most of these spoilage organisms are

TABLE 1: Fish catch by year in Kenya in metric tons.

	1974	1981	1982	1983	1984	1985
L. Victoria	17,175	38,179	60,958	77,327	71,854	88,589
L. Turkana	5,731	10,529	11,040	10,113	8,448	7,460
L. Baringo	122		401	352	297	653
L. Naivasha	39		411	692	320	245
Other lakes	572	2,677*	499	473	398	96
Rivers	1,526		268	1,526	2,172	1,972
Fish pods			440	584	711	1,085
Total	25,165	51,386	74,017	91,068	84,200	99,764
Marine products	3,416	5,967	7,116	5,798	6,069	5,777
Crustaceans				404	607	274
Grand total	28,581	57,352	81,133	97,270	90,876	105,815

* Total of other lakes, rivers and ponds.

Source: The study of integrated regional development master plan for the Lake Basin development area by Japan International Co-operation Agency, (1987)

acquired by fish and shellfish from their aquatic environments although some can be due to contamination after catch.

1.2 VIBRIO PARAHAEMOLYTICUS

Vibrio parahaemolyticus, the causative agent of acute gastroenteritis (Miwatani and Takeda, 1976) is now a very important organism in fish production because of outbreaks of food-poisoning that it causes. It was first isolated by Fujino et al. (1951) in Japan following an outbreak of gastroenteritis involving 272 people with 20 cases. However, its importance was not realised until a second outbreak occurred in 1956. From then on, it was recognised as a food-poisoning bacterium in Japan where Kudoh et al. (1974) indicated that from 1963 to 1974, it was responsible for 62.2% of all the bacterial food poisoning in Tokyo.

Occurrence of V. parahaemolyticus in certain marine animals and environments or its isolation from human patients has been reported in many parts of the world such as Germany (Nakanishi et al., 1968), U.S.A. (Molenda et al., 1972), India (Chatterjee et al.,1970), Togo (Bockemuhl et al., 1972) and in East Africa (Binta et al., 1982; Mhalu et al., 1982 and Ijumba, 1984).

Following the first reported clinical cases of V. parahaemolyticus infection in man, extensive research has been done on the isolation and characterization of the organism. It has been grouped amongst the halophilic marine vibrios, (Miwatani and Takeda, 1976). Work done on this organism has chiefly concentrated on isolating it from seafish, shellfish and sea waters as

it requires salt for its survival and growth.

However, recent literature has shown that despite their halophilic nature, these organisms do occur in freshwaters (Nair et al., 1986). It has been found that people living around such areas do carry the organisms in their gastrointestinal tracts. Studies on acute diarrhoeal diseases in those areas have shown that gastroenteritis caused by V. parahaemolyticus ranks second to cholera in terms of incidence (Chatterjee et al., 1970) In Kenya, Waiyaki et al. (1986) reported the isolation of this organism from fresh water streams and ponds in western Kenya in routine search for enteropathogens from community water sources. However, its existence in Kenyan lakes, where about 90% of total fish in Kenya is caught (Japan International Co-operation Agency Technical Report, 1987), has not been reported.

1.3 AEROMONAS HYDROPHILA

Aeromonas hydrophila is now recognised as a primary pathogen of humans, other mammals and cold-blooded animals capable of causing enteric and extra-intestinal infections in man (Holmberg and Farmer, 1984). It was first described by Zimmerman in 1890. It was, however, not until 1955, when Caselitz established human pathogenicity of Aeromonas strains which he called Vibrio jamaicensis, that medical

researchers became particularly interested in it.

It has now been established that some strains of Aeromonas actually cause enteric disease in immunocompromised patients (Gracey et al., 1982) as well as immunocompetent individuals (Chakraborty et al., 1986). These organisms can also cause extraintestinal infection, either independently (Joseph et al., 1979; Janda and Brenden, 1987) or in association with chronic conditions like liver disease and cancer (Harris et al., 1985).

Aeromonas species are ubiquitous water-borne organisms (Schubert, 1974) such that they can be recovered from most aquatic environments and fish. In Kenya, it has been isolated from Lake Victoria by Gathuma and Kaburia (1986). However, there has been no literature on it in respect to its enterotoxigenicity, neither has there been any report on clinical cases of disease due primarily to this pathogen.

The present study was undertaken for the following objectives:

i. To investigate the bacterial load by total bacterial count of fish, shell fish and water samples from various lakes in Kenya.

ii. To isolate V. parahaemolyticus and A. hydrophila group from fish, lakes and coastal waters of Kenya.

iii. To characterise the isolates by studying their toxin production, antimicrobial susceptibilities, plasmid profiles and antigenicities.

LITERATURE REVIEW

2.1 TOTAL BACTERIAL COUNT

Fish has been said to be more perishable than most other protein foods (Burgess and Shewan, 1970) such that even at chilling temperatures (0°C) rapid spoilage does occur in wet fatty fish. In lean fish, however, spoilage at this temperature is slow, but becoming progressively faster with increase in temperature. Although chemical reactions (oxidation and endogenous enzymic activities) also contribute, the dominant role in the process of spoilage is played by bacteria (Laycock and Regier, 1971).

In eviscerated whole cod stored on melting ice, autolytic enzyme reactions predominate for 4-6 days after which the products of bacterial activity become increasingly evident. Undesirable odours and flavours begin to appear, mostly resulting from bacterial metabolism of extractives (Hobbs, 1983). As spoilage proceeds, there is a gradual invasion of the flesh by bacteria from the outer surfaces. This was demonstrated histologically by Shewan and Murray (1979) who concluded that it was a slow process and that the development of objectionable odours and flavours resulted mainly from bacterial activity in the surface slime and in the integuments of the muscle. This agrees with the findings of Herborg and Villadsen

(1975) who established a direct correlation between organoleptic score and bacterial counts, particularly when the initial counts are higher than 100 colony forming units/g.

It is generally accepted that, when alive and healthy, the flesh of all fish is sterile (Shewan and Hobbs, 1967). The microbial flora developing at postmortem arises primarily from that present on the outer surfaces and in the intestinal tract of the live fish. Shewan and Hobbs (1967) also concluded that the natural flora of marine fish reflects, to a larger extent, that of its environment and feeds.

Both the species of fish and the method of catching may also affect the bacterial load. In a 27-month study, Liston (1956) found that the bacterial population of sole and skate caught in the same area vary considerably. With regards to method of catching, Shewan (1949) has shown that trawled fish usually carry loads 10 to 100 times heavier than lined fishes. The increased load in trawled fish is probably thought to be due to dragging along the sea floor, where the mud is known to harbour a lot of bacteria.

The initial bacterial flora of freshly caught marine fish mainly belong to the gram-negative genera and in addition freshwater fish usually have species of Aeromonas, Lactobacillus, Alcaligenes and Proteus (Frazier and Westhoff, 1978). While all these are inherent flora, species of the genera Salmonella,

Streptococcus and Escherichia may be acquired through contamination of the landed fish due to unhygienic handling of the fish and bacterial proliferation during storage.

Work done by Hobbs and Hodgkiss (1982) established that the total number of bacteria growing at 0-20°C increase systematically during storage in ice and that the gram-negative genera of Pseudomonas, Alteromonas Moraxella and Acinetobacter become increasingly predominant so that at the end of the shelf-life, they comprise most of the flora (see Table II). This has established the numerical significance of these genera in the spoilage process. However it has been noted that not all the types of bacteria found in fish bacterial flora contribute to the process. For example, Shewan and Murray (1979) reviewed a number of experiments where sterile muscle had been inoculated with pure cultures of bacteria. Moraxella and Acinetobacter strains did not produce typical spoilage odours and flavours whereas Pseudomonas and Alteromonas strains did.

Herbert et al. (1971) attempted to identify and enumerate the so called "active spoilers" rather than the total number of bacteria so that it could be used as an index of spoilage. Although some progress was made in identifying which components of the bacterial flora produce the spoilage changes, none has resulted into a practical method that can be used as an index.

TABLE II Changes in the flora of the skin of cod stored in melting ice.

	Counts after storage in melting ice for (days)							
	0		5		10		13	
	P	N	P	N	P	N	P	N
Pseudomonas/Alteromonas	26	24.49	33	162.49	84	11,088	82	45,805
Moraxella/Acinetobacter	33	31.09	26	128.18	7	924	13	7,262
Flavobacterium/Cytophaga	0	0	8	39.44	0	0	0	0
Coryneform bacteria	25	23.55	12	59.16	8	1,056	3	1,676
Micrococcus	14	13.19	21	103.53	0	0	0	0
Unidentified	2	1.88	0	0	1	132	2	1,117
Total count		94.20		49.3		13,200		55,860

P - percentage of total flora.

N - Number x $10^3/cm^2$ of skin

Source: Data from Shewan et al. (1960)

Other workers therefore suggested the use of accumulation of various chemical compounds in fish (Fields et al., 1968; Connel and Shewan, 1980). Most of these compounds are by-products of bacterial growth produced from extractive fractions. Amongst the substances produced, trimethylamine has been associated with bacterial spoilage of fish and used been used for some time, as an index of fish quality (Connel and Shewan, 1980). Most fish have considerable amount of trimethylamine oxide which is reduced directly to trimethylamine by bacteria. It has been shown that Alteromonas putrefaciens was mainly responsible for this reaction (van Spreekens, 1977) although other species of Alteromonas are also involved (Gibson et al., 1977).

Easter et al. (1982) showed that many spoilage bacteria, including A. putrefaciens are able to utilize trimethylamine oxide as a terminal hydrogen acceptor when oxygen becomes depleted. Thus the nonfermentative bacteria of the pseudomonad group are able to grow rapidly under the micro-aerophilic or anaerobic conditions that exist in spoiling fish tissues. The high level of trimethylamine oxide in marine fish was therefore offered as one of the reasons why they spoil more rapidly than freshwater fish and other animal flesh. Also the existence of lysozyme in the skin of certain species of fish (Murray and Fletcher, 1976) is

thought to prevent or retard bacterial invasion. Thus those species with lysozyme like plaice keep longer than those without it, e.g. cod, under the same storage conditions.

Because of differences in the microbial flora of tropical and temperate or cold waters, it has been reported that fish caught in tropical waters have longer shelf-life in ice than similar species caught in temperate or cold waters (Shewan, 1977). One reason advanced is that tropical fish do not carry a predominantly psychrotrophic bacterial flora while temperate fish do. Yet under chilling storage conditions, the same spoilage flora eventually emerge.

Spoilage process can be modified by handling and processing. Various methods of processing like heating, freezing and cold storage which aim at eliminating or limiting microbial activity, may be used. Other processes which extend shelf-life of fish include reduction of water activity by salt curing and drying or smoking and reduction of pH by fermentation (Hobbs, 1983). However, handling and processing especially if carried out under unhygienic conditions can introduce bacteria, including those that cause food poisoning.

The use of total bacterial counts as a measure of fish quality and hygienic standard has been controversial. Its major limitations have been that

very few genera of the bacterial flora actively cause fish spoilage (Herbert et al., 1971) and the initial total counts immediately after catching the fish can be quite variable, being influenced by the species and method of catch. Moreover, different values may be obtained when the total counts are done under different temperatures. Another reason is that other parameters such as total amount of volatile bases (TVB) can be used (Disney et al., 1975). Nevertheless, it has practical values and can easily be performed such that it is being increasingly applied (Shewan, 1977).

As the bacterial flora varies from one place to another, and between fresh water and marine fishes, it has been difficult to establish an internationally accepted bacterial standard counts (Disney et al., 1975). However, various workers have suggested certain general level of bacterial load to warrant fish to be acceptable for consumption. Amu and Disney (1973) reported a value of 10^5-10^8 organisms per gramme of flesh as a limit of acceptability while Disney et al. (1975) gave a value as low as 10^3-10^5 beyond which the fish should be declared inedible.

In Kenya, previous fish hygienic standards based on total counts have been done on fish from the coastal waters (Binta, 1978) and Lake Turkana (Gjerstad et al., 1987). Compared with the values obtained by Shewan (1949), Binta's (1978) counts were higher. In fresh marine fish sold at the city market, her counts ranged

from as low as $1.4x10^5$ to as high as $1.6x10^8$ and yet the fish was considered quite edible, whereas Shewan (1949) found that the initial counts were between 10^2 to 10^3 increasing gradually to maximum values of about 10^7 to 10^8 per gramme of muscle after about 10 to 12 days of storage on ice, at which time the fish was clearly stale and inedible.

2.2 VIBRIO PARAHAEMOLYTICUS

2.2.1 HISTORICAL REVIEW AND EPIDEMIOLOGY

Attention to the fact that V. parahaemolyticus is a human enteric pathogen was drawn in 1951 when Fujino and co-workers reported on a Shirasu food poisoning outbreak in Japan. This was later confirmed when a similar outbreak occurred in 1956 (Takikawa, 1956). From then on many investigators have examined the occurrence of this organism in natural environments and human patients. Disease outbreaks caused by this organism have subsequently been reported in many parts of the world, commonly associated with seafood (Miwatani and Takeda, 1976; Lawrence et al., 1979; Blake et al., 1980; Mhalu et al., 1982; Sarkar et al., 1983).

The epidemiology of this disease is influenced by a number of factors. Water temperature is an important

one in governing growth and survival of V. parahaemolyticus in the marine environment (Barrow and Miller, 1976). The number rapidly declines below 15°C and can hardly be isolated except in small numbers below 10°C. These authors found that Kanagawa positive as well as Kanagawa negative strains both survive well below 25°C in autoclaved seawater but at 37°C, the Kanagawa positive (K^{+ve}) strains lost their ability to cause β-lysis in Wagatsuma medium and would thus be regarded as Kanagawa negative (K^{-ve}). They, however, retained this ability below 25°C, although at this temperature K^{-ve} organisms were able to grow better. This evidence was further supported by the isolation, during seafood examination of several strains which were K^{-ve} on primary isolation at 37°C but rapidly became negative after subculture (Barrow and Miller, 1976). Examination of multiple colonies from primary cultures of various seafoods have, however, enabled the detection of occasional true K^{+ve} colonies, usually among multiple K^{-ve} serotypes (Peffers et al., 1973; Wagatsuma, 1974).

Marked seasonality of the disease has been noted in the USA (Barker, 1974) and Japan (Miwatani and Takeda, 1976). In these areas, these authors observed that outbreaks of the disease occurred almost exclusively during the warm summer and early fall months. This

seasonal variation of V. parahaemolyticus gastroenteritis therefore tallies with increased prevalence of the organism in the environment during warm weather and also enhanced opportunity for the organism to multiply in unrefrigerated food during the summer months (Blake et al., 1980).

Barker (1974) reported that several mechanisms, like keeping contaminated raw seafoods unrefrigerated for a long time and failure to cook foods at temperatures high enough, to kill vibrios, contribute to V. parahaemolyticus associated food poisoning.

Transmission from man to man has not been reported. However, Hobbs (1983) suggested that sewage from patients may pollute the coastal waters and keep a cycle of man-water-seafood-man in circulation until some other factors like variation in temperatures discourage the bacteria from growth in food and survival in water. Deb (1975) found many human carriers of V. parahaemolyticus in Calcutta throughout the year and implied their role in transmission of the pathogen. However, this was disputed by Blake et al. (1980) who pointed out that there is no evidence that infected foodhandlers have been a source of the organism in disease outbreaks. They, therefore, asserted that gastroenteritis caused by this organism is exclusively due to consumption of contamined food, usually raw or cooked seafood, although sometimes other

food presumably cross contaminated by raw seafood have been thought to transmit the infection. However, Sircar et al. (1979) reported in an interview of 60 patients with V. parahaemolyticus associated gastroenteritis, that one third denied having eaten fish or shellfish during the previous seven days, suggesting that other hitherto unknown, mechanism of transmission may exist.

The infective dose needed to cause illness has been determined in volunteers to be about 10^5-10^7 organisms (Sanyal and Sen, 1974). Although this appears to be rather large, the generation time of V. parahaemolyticus has been reported to be as short as nine minutes under ideal conditions (Aiiso, 1967) which enables the organism to multiply very rapidly in mishandled foods and quickly attain the ID50. The incubation period, therefore, can be correspondingly as short as 20 minutes or upto 9 hours depending on the number of organisms ingested (Hughes et al., 1978).

Clinically, this organism causes intestinal (Miwatani and Takeda, 1976) and extraintestinal (Twedt et al., 1969) infections in humans. Two clinical syndromes have been described for patients with V. parahaemolyticus associated gastroenteritis (Blake et al., 1980). The cardinal symptom of the commonly described syndrome is watery diarrhoea. The clinical manifestations of this type has been described as being diarrhoea, abdominal cramps, nausea, vomiting, headache, fever and chills; temperatures rarely exceed

38.9°C (Barker, 1974). This type is usually mild and self-limiting, with a medium duration of three days (Blake et al., 1980). However, severe cases with dehydration, hypotension and acidoses have been reported (Mazumdes et al. 1977).

The other syndrome described is a dysenteric syndrome which is characterized by mucoid or sanquinous stool. This type has been reported in 25% of the 60 patients hospitalised with V. parahaemolyticus associated gastroenteritis in India (Sircar et al., 1976) and six out of eight cases of an outbreak of the disease in Bangladesh (Hughes et al., 1978).

Vibrio parahaemolyticus associated gastroenteritis is usually mild or moderate, although severe cases requiring hospitalisation do occur (Blake et al., 1980). It can however be fatal. In Japan, the first reported outbreak (Fujino et al., 1951) had a fatality rate of 7% and subsequent cases reported between 1955 and 1974 had a rate of 0.04% fatality (Miwatani and Takeda, 1976). Most patients do not require antimicrobial therapy but severe and protracted cases may benefit from treatment with tetracycline (Farmer et al., 1985).

The first published indication that V. parahaemolyticus might cause extraintestinal infection was by Twedt et al., in 1969. Thereafter, a number of cases were reported by many investigators and although some of them were later proved to have been caused

actually by Vibrio vulnificus (lactose-fermenting vibrio) others were indeed authentic cases of V. parahaemolyticus infections (Blake et al., 1980; Farmer et al., 1985).

These extraintestinal infections may take the form of septicaemia (Tay and Yu, 1978), synovitis as a consequence of synovial puncture (Porres and Fuchs, 1975), wound infections progressing into gangrene and endotoxic shock (Rowland, 1970). Eye infections due to this organism such as panophthalmitis (Tacket et al., 1982) and endophthalmitis (Stenkuller et al. 1980) have been known to occur. Direct exposure to contaminated water seems to be the mode of transmission for all these extraintestinal infections.

2.2.2 ECOLOGY

Vibrio parahaemolyticus belongs to a group of halophilic vibrios, both psychrophilic and mesophilic (Barrow and Miller, 1976) and is present in coastal and estuarine waters, sediments and planktons as well as on and in marine fishes, crustaceans and molluses in many parts of the world (Thompson et al., 1976; Sayler et al., 1976). Baross and Liston (1970) observed that these vibrios prefer coastal waters to deep ocean waters because of the high organic content of inshore waters.

An annual cycle of V. parahaemolyticus in the estuarine environment was proposed by Kaneko and Colwell (1973). In their concept, the vibrio survives during winter in low numbers in the sediments. In late spring and early summer, they attach to zooplanktons, proliferating in the water with the rise of temperatures. During summer, the total bacterial population associated with zooplankton consists almost entirely of Vibrio species; the bacteria are predominantly on external surfaces of the zooplanktons.

The vibrios are capable of chitin digestion and Baumann et al. (1980a) demonstrated that all pathogenic Vibrio species elaborate an extracellular enzyme called chitinase which is chitinolytic. They also concluded that the organisms play an important role in the mineralization of the zooplanktons. During this mineralization process, the decaying plankton releases the vibrios which thus become free bacterial cells, actively swimming in the water during winter.

Under conditions of starvation, there is an increase in all numbers of marine vibrios as a survival strategy (Dawson et al. (1981). It was further suggested that these starved bacteria benefit from association with solid surfaces because of the accumulation of potential nutrients in the form of ions and macromolecules at solid-liquid interfaces (Baier, 1980). However, Karunasagar et al. (1986) observed that even without starvation, it is possible

under conditions of low temperature stress, that some factors present on the surface of chitin flakes stimulate the cells to multiply.

Kaneko and Colwell (1975) stated that efficiency of adsorption of vibrios onto chitin particle and crustaceans was dependent on pH and on concentration of sodium chloride and other ions found in seawater. Because of the role of sodium chloride in adsorption of this halophile on planktons, Natarajan et al. (1979) considered salinity of the water to be the principal parameter governing the distribution and abundance of V. parahaemolyticus and allied organisms in marine environs. However, very high salt concentration has adverse effect on this moderate halophile. Kaneko and Colwell (1975) demonstrated that very high salinity reduces the attachment efficiency of V. parahaemolyticus and other chitinoclastic vibrios to zooplanktons. Kaneko and Colwell (1975) considered the adsorption phenomenon to be one of the major factors determining the geographical and seasonal distribution and affecting the annual cycle of V. parahaemolyticus in the temperate estuary systems. Recently, there have been reports of isolation of this halophilic organism from freshwater fishes and environs which appears ecologically foreign (Sayler et al., 1976; De et al., 1977). This has led investigators to study its ecology in this apparently alien environment.

Sarkar et al. (1983) concluded that it does not form a part of the autochthonous microflora of freshwater ecosystems. However, the survival of the cells introduced by infected humans into freshwater systems appear to be prolonged by their association with freshwater plankton. Further study by Sarkar et al. (1985) confirmed the theory of adsorption onto planktonic substrate as a means of survival. They, however, found that this is also temperature related, occurring largely during summer months. In the post-monsoon and winter months, they could hardly isolate the organism from plankton, water or sediment samples. During the winter months, although the salinity profile remained relatively constant, V. parahaemolyticus was below detectable levels, apparently because the low water temperatures excludes even a transient survival of the organism in the freshwater environments. During summer months, despite extremely low salinity, water temperature is within the optimal limits, which perhaps aids the survival of the organism in the environment, particularly in association with plankton. These authors, therefore, considered adsorption onto plankton as a process which prolongs the survival of V. parahaemolyticus in the freshwater environment by confirming some kind of hitherto unkown protection.

Recent studies on the attachment of Vibrio cholerae to copepods have indicated the possibility that live

copepods may exercise growth-promoting or chemical attractant compounds which might enhance the attachment of chitinolytic vibrios to copepods (Huq et al., 1983). Furthermore, the association of V. cholerae with chitin has been found to confer resistance to acid pH on this organism (Nalin et al., 1979). Sarkar et al. (1986) therefore thought that the same mechanisms may apply to V. parahaemolyticus

They observed that although V. parahaemolyticus organisms might be released during the mineralization and disintegration of the planktonic substrate, they do not survive long in water or sediment in freshwater areas. Therefore, despite the presence of the organism in association with plankton and fishes of freshwater environs, no kind of flux of cells within the heterophilic niches could be conceivable.

From their experiments, Sarkar et al (1986) concluded that irrespective of their origin, (marine or freshwater) fishes provide an ideal substrate for the survival and proliferation of V. parahaemolyticus. Also unlike that in marine environment where sediment provide resevoir of V. parahaemolyticus. in fresh water environs, it is fishes and perhaps other acquatic animals that are reservoirs. The gastrointestinal tracts of freshwater fishes, in particular, provide unique microcosm for proliferation of V. parahaemolyticus in freshwater areas.

Another possible explanation of how V. parahaemolyticus can manage to survive in freshwater environs was advanced by Baumann et al. (1984). They reported that unlike other marine bacteria, V. parahaemolyticus and V. cholerae have their quantitative requirements for Na^+ for growth varying with the substrate serving as the carbon and energy source in the medium. This could imply that under certain specific nutrient conditions, the sodium ion (Na^+) requirement of V. parahameolyticus is not mandatory and that the halophile can well survive in conditions where the salt concentration may be equal to physiological concentration.

2.2.3 CLASSIFICATION AND MORPHOLOGY

Vibrio parahaemolyticus is known to belong to the order Eubacteriales, family Vibrionaceae, genus Vibrio and species V. parahaemolyticus. According to Baumann, et al. (1984), within a genus, a species comprises strains which are phenotypically similar and readily distinguishable from other species, genotypically similar in having DNA homologies of over 80% and closely related on the basis of similarities in the amino acid sequences. However, relatedness amongst the species of the genus Vibrio is not close in a phylogenetic sense (Brenner et al., 1983; Baumann and

Schubert, 1984), thus it is considered to be a heterogenous genus, similar to the Pseudomonas (Farmer et al., 1985).

On the basis of DNA relatedness, Brenner et al (1983) grouped Vibrio species into clusters. Species that cluster at 40% or more relatedness are: V. cholerae - V. mimicus; V. ordalli - V. anguillarum; V. fluvialis - V. furnissi; V. parahaemolyticus - V. alginolyticus - V. Campbelli - V. harveyi. If 30% or more relatedness was used to group species, they added V. natriegens, V. pelagius, V. splendidus, V. nereis and V. vulnificus to V. parahaemolyticus cluster. They suggested that this large cluster of V. parahaemolyticus group could in future be a candidate for a new genus.

Vibrio parahaemolyticus is a gram-negative pleomorphic (short or long, straight or curved) motile rod, about 0.5 - 0.8 um in width and 1.4-2.6 um in length with an ultrastructure typical of most other gram-negative bacteria (Baumann et al., 1984). In some strains, cell curvature is more pronounced in early stationary phase in liquid media than during exponential growth. In late stationary phase or under adverse conditions, involution forms generally predominate in the culture (Baumann et al., 1984).

In liquid medium, the organism has a single, sheathed, polar flagellum with a wavelength of 1.4-1.8

um (Baumann et al., 1980b). When grown on solid medium, V. parahaemolyticus synthesises additional unsheathed lateral flagella (Yabuuchi et al., 1974). However, upon transfer from solid to liquid media, there is an immediate cessation of synthesis of these flagella (Shinoda and Okamoto, 1977). Belas et al. (1986) found that the synthesis of these flagella is regulated by lateral flagella gene (laf) which is operated by changes in the viscosity of the medium.

Because of the lateral flagella, most strains of marine vibrios are able to swarm on solid media. This swarming is associated with the formation of long cells with many lateral flagella and is affected by a number of chemical and physical parameters including concentration of the agar, complexity of the medium and temperature (Baumann and Baumann, 1977).

The colonial morphology of V. parahaemolyticus on thiosulphate citrate bile - salt sucrose agar (TCBS) is smooth, moist, circular and opaque green with a dark raised centre (Sakazaki, 1968). However, mixtures of smooth and rough textured variants having serrated edges, a raspberry centre and a dark green appearance have been reported by Twedt et al. (1969).

It can grow readily on most general purpose media with added NaCl as the growth is stimulated by Na^+. This requirement for sodium ions can be reduced by levels of Magnesium ions (50 mM) and Calcium ions (10 mM) such as are in seawater (Reichett and Baumann, 1974).

2.2.4 ISOLATION AND IDENTIFICATION

2.2.4.1 Isolation

Vibrio parahaemolyticus can be isolated from clinical materials in the acute stage of illness, and environmental samples.

2.2.4.1.1 Collection and Trasportation

Farmer et al., (1985) recommended that clinical materials such as stool, urine, blood and wound specimen should be collected during acute stages of the disease, preferably before any antibiotic administration. The recommended transportation medium is Cary-Blair medium which maintains the viability of vibrio cultures for upto 4 weeks (Farmer et al., 1985).

2.2.4.1.2 Enrichment

The commonest enrichment medium for this organism is alkaline peptone water (APW) which contains 1% (W/V) peptone and 1% (W/V) Nacl at pH 8.6 (Baumann et al., 1984). Other enrichment media include salt-colistin broth (Sakazaki, 1969) and glucose-salt-teepol broth (Sakazaki, 1973). Like alkaline peptone water, all these media depend partly on a high pH value for their selective action, and current trends suggest that this may be further enhanced by incubation at 43°C, by the addition of chitin, starch or mineral salts as well as by the use of fish based media (Barrow and Miller, 1976). Kampelmacher et al. (1972) found freshly

prepared meat broth with 6% Nacl an acceptable enrichment fluid for V. parahaemolyticus while Vanderzant and Nickelson (1972) proposed enrichment in trypticase soy broth with 7% Nacl and 18hrs incubation at 42°C.

2.2.4.1.3 Cultural Media

Many selective media have been described for the isolation of this organism either directly from specimens or after enrichment. Thiosulphate citrate bile-salt sucrose (TCBS) agar (Kobayashi et al., 1963) is the most suitable selective agar being easy to prepare for use, and highly selective. Various modifications of TCBS-agar have been recommended by other authors such as Sakazaki,(1973) and Beuchat (1977) in order to increase its ability to inhibit other bacteria.

After incubation at 37°C for 24 hrs, isolated colonies of V. parahaemolyticus on this medium, appear round 2-4 mm in diameter with blue-green centres (alkaline colour of indicators). In contrast, V. alginolyticus which is frequently present in seawater and seafoods form smooth, raised, yellow colonies, 3-4 mm in diameter, due to the acid formed by sucrose fermentation. Vibrio cholerae is also sucrose-positive and forms colonies similar to those of V. alginolyticus. Few other bacteria are capable of growing on TCBS-agar and can be differentiated from the

vibrios by colonial morphology, colour and opacity (Baumann and Schubert, 1984). These authors stated that since the yellow colour of colonies of V. alginolyticus and V. cholerae changes to green blue on prolonged incubation, plates should preferably be inspected after 15hrs incubation.

Another selective agar medium suggested by Sakazaki (1969) as an alternative to TCBS was Bromothymol blue-salt Teepol. After 24h incubation at 37°C on this medium, V. parahaemolyticus and V. alginolyticus form colonies similar to those on TCBS agar. A modified BTB-agar containing polymyxin B-sulphate and tylosin for higher sensitivity showed low productivity (Kampelmacher et al., 1970).

The above selective media have the disadvantage of selecting for V. parahaemolyticus and V. alginolyticus equally and especially in seawater samples containing large numbers of V. alginolyticus, it has the tendency of overgrowing V. parahaemolyticus thus making the separation difficult. As an alternative, Kourany (1983) introduced Triphenyltetrazolium chloride soya tryptone agar (TSAT) to enable easy differentiation of V. parahaemolyticus from V. alginolyticus. On this medium, differentiation is largely based on the colour change and size of the colonies; V. parahaemolyticus colonies are bright red and about 2-4 mm in diameter, whereas V. alginolyticus colonies are white and smaller

and occassionally have tiny pink centres. The author concluded that although it is slightly more inhibitory than TCBS, TSAT offers a definite advantage due to its more consistent differentiation qualities when used as a primary isolation medium for V. parahaemolyticus, especially in samples containing large numbers of V. alginolyticus and that it is of particular value in direct plating of seawater and other specimens from the marine environments.

Baumann et al. (1984) states that isolation of this organism from pathological material such as wound swabs, urine and blood does not present any problem and selective procedures are unnecessary as the causative agents usually grow luxuriously in almost pure culture on ordinary medium. Since some strains of V. parahaemolyticus swarm on complex solid media such that isolated colonies become difficult to obtain during cultivation, Reichett and Baumann (1973) suggested that this can be abolished or reduced by increasing the concentration of agar to 4% (W/V), or streaking the organism onto minimal medium.

2.2.4.2 Identification

Identification of characteristic colonies from the selective media can be done by biochemical methods. Biochemically, V. parahaemolyticus, like most other species of Vibrio is oxidase-positive, a property which correlates with the presence of cytochromes of the C

type (West et al., 1978). These authors asserted that the oxidase reaction is due to C type cytochrome which is soluble and has the capacity to bind carbon dioxide. and a, which occur in most species of Vibrios (Unemoto and Hayashi, 1979). According to West et al. (1978), Vibrio metschnikovii, the only oxidase-negative Vibrio species contain cytochromes b, d, o and a, but lacks cytochromes of the C type. The oxidase reaction is an important test in differentiating Vibrio species among colonies of Enterobacteriaceae in clinical specimens, as all the human vibrios are strongly oxidase positive, with the lone exception of V. metschnikovii (Farmer et al., 1985).

Another important test in differentiating the genus Vibrio from other genera is the vibriostatic agent O/129(2,4-diamino-6,7-di-isopropylpteridine phosphate) test (Merkel, 1972). This compound inhibits the growth of Vibrio species plus Plesiomonas and Photobacterium while allowing growth of Aeromonas species and all the genera of the family Enterobacteriaceae and Pseudomonadaceae. Other important biochemical characteristics of V. parahaemolyticus have been summarised by Barrow and Miller, (1976).

Farmer et al. (1985) reported that the majority of vibrio cultures isolated from clinical specimens could be easily identified as one of the following ten species: V. cholera, V. minicus, V. metschnikovii, V.

hollisae, V. damsela, V. fluvialis, V. furnissii, V. alginolyticus, V. parahaemolyticus, and V. vulnificus. They further listed six tests which could be used in dividing the 10 species into 5 groups as:- requirement for Na^+, oxidase, nitrate reduction to Nitrite, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase. The members of group 5 which includes V. parahaemolyticus could then be differentiated using the properties in Table III.

Urea-positive strains of V. parahaemolyticus

Until recently, V. parahaemolyticus was considered negative for urea hydrolysis. Sakazaki and Balows (1981) reported that none of their 2,354 strain was urea positive. However in the last few years, a number of strains which were otherwise typical of V. parahaemolyticus but were urea positive were isolated from acute gastroenteritis cases (Lam and Yeo, 1980; Oberhofer and Podgore, 1982). Farmer et al. (1985) stated that the number of urea-positive V. parahaemolyticus is increasing steadily such that 50% or more of strains from California and from outbreaks investigated by Centres for Diseases Control (CDC) were found to be urea-positive. They reported that these urea-positive strains were confirmed by DNA hybridization as V. parahaemolyticus.

TABLE III Differentiation of the Group 5 vibrios

Property	V. alginolyticus	V. parahaemolyticus	V. vulnificus
Fermentation of cellobiose	-	-	+
Lactose	-	-	(+)
Salicin	-	-	+
Zone sizes around			
Colistin	large	large	small
Ampicillin	small	small	large
Carbenicillin	small	small	large
Growth in			
8% NaCl	+	+	-
10% NaCl	V	-	-
Voges Proskaeur	+	-	-
Sucrose fermentation	+	-	(-)
L-Arabinose fermentation	-	(+)	-

Source: Vibrio from Manual of Clinical Microbiology, 4th ed., 1985.

+ = almost all positive, usually 90% or more.

(+) = most strains positive, usually 75 to 89%.

(-) = few strains positive, usually 11 to 15%.

- = almost no strains positive 0 to 10% positive.

2.2.5 PATHOGENESIS

2.2.5.1 General consideration

The ability of V. parahaemolyticus to cause haemolysis on Wagatsuma agar (the Kanagawa phenomenon) was associated with gastrointestinal illness by Sakazaki et al. (1968). They observed that 96.5% of isolates from patients with diarrhoea were K^{+ve} while less than 1% of the isolates from sea-foods and seawater were K^{+ve}. Similar results were obtained by Sircar et al. (1979) and De et al. (1977).

Vibrio parahaemolyticus isolates from extraintestinal infections tested at Centres for Disease Control, USA have been found to be all K^{-ve} (Blake et al., 1980). The only extraintestinal K^{+ve} isolate was from a patient with septicaemia whose illness began with diarrhoea (Tay and Yu, 1978).

Ironically, the source of intestinal infection for humans is from seafoods and the environment and yet there is the apparent non-identity between stool and food or environmental isolates. Various explana-tions have been advanced by investigators as to why environmental isolates are mostly K^{-ve}, whereas clinical ones are K^{+ve}. One is that there may be a failure of laboratory studies to detect pathogenic strains (K^{+ve}) which are actually present in the food, but are obscured by the presence of many non-

pathogenic strains (K^{-ve}) (Barker et al., 1974). Furthermore, there is a suggestion by Sakazaki et al. (1974) that while in the human gut, K^{+ve} strains multiply more rapidly than K^{-ve} ones, thus outgrowing them. This suggestion agrees with the experimental evidence obtained by Barrow and Miller (1976) from growth studies in digest broths prepared from various fish and crustacea. They found that when they inoculate approximately equal numbers of a K^{+ve} and K^{-ve} strains, conditions simulating the alimentary tract, the number of K^{+ve} organisms greatly exceeded that of K^{-ve} organisms within 12 hrs. The presence and survival of K^{+ve} strains in the environment was recently reported by Kumazawa and Kato (1985). They observed that these haemolysin producers are preserved principally in sediments and some shellfish and may be difficult to pick in routine survey.

Another reason put forward is that ingested K^{-ve} organisms may undergo transformation with regard to their genetic and antigenic structure hence the capacity to produce Kanagawa haemolysin during passage through the intestinal tract (Barker et al., 1974). Such transformation might be mediated by bacteriophages or by effects of the chemical millieu of the intestines upon the organisms. Since it was suggested that genetic exchange through plasmids might occur among

marine vibrios in their natural habitats which might include the transfer of virulence factors (Baross *et al.*, 1978), it is highly probable that the same exchange could occur in the human alimentary tract, not only between *V. parahaemolyticus* and other vibrio species but also between *V. parahaemolyticus* and the *Enterobacteriaceae* group which are normal resident microflora, especially *E. coli* (Barker and Gangarosa, 1974).

Another possible explanation was advanced by Burstyn *et al.* (1980) who suggested that the interconversion of K-phenomenon may be related to transposable genetic elements like insertion sequence (IS) and transposons (Tn). It was found in *E. coli* that some galactose and lactose mutants are due to the integration (IS) elements into the respective *gal* and *lac* operons (Kleckner, 1977). Excision of these elements leads to restoration of the wild-type gene. Insertion of (IS) elements tends to occur at specific loci, with some sites being hot spots where integration is favoured over other sites (Pfeifer *et al.*, 1977). Burstyn *et al.* (1980) therefore speculated that if insertion sequence prove to be the explanation for phenotypic conversion, then *V. parahaemolyticus* would possibly have insertion sites in genes responsible for TDH on the chromosomes such that insertion and excision of the insertion sequence elements may lead to the

expression of the hemolysin gene.

The actual mechanism of pathogenesis of this organism still remains debatable, despite the excellent correlation between TDH and human disease, since other toxins or virulence factors may be involved (Farmer et al., 1985). Furthermore, it has been found that some strains of both K+ve and K-ve isolates could cause dilation and inflammation of ligated rabbit ileal loop (Sakazaki et al., 1974). Donta and Smith (1974) found that filtrates from cultures of V. parahaemolyticus strains from patients with gastroenteritis did not effect either morphologic changes or steroidogenesis in Y-1 adrenal cells. However, Honda et al. (1976b) detected a heat liable factor, from a culture filtrate of a K+ve strain that causes morphologic changes in Chinese hamster ovary (CHO) cells similar to those caused by cholera toxin and heat-labile E. coli enterotoxins. This heat-labile factor has been suggested to be responsible for the typical watery diarrhoea syndrome (Hughes et al., 1978).

The second syndrome of bloody diarrhoea and systemic reaction caused by V. parahaemolyticus has been attributed to invasion of the intestinal tissue of humans (Boutin et al., 1979). Polymorphonuclear leukocytes and blood in the stools of patients (Hughes et al., 1978) and ulceration of the

rectosigmoid (Bolen et al., 1974) have been observed during episodes of such syndrome.

Carruthers (1975) observed that while both K^{-ve} and K^{+ve} V. parahaemolyticus can cause cytotoxic effects on the HeLa cell cultures, K^{+ve} organism act more rapidly; and non-viable preparations have no effect on HeLa cells. Invasiveness in suckling rabbits (Calia and Johnson, 1975) and cytotoxicity of TDH to human cells from allantoic membrane in cell cultures (Sakurai et al., 1976) suggest that cytotoxicity with tissue destruction and invasion may occur under some circumstances in man resulting in the bloody diarrhoea syndrome (Hughes et al., 1978). This was further supported by work of Boutin et al., 1979, who reported that the bloody swollen adult rabbit ileal loops inoculated with K^{+ve} strains were comparable to severe gastroenteritis in humans.

2.2.5.2 Toxins Produced

2.2.5.2.1. Thermostable Direct Haemolysin

Among the exotoxins produced by V. parahaemolyticus, the thermostable direct hemolysin (TDH) associated with Kanagawa positivity, has been extensively studied (Honda et al., 1980). Honda et al. (1976a) identified, purified and determined the physicochemical properties of this toxin. They described it as a simple protein with a molecular

weight of about 42,000 daltons. It has marked cardiotoxicity in mammalian heart. Low doses stop the spontaneous contraction of cultured mouse and rat heart cells while high doses cause degeneration of cell shape (Goshima et al., 1977).

This cardiotoxicity is prevented by preincubation of the haemolysin with neurominidase-sensitive ganglioside, (Honda et al., 1976c) suggesting that these gangliosides may be intestinal mucosal receptors for the cytotoxic haemolysin. However, doubts about this were raised by Honda et al. (1985) because their ganglioside ELISA failed to detect TDH.

2.2.5.2.2 Thermolabile Haemolysin

The thermolabile haemolysin (TLH) is one of the toxins elaborated by V. parahaemolyticus organism and can be detected in culture filtrates of Kanagawa positive strains (Honda et al., 1976b). These authors reported that this TLH causes morphologic changes in Chinese hamster ovary (CHO) cells similar to those caused by cholera and heat-labile E.coli enterotoxins. Hughes et al., (1978) suggested that this toxin might induce the typical watery diarrhoea syndrome.

2.2.5.3 Kanagawa Reaction

Kanagawa reaction is a test done on strains of V. parahaemolyticus to determine the ability of the strains to produce a haemolysin for human group 'O'

erythrocytes when it is grown on a Wagatsuma medium (Farmer et al., 1985). K^{+ve} strains produce clear zones of beta-haemolysis on agar plates whereas K-ve strains produce alpha-haemolysis or discolouration of red blood cells under the growth (Wagatsuma, 1968). A TDH believed to be an exotoxin, mediates the haemolytic reaction on Wagatsuma agar, and it is said to be partially responsible for the human pathogenicity (Obara et al., 1967).

The Kanagawa reaction of V. parahaemolyticus is routinely determined on Wagatsuma agar (Nair et al., 1985). However, false positive haemolysin reactions occasionally occur owing to pH changes around the colonies, fragility of erythrocytes, or haemolysis caused by haemolysins other than the TDH (Honda et al., 1980). To overcome the variables associated with Wagatsuma agar, Honda et al. (1980) developed a purportedly refined immunological technique of the modified Elek test (Elek, 1948) and the immuno-halo test. These immunological techniques were further modified by Honda et al. (1982) by using media which can select for V. parahaemolyticus to make it possible to isolate the organism and at the same time identify the K-phenomenon on a single plate. The selective media they used were BTB-teepol agar and modified arabinose-ammonium sulphate chocolate agar.

Another method for detection of TDH, hence the K-

phenomenon was recently developed by Honda et al. (1985) using Enzyme Linked Immunosorbent Assay (ELISA) methods. They tested both direct and sandwich ELISAs. They found that of the two methods, sandwich ELISA was better. Compared with the modified Elek and others discussed previously, they found that ELISA methods were far more superior for detection of TDH as some K^{-ve} strains judged by modified Elek test were K^{+ve} by ELISA. They therefore concluded that most of the isolates, previously labelled K^{-ve} by other methods from clinical cases produce TDH which tend to support their theory that TDH is the only toxin of V. parahaemolyticus responsible for the pathogenicity of this bacterium.

2.2.6 PLASMIDS OF V. PARAHAEMOLYTICUS

Plasmids are of great importance to microbiologists as primary virulence factors because they can code for toxins and other proteins which directly increase the virulence of pathogenic micro-organisms and also be responsible for specific surface antigens permitting attachment of bacteria to host cells or as secondary factors because they confer antibiotic resistance on these pathogens of animals and man, thus enhancing their survival (Evans et al., 1975; Gemski et al., 1980; Toranzo et al., 1983). The presence of plasmids in V. parahaemolyticus was first reported by Guerry and Colwell in 1977. They found plasmid DNA in four of the

nine K^{+ve} isolates from patients while none of the three K^{-ve} environmental strains of V. parahaemolyticus tested had any. These workers were unable to assign phenotypic characteristics to any of the plasmids identified. They found no association between the presence of plasmid DNA and K-reaction, thus they called them cryptic. The possibility that TDH might be encoded on a plasmid whose loss would give rise to the K^{-ve} characteristics was investigated by Burstyn et al. (1980). They examined some K^{+ve} strains for the presence of covalently closed circle (CCC) DNA but could not detect any.

Further work on this subject was done by Twedt et al. (1981) who investigated the correlation of the presence of plasmid DNA with drug resistance, haemolysin production and indices of pathogenicity. They examined a total of 31 strains of V. parahaemolyticus. They were able to detect plasmids in only three clinical strains; two were K^{+ve} and the other one was K^{-ve} strain. All the environmental isolates and the rest of clinical strains did not have any plasmids. They concluded that plasmids do not appear to control the Kanagawa reaction.

2.2.7 ANTIGENIC STRUCTURE AND TYPING

Vibrio parahaemolyticus possesses three groups of antigenic components, O, K and H antigens. The current

antigenic schema for V. parahaemolyticus now comprises from O1 to O13 and K1 to K71 (Nair et al., 1985). The H-antigens of the different strains so far examined are regarded as serologically identical within the species, thus not useful in serotyping (Barrow and Miller, 1976).

The present typing scheme is based only on isolates from cases of human infection, and for this reason, the incidence of yet untypable isolates from marine sources and seafoods may vary considerably from one area to another (Barrow and Miller, 1976).

In making serological survey, it has been found that certain serotypes are isolated more frequently than others (Kudoh et al., 1974), although the actual serotypes vary with time and place. In a WHO Bulletin (1974), Chatterjee and Sen reported that their serotyping of isolates from different sources in Calcutta showed the following:- human diarrhoea isolates - 80.2% typable and 19.8% untypable, marine fish and water isolates 40.7% typable and 59.3% untypable and non-marine fish and water isolates 32.6% typable and 67.4% untypable. They therefore agreed with Barrow and Miller (1976) that environmental strains have higher percentage of untypable strains than human strains. Nair et al. (1985) indicated that the dominant serotype among diarrhoeal cases is O1:K56 which has not been isolated from the environment.

Conversely, the dominant environmental serotype O2:K28 was not recorded among serotypes recovered from either human diarrhoeal cases or carriers in Calcutta. Such a difference in serotypes of human and environmental origin was also observed in Togo (Bockemuhl and Triemer, 1975).

Nair et al. (1985) stated that the inability to isolate the same serotypes from patients and incriminated food items in the same outbreak in several epidemics also suggests that some differences exist between serotypes found in the environment and those associated with gastroenteritis. They found from their studies that similar serotypes occur in freshwater and brackish environs. However, they noted that even in areas where there is large-scale interaction between humans and environment and where incidence of V. parahaemolyticus gastroenteritis is high, the enteropathogenic serotypes remained elusive in the environment. They, therefore, concluded that some serotypes of V. parahaemolyticus can survive and proliferate better in natural environs than others.

The method commonly used for identification of K and O antigens in serotyping strains of V. parahaemolyticus is the slide agglutination test recommended by Sakazaki (1973).

The antigenic structure of the O-group has been described by Hisatsume et al. (1980) who showed that

the lipopolysaccharides of V. parahaemolyticus antigen 'O' do not have the 2-keto-3-deoxy-octonic acid in their polysaccharide core unlike other marine vibrios. The living vibrio is usually not O-agglutinable due to the presence of K-antigen on the outside of the cells (Baumann et al., 1984). The O-agglutinability may be improved by heating the cells for 1 hr at 100°C, as they are heatstable, or by washing centrifuged cells repeatedly with 1% saline.

K-antigens do not appear under routine cultural conditions in V. parahaemolyticus, although Chatterjee and Neogy (1972) reported having detected visible capsules. The organism possesses two kinds of flagella, lateral (L) and polar monotrichous (M) which differ morphologically, functionally and antigenically from each other (Shinoda et al., 1979). Antigenicity of M-flagellum is common among the genera Vibrio (Shinoda et al., 1976) while L-flagellar, which is divided into three serotypes of HL1, HL2 and HL3 are known to be species specific (Shinoda and Nakahara, 1977). This species specificity of H-antigens of L-flagella was used by Shinoda et al. (1983) for simplifying identification of V. parahaemolyticus.

2.3 AEROMONAS HYDROPHILA GROUP

2.3.1 HISTORIAL REVIEW AND EPIDEMIOLOGY

When Zimmerman first described Aeromonas in 1890, it was generally considered to be a pathogen of cold-blooded animals. This concept was maintained until the sixties when pioneer studies by Caselitz (1960) showed that this member of family Vibrionaceae could be a human pathogen.

It is now considered to be an important organism in intestinal infection (Holmberg and Farmer, 1984; Holmberg et al., 1986; Moyer, 1987); and hence its role in gastroenteritis has been reported from various parts of the world (Cumberbatch et al., 1979; Rahman and Willoughby, 1980; Figura et al., 1986; Sack and Lanata 1986; Kuijper and Peeters, 1986a).

Infection by this organism is thought to be as a result of a direct exposure to water harbouring the organism (Daily et al., 1981; Holmberg and Farmer, 1984). Infections of this type have been reported in divers operating in polluted waters (Joseph et al., 1979; Seidler et al., 1980) where open wounds become infected by the organisms. Seidler et al (1980) found that a short exposure to river water containing Aeromonas and coliforms altered the skin microfloral composition sufficiently to reflect the kinds of bacteria present in the water. This ability of Aeromonas to attach rapidly to skin surface is the

first step in pathogenesis. This adherence to skin surface is promoted by pilli (Joseph et al., 1978) and the production of adhesive factors (adhesin) in some strains of A. hydrophila (Krovacek et al., 1987).

Enteritis could also occur as a result of drinking contaminated water. Kuijper and Peeters (1986b) found that in 21% of 29 patients with diarrhoea due to A. hydrophila or A. sobria, there was an epidemiological association between the symptoms and recent contact with surface water. Rahim and Aziz (1986) also indicated the possibility that infection could be due to contamination after handling infected materials like fish and shellfish. Other foods of animal origin are also likely to be a significant potential source of the organism (Palumbo et al., 1986) as it has been isolated from products such as dairy products, poultry, and red meat (Pittock and Donovan, 1986; Palumbo et al., 1986).

Clinically, Aeromonas species have been known to cause intestinal (Burke et al., 1983; Goodwin et al., 1983; Agger, 1986) as well as extraintestinal (Joseph et al., 1979; Daily et al., 1981) infections. Acute gastroenteritis in both adults and children has been described ranging from watery to bloody diarrhoea of either short or prolonged duration (Rahman and Willoughby, 1980; Gracey et al., 1982; Aziz et al., 1986). A typically cholera-like illness due to A. sobria was reported by Champsaur et al. (1982) where

the clinical outbreak was indistinguishable from that of severe cholera.

Extraintestinal infection have been reported (Janda and Brenden, 1987; Kindschuh et al., 1987). The type of infections so far reported were listed by Freij (1986) as including sepsis, meningitis, cellulitis, necrotising fascitis, ecthyma gangrenosum, pneumonia, peritonitis, conjunctivitis, corneal ulcers, osteomyelitis, arthritis, myositis, liver abscess, cholecystitis, urinary tract infection, endocarditis, ear nose and throat infection, balanitis and vaginal infection.

2.3.2 ECOLOGY

The members of the genus Aeromonas have been considered autochtonous inhabitants of aquatic environments (Nishikawa and Kishi, 1987) and hence taken to be ubiquitous in this ecological system (Daily et al., 1981). They have been grouped conveniently into two for systematic and ecological reasons by Schubert (1967), that is, aeromonads of the hydrophila group comprising of the species A. hydrophila. A. sobria and A.caviae and aeromonads of the salmonicida group. The hydrophila group is normally present in all surface waters, but particularly abundant in waste water. It has therefore been suggested that it can serve as a water pollution indicator organism (Schubert, 1967; Schubert, 1975). Other investigators

have observed that the number of motile aeromonads often approached or exceeded E. coli in surface waters and sewage (Rippey and Cabelli, 1980; Seidler et al., 1980; Kaper, et al., 1981). Aeromonads of salmonicida group, the other hand, have mostly parasitic habits and are normally found in surface waters and in bottom sediments whenever certain diseases of fish are prevalent.

Aeromonas hydrophila is not generally considered to be a normal inhabitant of the human gut (von Graevenitz and Mensch, 1968; Figuraet al., 1986). Therefore a source other than human sewage appears to be responsible for the number of A. hydrophila found in estuarine environment (Kaper et al., 1981). They concluded that fish may act as a reservoir for this organism because the organism naturally parasitises on fish, forming part of the microflora of the gut.

Seidler et al (1980) and Kaper et al (1981) observed an inverse correlation between dissolved oxygen in the water and the incidence of Aeromonas species. They therefore proposed that A. hydrophila be used as an indicator of lake deterioration or recovery.

Lupo and coworkers (1977) showed that aeromonads which produced acid and gas from glucose gave false positive reactions in the test for coliforms; resulting in overestimation of the number of coliforms by two to 33-fold. The frequency of occurrence of inaccurate estimates of coliforms was greater in summer than in

winter.

Water temperatures have been found to affect significantly the population size of A. hydrophila such that bacterial counts were highest in summer and lowest in winter (Kaper et al., 1981). Aeromonas-induced infections in both fish and man were noted to occur mainly in the spring and summer months (von Graevenitz and Mensch, 1968; Hazen et al., 1978).

2.3.3. CLASSIFICATION AND MORPHOLOGY

The genus Aeromonas was proposed by Kluyver and van Niel (1936) to accommodate enteric bacteria-like microorganisms which were associated with fresh-water aquatic environments. In 1966, Veron grouped this genus under the family Vibrionaceae, order Eubacterioles. However, Colwell et al. (1986) argued that substantial molecular genetic evidence exists which suggests that species currently assigned to this genus possess a phylogenetic history sufficiently distinct from the histories of the families Enterobacterioceae and Vibrionaceae to warrant exclusion of the genus Aeromonas from the Vibrionaceae and creation of a new family Aeromonadaceae. However, this proposal has yet to be approved.

Two basic sub-divisions of the genus Aeromonas can be defined based on phenotypic differences and pathogenicity (Hickman-Brenner et al., 1987). The first

group is non-motile, does not grow at 35 to 37°C and is pathogenic for fish. This is the psychrophillic group known as Aeromonas salmonicida. The second group grows at 35 to 37°C, is usually motile, and has been isolated from human clinical specimens and other sources. This is the mesophilic group that is known as Aeromonas hydrophila (Janda and Brenden, 1987; Hickmann-Brenner et al., 1987). Strains isolated in clinical microbiology laboratories belong to this group (Sakazaki and Balows, 1981).

Although the classification of the motile aeromonads into species has been controversial (Ewing et al., 1961; Eddy and Carpenter, 1964; Schubert, 1969; McCarthy, 1975), Popoff et al. (1981) asserted that they could be divided into three species, that is, A. hydrophila, A. sobria and A. caviae.

Two new species of A. hydrophila group have recently been suggested. Allen et al. (1983), proposed A. media sp. nov. to be a distinct species isolated from river water, while Hickmann-Brenner et al. (1987) proposed A. veronii to be another distinct species.

Morphologically, these motile species of Aeromonas are Gram-negative cells which show considerable variation in shape and size. Popoff (1984) described some strains as short rods whereas others produce thin and filamentous forms. McCarthy (1975) observed that rare cells may exhibit a somatic curvature but this is by no means as marked as that shown by some Vibrio

species. Generally they are 0.3-1.0um in diameter and 1.0-3.5um in length. They can be arranged in singles, pairs or short chains.

Cells of the motile species possess a single polar flagellum, generally with a wave length of 1.7um. Most of these flagellated strains form lateral flagella in young cultures which have a shorter wavelength than the polar flagellum (Popoff, 1984); however, when incubated past the logarithmic phase of growth, cells exhibit only polar flagellum.

The optimum growth temperatures for motile Aeromonas species is 28°C. Some strains can grow at 5°C, while the maximum temperature at which growth occurs is usually 38-41°C (Popoff, 1984). Their colonial morphology on nutrient agar is round, raised, with an entire edge and smooth surface; which is transluscent and white to buff in colour. The culture odour varies from extremely strong to absent (McCarthy, 1975).

2.3.4 ISOLATION AND IDENTIFICATION

2.3.4.1 Isolation

2.3.4.1.1. Enrichment

Enrichment media that have been recommended for isolation of Aeromonas species are alkaline peptone water (Moulsdale, 1983; Millership and Chattopadhyay, 1984), nutrient broth (Robinson et al., 1986) and cold

enrichment (0°C) in phosphate buffered saline (Altwegg, 1986). Robinson et al. (1986) pointed out that stools from patients with acute diarrhoea should be plated directly on media for the isolation of Aeromonas species without enrichment as routine use of enrichment media would lead to recovery of Aeromonas species not in any way associated with diarrhoeal disease.

2.3.4.1.2 Culture media

Both ordinary and selective media can be used to isolate the motile Aeromonas species. Among the common non-selective media, nutrient, tryptic soya, blood and MacConkey agars could be effectively used (Popoff, 1984).

Various selective media have been proposed for the isolation of this organism. For stool specimens, blood agar containing ampicillin (Kay et al., 1985) has been used. However, it has been found to have low selectivity; in addition, ampicillin-sensitive Aeromonas strains have been described (Rahim et al., 1984). An alternative proposal was Celsulodin-Irgasan-Novobiocin (CIN) agar, a medium which allows simultaneous screening for Aeromonas as well as Yersinia species (Altorfer et al., 1985). Altwegg (1986) pointed out that although this medium is highly selective it does not allow direct oxidase testing, unlike the blood-ampicillin agar, due to the mannitol

fermentation.

Other selective media for isolation of faecal aeromonads are xylose deoxycholate citrate agar (Shread et al., 1981) and Bile salts brilliant green agar (Millership and Chattopadhyay, 1984). Aeromonas can readily be differentiated from coliforms on these media by the oxidase positive reactions or absence of xylose fermentation (Millership and Chattopadhyay, 1984).

However, during the examination of environmental specimens, neither of the above characteristics can be used as a differential marker because many of the differential flora are oxidase positive and do not ferment xylose either. Palumbo et al. (1985) reported that in Gram-negative bacterial species, association with food starch hydrolysis is largely restricted to Aeromonas and Vibrio species. They recommended the use of starch Ampicillin agar (SA) for the examination. Nishikawa and Kishi (1987) however, noted that colonies were very often obscured by the swarming Proteus present in samples.

As a modification, Nishikawa and Kishi (1987) added starch into bile salts brilliant green (BBG) agar for environmental specimens. They found that the modified bile salts brilliant green starch agar (BBGS) was far superior to SA agar for quantitative recovery of motile Aeromonas. The species could easily be differentiated from other organisms. They, therefore, proposed the use of BBG for faecal and BBGS for environmental

specimens. Deoxyribonuclease agar supplemented with ox-gall and crystal violent as a primary isolation medium, has been found to be useful as all the strains of Aeromonas produce DNase (Morgan et al., 1986). It has the advantage of direct oxidase testing.

Biochemically, all species of Aeromonas produce acid from glucose and maltose, but not from xylose, dulcitol, inositol, adonitol, malonate and mucate (McCarthy 1975). All strains of Aeromonas possess gelatinase, deoxyribonuclease, ribonuclease and Tween 80 esterase (Popoff and Veron, 1976).

Popoff (1984) summarised the physiological tests that are universally positive for motile Aeromonas species as being catalase, starch hydrolysis, lecithinase, phosphatase, Arginine, dehydrolase, hydrolysis of O-nitrophenyl-B-D-galactopyranoside (ONPG), growth in nutrient broth without NaCl, and fermentation of mannitol, trehalose, fructose, galactose and dextrin; while the universally negative ones are pectinase, ornithine decarboxylase tryptophan and phenylamine deaminase, growth on centrimide agar, growth in nutrient broth containing 5% NaCl and acid production from sorbose, erythritol and raffinose. An important exception to this general rule is the fact that Hickmann-Brenner et al (1987) reported strains that are consistently ornithine decarboxylase positive. They, therefore, proposed the name A. veronii for this group.

The importance of identifying motile Aeromonas to species level has been emphasised (Barer et al., 1986a) because the three established species (Popoff et al., 1981) vary in their enteropathogenicities. Because quick identification to species level is sometimes required in clinical cases, Barer et al., (1986a) proposed four simple and rapid tests for identification, that is, haemolysis, growth at 42°C, production of gas from glucose and breakdown of aesculin. They found that they could correctly identify 73% of all their isolates for acetoin production and fermentation of salicin and arabinose, increased their accuracy to 80%.

Use of phenotypic identification scheme to determine enterotoxigenicity of strains has been suggested (Burke et al., 1982; Turnbull et al., 1984; Kirov et al., 1986). Turnbull et al. (1984) stated that strains that produce gas from glucose, are Voges-Proskauer and lysine decarboxylase positive, haemolyse human erythrocytes and oxidise glucomate are likely to be enterotoxigenic, while the markers used by Burke et al. (1982) were gas production from glucose, Voges-Proskauer positivity, fermentation of arabinose and glucomate oxidation. However, Gosling (1986) cautioned that the two schemes could provide both false positive and false negative predictions of enterotoxigenicity. He observed error rates of 14% and 9% respectively.

Studies carried by Kindschuh et al. (1987) also showed no correlation between toxin production and lysine decarboxylase or Voges-Prokauer reaction.

Another method for rapid identification to species level was recently proposed by Figura and Guglielmette (1987). This method employs the detection of CAMP-like (Christie, Atkins and Munch Peterson) factor produced by A. hydrophila and A. sobria species in sheep blood agar. These authors showed that A. hydrophila strains produce the CAMP-like factor either aerobically or anaerobically, A. sobria strains produced it only aerobically and A. caviae strains did not produce it at all.

2.3.5 PATHOGENESIS

2.3.5.1 General Considerations

In recent years the large number of reports of gastrointestinal and extraintestinal human infections caused by Aeromonas have established this organism as a primary human pathogen. Mechanism of infection has, however, not been clearly established as yet (Wadstrom, 1986), although a number of potential virulence factors related to the pathogenicity have been described (Janda et al., 1985).

These factors are biologically active extracellular substances such as haemolysins, cytotoxins and enterotoxins (Kindschuh et al., 1987) that are known to

mediate the clinical manifestation of Aeromonas-associated gastroenteritis in man (Daily et al., 1981; Pitarangi et al., 1982; Janda et al., 1985; Chopra et al., 1986).

Other factors that may play a role in pathogenesis of Aeromonas species are the adherence mechanisms which include those that are pilli mediated (Atkinson and Trust, 1980). These authors noted that none of the less virulent, non-cytotoxic environmental strains was pilliated.

2.3.5.2 Toxins produced by Aeromonas hydrophila group

Aeromonas species produce a range of extracellular enzymes and toxins, some of which are potential virulence factors (Cumberbatch et al., 1979; Ljungh, 1986; Kindschuh et al., 1987). Characterization of these toxins has been difficult because of the complex extracellular protein profile of this species and the instability of these toxins during purification (Potomski et al., 1987a). Hence attempts by investigators have produced different results. However, using immunological techniques these enterotoxins have been purified, characterized and divided into cytotoxic and cytotonic enterotoxins (Burke et al., 1986; Potomski et al., 1987a).

2.3.5.2.1 Cytotoxic enterotoxins

Potomski et al. (1987a) using monoclonal antibodies to purify and characterise this toxin stated that it is haemolytic, cytotoxic and enterotoxic, and that it is a single protein. This was in agreement with earlier reports (Hostacka et al., 1982; Asao et al., 1984) although it conflicted with research done by some workers (Chopra et al., 1986) who claimed that Aeromonas species produce haemolysin and cytotoxin as separate entities apart from the enterotoxin.

The molecular weights of this enterotoxin was determined by Potomski et al. (1987a) to be 63,000 daltons with isoelectric point (pl) of 6.2. Biological activities of this toxin in rat ileal loops, suckling mice, cell culture and haemolysin assay were all positive (Asao et al., 1984; Potomski et al., 1987a).

Bunning et al. (1986) stated that this toxin is one of the principal virulence factors of Aeromonas species. Because of the characteristic of this enterotoxins, it has been reported by various workers (Asao et al., 1984; Stelma et al., 1986; Bunning et al., 1986) that this toxin is the same as hemolysin or aerolysin (Chakraborty et al., 1986).

2.3.5.2.2 Cytotonic enterotoxin (Cholera-toxin cross-reactive - CTC factor)

The fact that a family of cholera toxin (CT)-like

enterotoxins may be produced by bacteria other than Vibrio cholerae led to the search for the same in aeromonads. Wadstrom et al. (1976) initially reported partial inhibition of Aeromonas enterotoxin by CT or heat-labile (LT) of E. coli. James et al. (1982) demonstrated cross reactions between the exotoxins of Aeromonas species and CT or LT. Now it has been established that Aeromonas species, apart from cytotoxic enterotoxin also produce a cholera toxin cross-reactive (CTC) factor which is a cytotonic enterotoxin (Chopra et al., 1986; Potomski et al., 1987b).

Ljungh et al. (1982) described a cytotonic enterotoxin produced by A. hydrophila as a heat-labile (60°C for 20 min) molecule having a molecular weight of 15,000. This enterotoxin is capable of activating adenylate—cyclase, resulting in increased levels of intracellular cyclic AMP. Like the cytotoxic enterotoxin, it is capable of producing fluid accumulation in suckling mice and rat ileal loops, thus the use of such models would not allow detection of neutralization of one toxin in the presence of the other (Potomski et al., 1987b). Unlike the cytotoxic enterotoxin, it causes rounding in adrenal Y1 cells. All these effects were neutralised by cholera antitoxin.

Burke et al. (1986) reported CTC factor, detected

in ELISA, in 25.8% of A. sobria, 20.0% A. hydrophila and 24.3% of A. caviae, but cytotoxic enterotoxin was most commonly associated with A. sobria, less frequently with A. hydrophila and never with A. caviae. Strains of A. sobria and A. hydrophila producing CTC factor usually produced cytotoxic enterotoxin as well.

Potomski et al. (1987b) reported that diarrhoea is associated with cytotonic enterotoxin only if the Aeromonas strain also possesses the required colonisation factor analogous to the situation with enterotoxigenic E. coli. They observed that none of the A. caviae strains that gave positive results in ELISA showed frucose-resistant hemagglutination, the pattern most commonly produced by strains isolated from patients with Aeromonas-associated diarrhoea. These authors concluded that cytotonic enterotoxin alone is not a virulent factor for aeromonads.

Other extracellular substances produced by Aeromonas species are haemolysins (Notermans et al., 1986) and proteases (Pansare et al., 1986). However, their role in pathogenesis of infection, if any, is thought to be minor (Ljungh, 1986).

2.3.5.3 Toxicity assay

Various models have been used for assaying virulence properties of A. hydrophila with varying degrees of success.

2.3.5.3.1 Baby-mouse test (Suckling mouse test)

This model was developed by Dean et al. (1972) for the detection of heat-stable enterotoxin (ST) of E. coli (Stavric and Jeffrey, 1976). The suitability of this model for detecting the presence of enterotoxins of A. hydrophila was evaluated by Burke et al., 1981a). They reported that it was satisfactory for larger scale of strains than is possible with the use of systems such as the rabbit ileal loop or perfusion of rat jejunum in vivo. They recommended it in epidemiological studies to clarify the role of enterotoxigenic strains of Aeromonas in diarrhoeal diseases.

Subsequent investigators used this model with success (Johnson and Lior, 1981; Turnbull et al., 1984; Chopra et al., 1986; Potomski et al., 1987a) and it was observed that unlike in E. coli where the assay is for detection of heat-stable enterotoxins, in A. hydrophila, this model detects heat labile enterotoxin (Turnbull et al., 1984; Chopra et al., 1986). Potomski et al. (1987a) also observed that this heat-labile enterotoxin is not as potent as ST of E. coli when detected in this model. In this assay, the intestinal weight over body weight (IW/BW) ratio was the sole criterion and an IW/BW ratio of >0.08 was considered a positive result (Potomski et al., 1987a). They defined one mouse unit as the minimum amount of protein that caused IW/BW ratio of 0.08.

2.3.5.3.2. Rabbit and rat ileal loop test

This model was described by De and Chatterjee (1953) who used it for testing cholera toxin. It has now been found reproducible and reliable for A. hydrophila (Boulanger et al., 1977; Barer et al., 1986b; Potomski et al., 1987a). Potomski et al. (1987a) reported that purified Aeromonas enterotoxin causes fluid accumulation with damage to intestinal mucosa, induction of an inflammatory response and haemorrhage in rat ileal loop after 18hrs of incubation.

2.3.5.3.3 Cell culture assay

Cytotoxic and cytotonic effects on cell cultures have been used by Sack and Sack (1975) for assaying E. coli toxins. The cell culture method was later found useful in detecting cytotoxic and cytotonic effects of Aeromonas enterotoxin (Daily et al., 1981; Barer et al., 1986a; Potomski et al., 1987a). . Barer et al. (1986a) used five cell lines in monolayer microtiter plates. They found that vero cell cultures were the most sensitive in detecting the cytotoxic effects of Aeromonas species. However, the commonly used cell lines have been the adrenal YI and CHO-K1 cells (Potomski et al., 1987a). These authors used these cell lines to test the cytotoxic activity of purified enterotoxin and they considered death of >50% of the cells to be a positive result. Bunning et al. (1986)

reported that when using CHO cells, morphological changes of the cultures after 18hr can differentiate cytotoxic enterotoxins from the cytotonic or CT-like enterotoxins of Aeromonas. They recorded that severe cell rounding, cell detachments, cell ruffing and growth inhibition are indicators of cytotoxicity while growth inhibition and cell elongation are indicative of cytotonic or CT-like effects. These authors using several cell lines including B16C3 and mouse melanoma cell lines concluded that generally cell culture models are more sensitive to extracellular toxic factors than animal models.

2.3.5.3.4 Haemolysin activity assay

This was described by Burke et al. (1981b) for detection of haemolysin of Aeromonas species. They used 1% rabbit red blood cells taking 100% lysis as the end point. Potomski et al (1987a) employed the same technique, however, using human group O erythrocytes. They considered lysis of at least 50% of erythrocytes as positive and defined one haemolytic unit as the minimum amount of toxin that gave a positive result.

2.3.6 ANTIMICROBIAL SUSCEPTIBILITY OF AEROMONAS SPECIES

Prior to 1980, antimicrobial susceptibilities of Aeromonas were determined primarily by agar diffusion method using discs impregnated with the antimicrobials

(Fass et al., 1986). Recently testing of the in vitro activities of antimicrobial agents against Aeromonas species have been done using Mueller-Hinton broth, in which antimicrobials have been incorporated. Minimum inhibitory concentrations (MIC) of antimicrobial agents for aeromonads were also used by Richardson et al., 1982; Motyl et al., 1985; Gosling, 1986).

Results obtained by various workers for the antimicrobial susceptibility patterns for aeromonads tend to agree that most strains are susceptible to gentamycin, amikacin, chloramphenicol, trimethoprim-sulphamethoxazole, tetracycline and nitrofurantoin and uniformly resistant to methicillin, erythromycin, clindamycin and vancomycin (Richardson et al., 1982; Motyl et al., 1985). All strains are resistant to beta-lactam antibiotics, i.e. penicillin, ampicillin and carbenicillin, although 50% of all strains were susceptible to piperacillin and mezlocillin (Motyl et al., 1985). This resistance to β-lactams is attributed to the production of β-lactamase by majority of Aeromonas species (Richardson et al., 1982).

Generally A. hydrophila has been reported to be more resistant to the penicillins and cephalosporin than either A. caviae or A. sobria (Motyl et al., 1985; Chang and Bolton, 1987). Therefore identification of Aeromonas isolates to the species level is important in the selection of definitive

species-oriented therapy.

The fact that resistance may be plasmid mediated was indicated by McNicol et al. (1980) when they reported that 57% of their environmental Aeromonas isolates were resistant to multiple antibiotics including tetracyclines and chloramphenicol. However, Motyl et al. (1985) did not see similar antibiograms from their clinical isolates as all of them were susceptible to both drugs, thus they concluded that these differences may be related to the source of the species of Aeromonas, methods of isolation and most importantly, the frequency of use of certain antimicrobial agents in a specific geographical area.

Plasmids that code for resistance to antibiotics in aeromonads have been isolated (Aoki et al., 1971; Hedges et al., 1985), some of which were found to be transferable to E. coli (Mizon et al., 1978; Toranzo et l., 1984). Chang and Bolton (1987) also detected a conjugative plasmid in A. sobria which codes for multiple resistance to antibiotics and was transferable to E. coli, A. hydrophila and S. typhimurium. Toranzo et al. (1983) while characterizing plasmids in bacterial fish pathogens found that the majority of the strains they examined including A. hydrophila harboured at least one conjugative (heavy) plasmid which suggested a possibility of direct transfer of these plasmids within the marine bacterial population and also their use in mediating mobilization of smaller

plasmids.

2.3.7 PLASMID PROFILES OF AEROMONAS SPECIES

Plasmid profiling is one of the methods, like serotyping, biotyping, phagetyping and antimicrobial susceptibility testing, which is now being used as an epidemiological tool for tracing outbreaks and transmission of certain diseases like salmonellosis, shigellosis and Aeromonas salmonicida infection (Holmberg et al., 1984; Olsvik et al., 1985; Kvello et al 1987). Toranzo et al. (1983) noted that there were similarities between strains of A. hydrophila and fish bacterial pathogens isolated from different geographical areas in their plasmid pattern.

2.3.8 ANTIGENS AND TYPING OF AEROMONAS SPECIES

In drawing the scheme for mesophilic aeromonads, Sakazaki and Shinoda (1984) suggested that since O and H antigens of S. sobria and A. caviae are able to be determined with antisera prepared with A. hydrophila, serovars of these three species could be included in the single scheme.

Serological typing, though possible, has limited application because of heterogeneity amongst strains such that it is difficult to raise a useful bank of sera (Stephenson et al., 1987). von Graevenitz (1984) reported that as many as 72% of strains of Aeromonas are untypable.

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 FISH SAMPLES

Fish were obtained directly from the fishing sites of the lakes and the coast. In Lake Victoria, the fish were freshly caught using a trawling boat. Those that were to be sampled were immediately separated and put in clean tray. The various species of fish sampled are shown in Table IV.

Swabs were made using charcoal swabs from the surface, gills, peritoneum, intestine and kidneys. The swabs from the gills were obtained by opening the operculum and inserting a charcoal swab and rotating it several times. The abdomen was opened using a clean scapel blade and the peritoneum and kidney swabbed. The stomach and the intestine were also opened and swabbed. All the swabs were placed in bijou bottles containing Stuart's transport medium (lab M).

3.1.2 WATER SAMPLES

Water from the sampling sites was collected in sterile universal bottles at different points about one foot from the surface.

Table IV Fish species sampled in the survey

Area	Fish species	English name	Local name
L. Victoria	Lates niloticus	Nile perch	Mbuta
	Tilapia niloticus	Tilapia	Ngege
	Mormyrus kannumme	Elephant snout fish	Suna
	Parpus alternialis	-	-
	Barbus apleurograma	-	-
	Engraulicypris argenteus	-	Omena
	Clarias mossambicus	Cat fish	Mumi (ASC)
	Bagrus docmac	Cat fish	Seu
L. Naivasha	Oreochromis niloticus	Tilapia	Ngege
	Barbus species	-	Adei
L. Turkana	Oreochromis niloticus	Tilapia	
	Synodontis victoriae		
	Bagrus docmac	Cat fish	Seu
	Barbus species	-	Adei
	Clarias mossambicus	Cat fish	Mumi
Sombeli River (L. Magadi)	Clarias mossambicus	Cat fish	Mumi
Masinga Dam	Barbus species	-	Adei
Indian Ocean (Mombasa)	Chorinemus lysan	Queenfish	Pandu
	Monodactylus argentiens	Finger fish	-
	Kyphosus cinerascens	Rudder fish	Kufi
	Lutianus bohar	-	Kunguwale
	Gaterin species	-	-
	Caesio lunaris	-	-
	Tripterodon orbis	Batfish	-

3.1.3 SAMPLE AREAS AND SIZE

Fish and water samples were collected from the areas shown in Table V.

Table V Places and sizes of samples collected

Place	Number of samples collected	
	Fish	Water
Lake Victoria	347	15
Lake Turkana	98	5
Lake Naivasha	107	5
River Sombeli (L. Magadi)	4	5
Masinga Dam	74	5
Coastal waters	93	5
Total	723	40

3.1.4 TRANSPORTATION AND MAINTENANCE

Samples were transported in cool boxes and stored at 0C before analysis. Quantitative bacterial analyses was carried out within 10 hrs of collection, whereas those for bacterial identification were analysed within three days. Whole fish to be transported to the laboratory were put in plastic bags.

3.2 METHODS

3.2.1 STERILIZATION OF GLASSWARE AND MEDIA

All the glasswares used in the experiments were cleaned and dry sterilized at 160°C for 2 hrs. The media were sterilized according to the recommendations of the manufacturers. Generally, this involved autoclaving at 121°C for 15 min or filtration using membrane filters of 0.22 um pores (Millipore Corporation, Bedford, Mass., USA).

3.2.2 BACTERIOLOGICAL AND BIOCHEMICAL METHODS

3.2.2.1 Total bacterial count

Dehydrated Plate Count Agar (PCA) (Oxoid) was reconstituted and prepared according to the directions of the manufacturers and dispensed in disposable petri dishes (90 mm).

A homogenate of the fish sample was obtained by cutting 10 gm of fish material and adding to 90 ml of sterile physiological saline (P.S.) solution (0.85% sodium chloride) and homogenizing for 5 minutes in an homogenizer. Serial dilutions were made from 10^{-1} to 10^{-10} with sterile P.S. in culture tubes. Decimal dilutions were performed by transferring one ml of homogenate to nine ml of diluent. Each dilution tube was agitated to resuspend the material and one ml

of each dilution was pipetted into each of appropriately marked duplicate petri dishes. Twelve ml of melted PCA previously cooled to 45°C in a water bath was added to the inoculated plates. The inoculum and the molten PCA agar medium were mixed immediately by rotating and tilting. These were allowed to set for 15 min and then incubated at 37°C for 48 hrs. Water samples were similarily inoculated and incubated.

After the incubation, all colonies, on plates containing countable colonies (1-300), were selected and counted using a counter and the average counts per dilution were recorded. The geometric means were calculated for each sample and recorded as the total bacterial count per gram of fish material or per millilitre of water sample.

3.2.2.2 Isolation of *Vibrio parahaemolyticus* and *Aeromonas* species

Unless otherwise stated, sodium chloride was added to all culture media used for isolation of *V. parahaemolyticus* to a concentration of 3%.

3.2.2.2.1 Enrichment medium

Enrichment was only done for *V. parahaemolyticus* isolation while samples for *Aeromonas* were plated directly.

Two liquid selective enrichment media were used in parallel for *V. parahaemolyticus*.

3.2.2.2.1.1 Alkaline peptone water (APW)

This was prepared as a 1% bacteriological peptone (lab M) supplemented with 2% sodium chloride. The pH was adjusted to 8.6. This preparation was then dispensed into universal bottles in 10 ml volumes and sterilised.

3.2.2.2.1.2 Glucose salt-teepol broth (GSTB)

The preparation of this broth is as shown in appendix 2.1. It was distributed in culture tubes in volumes of 10 ml.

The fish homogenates and swabs for V. parahaemolyticus isolation were then inoculated in the two enrichment media and incubated at 37°C overnight. Loopfuls of the broth were inoculated on Thiosulphate citrate bile-salts sucrose (TCBS) agar. Both enrichment and direct culture procedures were used for water samples. In enrichment, 25 ml of water was mixed with an equal amount of single strength of GSTB. This suppresses the growth of Vibrio alginolyticus. The mixture was incubated at 37°C for 48 hrs after which loopfuls of the growth were directly plated on to TCBS. Part of this broth and the remaining water were concentrated by membrane filtration procedure. They were filtered through membrane discs of size number 0.45 um (Millipore Corporation, Bedford, Mass., USA). The membrane filters were asceptically placed onto TCBS agar plates for incubation at 37°C for 24 hrs.

Water samples for Aeromonas isolation were similarily concentrated and the filters were placed on A-Tween 80 plates and incubated at 37°C for upto 48 hrs. suspect colonies of V. parahaemolyticus and Aeromonas species on the respective plates were checked and subcultured on fresh media.

3.2.2.2.2 Direct culture medium

The two selective agar media used for V. parahaemolyticus isolation were:-

Thiosulphate citrate bile-salts sucrose (TCBS) agar. This medium was prepared according to manufacturer's instructions. The surfaces of the plates were dried before use to prevent excessive swarming of the bacterial colonies.

Triphenyltetrazolium chloride soya tryptone (TSAT) agar. This medium was prepared according to the recommendation of Kourany (1983) as shown in appendix 2.3. The plates were stored at 4°C and used within two weeks.

Primary isolation of Aeromonas species was also done on two agar media:-

Trytone soya agar (AGAR)

Swabs from fish were directly streaked on TSA and incubated for 24 hrs at 37°C. All the discreet colonies were picked and tested directly for their oxidase reactions. Those that were oxidase positive

were further tested for their biochemical reactions to differentiate Aeromonas species from other oxidase positive organisms.

A-Tween 80

This medium was prepared as shown in appendix 2.6. The plates were used in parallel with TSA such that samples streaked on TSA were also inoculated here. The plates were incubated for up to 48 hrs at 37°C and colonies that formed halos around them were further identified by biochemical methods.

3.2.2.2.3 Compound 0/129 (Vibriostatic agent) test

This vibriostatic agent (2,4-diamino-6,7,di-iso-propyl pteridine) (Sigma Chemicals Ltd.) was used for differentiating vibrios from other oxidase positive species, particularly Aeromonas. Briefly, the procedure followed was that Tryptone Soya Agar was prepared in disposable petri dishes and isolates to be tested were inoculated on to them. A few crystals of the pteridine compound were placed on the surface of the inoculated plates and incubated overnight at 37°C. They were examined for inhibition of growth around the crystals.

3.2.2.2.4 Oxidation-Fermetation test: (O/F test)

This was performed according to Hugh and Leifson (1953). All suspect colonies of V. parahaemolyticus and Aeromonas species were tested. Briefly, the O/F

basal medium was prepared as shown in appendix 2.8. One per cent glucose was incorporated into the medium aseptically. The tubes were inoculated in duplicates by stabbing with a straight wire. To one sect of tubes was added a layer of melted soft paraffin (petrolatum) to a depth of about one cm to provide anaerobic conditions, while the other one was not covered so that growth could occur aerobically. Both were incubated at 37°C and examined daily for upto 14 days.

3.2.2.2.5 Other biochemical tests

Presumptive isolates of the two organisms were confirmed by testing for their reactions in sugars and amino acids. The sugars were prepared in Andrade's peptone water (Oxoid) in 1% concentration after they were sterilised by filtration using 0.22 um filters (Millipores). Moeller's method (1955) was used for testing the amino acid reactions. One per cent of sodium chloride was incorporated in Moeller's medium for testing V. parahaemolyticus isolates.

3.2.2.2.6 Determination of salt requirement for V. parahaemolyticus

Influence of salt concentration on the growth and survival of V. parahaemolyticus was studied. Optimal salt concentration determination was performed by inoculating the isolates into tubes containing peptone

water (1% Bacteriological peptone; (lab M) of different sodium chloride concentrations. The final salt concentrations used were 0%, 1.0%, 3.0%, 8.0%, 9%, 10% and 13%. The broth tubes were then incubated at 37°C for 18 hrs after which growth was monitored in terms of turbidity using spectrophotometer (Spectronic 20, Bausch and Lomb).

3.2.2.2.7 Differentiation of Aeromonas isolates to species level

Differentiation of Aeromonas isolates to species level was done by both biochemical tests and testing for the CAMP-like factor.

3.2.2.2.7.1 Biochemical tests

The procedure of Barer et al. (1986a) for rapid identification of Aeromonas species was followed. In these tests, the ability of the isolates to cause beta-haemolysis in bovine blood agar, growth at 42°C, production of gas from glucose, breakdown of aesculin, acetoin production and fermentation of salicin and arabinose were tested.

3.2.2.2.7.2 CAMP-Like factor detection

This test was performed according to the descriptions of Figura and Guglielmetti (1987). Briefly, 5% sheep blood was incorporated into Trypticase soy agar (Gibco, Europe) during preparation

of the plates. A beta-haemolytic strains of Staphylococcus aureus was used to make streaked across the middle of the plates. The Aeromonas isolates were streaked perpendicular to the S. aureus line on duplicate plates. Streptococcus agalactiae and S. dysgalactiae were used as positive and negative controls respectively. One plate was incubated aerobically and the other both anaerobically at 37°C overnight.

3.2.2.3 Pathogenicity assay

The pathogenicity of V. parahaemolyticus isolates was assayed by testing for the Kanagawa phenomenon while that of Aeromonas isolates was done by testing for the production of toxins in baby mice and cell-culture systems.

3.2.2.3.1 Kanagawa phenomenon for V. parahaemolyticus

In this study, three reference strains kindly provided by Dr. Nair, G.B. of National Institute of cholera and Enteric Diseases, Calcutta, India, were used as controls. The medium used for the reaction was the modified Wagatsuma blood agar (WBA) of Sakazaki (1968), whose composition is described in appendix 2.4. The hemolytic activity was determined according to the description of Miyamoto et al. (1969). Cultures of the test strains in 3% sodium chloride nutrient broth were

inoculated on WBA plates and the results read after incubation for 24 hrs at 37°C. Well defined clear beta-hemolysis around the bacterial growth was recorded as positive, whereas a very narrow zone of haemolysis was doubtful and no haemolysis at all was considered as negative.

3.2.2.3.2 Toxigenicity assay for Aeromonas Isolates

3.2.2.3.2.1 Preparation of the toxic supernate

The growth broth of tryptic soy-broth-yeast extract (TSB-YE) was prepared by supplementing Tryptic soy broth (Gibco-Europe) with 0.6% (W/V) of yeast extract (Gibco-Cult Diagnostics); and five ml volumes dispensed into screw-capped tubes before sterilization. The isolates were inoculated into the tubes and incubated at 37°C overnight on a roller-drum. One and a half ml of the growth were transferred into Eppendorf tubes and centrifuged in an Eppendorf centrifuge at 10,000 g for three min. The supernates obtained were filtered using 0.22 um filters (Millipore Corporation, Bedford, Mass., USA) to get cell-free filtrates.

3.2.2.3.2.2 Testing with the suckling mice

For this test, a drop of 2% sterile solution of Evans Blue Dye was added to the 1.5 ml cell-free filtrates and thoroughly mixed. These preparations were kept at -20°C and used within one week.

Three day old Balb/c mice were used for the test. An Aeromonas supernate preparation was taken in a syringe fitted with a flexible plastic tubing of external diameter of 0.5 mm and introduced gently into the mouth of a mouse. An amount of 0.2 ml of supernate/dye mixture was gently introduced into the stomach of the mouse. Duplicate mice were inoculated with each test supernate and appropriately labelled.

Following the intragastric inoculation the mice were kept at room temperature for a period of three hours after which they were sacrificed by putting them in a chloroform chamber. The abdominal cavity of each mouse was opened and the entire small intestines dissected out and weighed (IW). The rest of the body weight (RBW) was also recorded for each mouse. Ratios of IW and RBW were calculated and recorded. Means of IW/RBW for each pair of mice which received a given supernate were calculated. Valves of 0.08 and above were considered to be positive. Mice which died during the incubation period as well as mice whose stomachs burst during feeding with supernate were discarded, and the corresponding supernates were retested. Positive control was Aeromonas strain (lab No. 65(2)) kindly donated by the Kenya Medical Research Institute (KEMRI).

3.2.2.3.2.3 Cell-culture assay

This assay was performed using monolayer cell-cultures of vero cells, strain 76, passage 149 kindly

provided by the Kenya Medical Research Institute. This was prepared by trypsinizing the cells from the bottle, with 0.25% of 1:250 trypsin (Gibco, Europe), and suspending them in minimal essential medium (MEM) with Eagle salts (Gibco, Europe), 10% faetal calf-serum, 1% L-glutamine, 3% sodium bicarbonate and 0.4% antibiotic. These were then seeded into flat bottomed 96 well-microtitre plates (Falcon Tissue Culture plates, B.D., Calf., USA) by adding 100 ul of the suspended cells. The plates were covered and incubated in a CO_2 incubator at 37°C until the cells formed complete confluent monolayers in the wells.

The Aeromonas cell-free filtrates prepared as described previously were serially diluted with sterile PBS in a new microtitre plate. The titration was done in a two-fold dilution, while leaving the last well with only PBS for control. The filtrates thus diluted were inoculated on the corresponding wells of the monolayers each well receiving 100 ul. Controls were performed with fresh sterile preparation of TSB-YE and sterile PBS. The plates were again incubated in CO_2 incubator at 37°C and the results scored after 18 hrs. A positive control was a verotoxigenic E. coli strain 0157 H7.

3.2.2.4 Antimicrobial drug sensitivity of the isolates

The antibiograms of 20 isolates of V. parahaemolyticus and 16 isolates of Aeromonas species

were tested.

The test was carried out on Mueller-Hinton agar (Oxoid) plates using single antibacterial tablets. The concentration of the inoculum used was 10^5 cfu/ml as recommended by Casals and Pringler (1985). Standardization of the inoculum was done by taking the optical densities of the broth cultures at regular intervals of 30 min starting from two hours of incubation at 37°C on roller-drum, and then determining the cell concentrations at each reading by plate count agar after incubation at 37°C overnight.

The optical density corresponding to 10^5 cfu/ml was found to be 1.09 at a wave length of 490 nm. Hence, the isolates were grown in Tryptic soy broth upto the time when the turbidity had reached an optical density of 1.09. Both the culture broth and Mueller-Hinton agar used for V. parahaemolyticus isolation were supplemented with 3% sodium chloride. The broth culture was then spread on the surface of Mueller-Hinton agar using a sterile bent glass rod in order to achieve a uniform distribution of the organisms. Six single antibiotic discs were placed on a plate. The plates were incubated at 37°C for 24 hrs and the diameters of zones of inhibition measured.

The antimicrobial tablets used were Neo-sensitabs supplied by A/S Rosco Taastrup, Denmark. The tested

agents were:- penicillin / low (5 ug), ampicillin (33 ug), oxacillin (5 ug), bacitracin (40 IU), chloramphenicol (60 ug), fucidin (400 ug), nalidixic acid (130 ug), neomycin (120 ug), nitrofuran (200 ug) streptomycin (100 ug), gentamicin (40 ug), sulphonamides (240 ug), trimethoprim (5.2 ug), trimethoprim + sulpha (5.2 + 240 ug), tetracycline (80 ug) and doxycycline (80 ug). Reference strains of both V. parahaemolyticus and Aeromonas species were included in the tests.

3.2.2.5 Plasmid profiles

Plasmid profiles of the isolates of V.parahaemolyticus were studied using a modified Birnboim (1983) procedure. Briefly, the isolates were grown in 3 ml Mueller-Hinton broth (lab M) and incubated at 37°C overnight on a roller drum. Aliquots of 1.5 ml of the broth culture were transferred into appropriately labelled Eppendorf tubes (Treff Lab, Switzerland). The cells were harvested by centrifuging at 10,000 g for 3 min in an Eppendorf centrifuge and the pellets were resuspended in 100 ul of aqueous solution containing 4.0 mg lysozyme/ml, 0.05 mol glucose/l, 0.01 mol EDTA/l and 0.025 mol Tris-Hcl/l using a whirlmixer (Fission Scientific apparatus, Leciestershine, England). After incubating the suspension in an ice-water bath for 30 min, 200 ul of freshly prepared 0.2 ml NaOH solution with 1% SDS was added to them and the samples were

mixed by inverting the tubes several times until the solutions cleared. One hundred and fifty ul of 3.0 M . NaAcetate solution pH 4.8 was added to the lysates and mixed thoroughly. They were kept in ice-cooled water bath for 60 min. after which they were centrifuged for 15 min. at 10,000 g and the supernatants transferred into new Eppendorf tubes (about 450 ul) without disturbing the sediment. One ml of cold (-20°C) absolute ethanol was added to each tube, thoroughly mixed and kept in a freezer at -20°C for at least 60 min. The samples were again centrifuged for 20 min at 10,000 g, the supernatants discarded and the sediments allowed to stand at room temperature with the lids open for at least 20 min. After complete evaporization of the alcohol, the DNA precipitates were dissolved by adding 25 ul of 10 M Tris-Hcl buffer in ImM EDTA (pH 8.0). The tubes were allowed to stand for 15 min. and then gently tapped to mix the contents. A loading buffer (0.25% bromothymol blue, 0.25% xylene cyanol and 30% glycerol) of 25 ul was added and mixed gently.

The plasmids were separated by electrophoresis in a 1% agarose NA gel (Pharmacia Fine Chemicals, Uppsala, Sweden) in horizontal apparatus through a voltage of 20 V/cm and current of 15 mA for 15 hrs. The running buffer used was Tris-Acetate EDTA at pH of 7.4-8.0. Plasmids were stained in an ethidium bromide solution for 25 min and photographed under fluorescent light using an ultraviolet illuminator.

Standard strain of E. coli V157 with eight plasmids of 1.4, 1.8, 2.0, 2.6, 3.4, 3.7, 4.8 and 35.8 M daltons was used as a marker. Molecular weights of the plasmids of the isolates were calculated by plotting a standard graph of the relative mobility of the reference plasmids of E. coli V157 against log molecular weights.

3.2.2.6 Coagglutination test for the detection of somatic O antigen

3.2.2.6.1 Preparation of 'O' agglutinable bacterial suspension

The somatic 'O' antigens were prepared from reference strains of V. parahaemolyticus, NICED 42, serotype O7:K19 and A. hydrophila, ATCC 7966. The method described by Talwar (1983) was adapted with modifications. The two strains were grown in Tryptic soy broth (Gibco, Europe) overnight. Each of these culture broths was inoculated on ten plates of Tryptone soy agar (Oxoid) and incubated at 37°C for two hrs. The cells were harvested by adding one ml of sterile physiological saline solution and scraping them off from the surface using a sterile bent glass rod. These bacterial suspensions were taken with sterile pasteur pipettes and transferred into culture tubes and boiled for two and a half hours in a boiling waterbath, with agitations; after which they were centrifuged in a

refrigerated centrifuge (Minifuge, Hereus Christ, Germany) at 4°C and 2,000 g for 20 min. The supernatants were discarded and cells resuspended in 0.3% formalised saline. The sterility of the bacterial suspensions was checked by inoculating plates of TSA and incubating them for upto 48 hrs at 37°C. They were examined daily for growth.

3.2.2.6.2 Raising of antiserum to 'O' agglutinable antigen

For each of the somatic antigens of V. parahaemolyticus and hydrophila two adult New Zealand large white rabbits were used. These rabbits were bled prior to immunization. The concentrations of the bacterial suspensions were adjusted to the required concentrations of 10^8 cells/ml by the method of McFarlands nephelometer and used as the inocula. Half a millilitre of each inoculum was emulsified with 1.5 ml of Freud's complete adjuvant (Gibco Laboratories, Grand Island, N.Y. USA). Injections were made intramuscularly and into superficial lymph nodes as described by Newbould (1965). The rabbits were given boosters of the antigen in Freud's incomplete adjuvant at fortnightly intervals. Each rabbit was bled prior to immunization.

3.2.2.6.3 Testing for antibody production

Antisera to V. parahaemolytucs and A. hydrophila 'O' agglutinable antigens were tested using the microtechnique of Ouchterlony double diffusion as described by Crowle (1973). One per cent (w/v) of purified agar (Oxoid) was dissolved in distilled water and PBS in the ratio of 3:1 and preserved by adding 0.1% of sodium azide. A microscope glass slide was flooded with about 4 ml of the molten agar and after the gel had set, wells of 4.0 mm diameter were made with a gel-punch.

Each central well was filled with 20 ug of the V. parahaemolyticus and A.hydrophila antigens while the peripheral wells were each filled with serum obtained from different bleedings of the rabbits. Diffusion was allowed to take place in a humid chamber at room temperature. The wells were refilled twice at one hour intervals. The lines of precipitations were checked after 24 hrs incubation. When the precipitation lines became clearly visible, the gels were pressed and washed in 3% (w/v) trisodium citrate (Koch-Light Laboratories Ltd., England) buffer of pH 8.5 overnight. The slides were rinsed in tap water, pressed a second time, dried and stained with Coomasie brilliant blue dye (Sigma Chemicals, St. Louis, USA) for 20 minutes. The slides were then destained in a destaining solution until the background was clear. The slides were rinsed and dried.

3.2.2.6.4 Coagglutination test for the detection of V. parahaemolyticus and A. hydrophila somatic 'O' antigens

3.2.2.6.4.1 Growth and stabilization of Staphylococcus aureus

The method used was an adaptation of that described by Kronvall (1973). S. aureus strain Cowan I was used. An overnight growth of this strain was inoculated and spread on TSA plates and harvested by adding sterile PBS and scraping the cells from the plates using sterile bent glass rod. More PBS was added to the bacterial suspension and centrifuged at 4°C at 2,000 g for 15 min. The supernatant was discarded and the bacterial sediment resuspended in PBS. This was washed three times in PBS and finally resuspended in 0.5% (w/v) of 10% formaldehyde solution and kept at room temperature for three hrs. After this, it was centrifuged as before and washed 4 times in PBS, resuspended in PBS to 10% (w/v) heated to 80°C and maintained at that temperature for 10 min to kill the bacteria. The bacterial cells were washed twice in PBS and suspended to final concentrations of 10% (w/v) in 0.5% formaldehyde solution and stored at 4°C until required.

3.2.2.6.4.2 Coating of *Staphylococci* with rabbit antisera

The stock of the stabilised *S. aureus* was mixed thoroughly and 2.0 ml was taken and centrifuged. The supernatant was discarded and the sediment was washed five times in PBS and finally resuspended in 20 ml PBS. Two hundred microlitres of rabbit anti *V. parahaemolyticus* serum was slowly added to the suspension while shaking the suspension on a whirlmixer for five min. The suspension was centrifuged and the sediment saved. It was washed four times in PBS to remove excess serum proteins and resuspended in 2% (v/v) PBS containing 0.1% sodium azide. Antiserum against *A. hydrophila* was similarly coated to *Staphylococcus* cells.

3.2.2.6.4.3 Coagglutination test

The test was carried on a clean dry microscope glass slide. A drop of sterile PBS was placed on the slide, and using a sterile wire loop, part of the colony of the isolates was emulsified to give a uniform suspension. A drop of well mixed 2% suspension of stabilised *S. aureus* coated with rabbit anti-serum was added to the suspension and mixed. The slide was then rocked from side to side for about five minutes and the result read against a dark background in adequate lighting. All the isolates of *V. parahaemolyticus* and *A. hydrophila* were tested with the corresponding coated antisera.

RESULTS

4.1 TOTAL BACTERIAL COUNTS

The ranges of total bacterial counts obtained for samples from Lake Naivasha, Lake Magadi and Masinga Dam are shown in Table VI. They vary from 10^5 to 10^8/gm of fish samples and 10^1 to 10^5/ml of water. Water samples from L. Magadi have the least bacterial number per ml. Counts obtained in L. Naivasha water are generally low and constant as compared with Masinga Dam and Sombeli River.

4.2 ISOLATION OF *VIBRIO PARAHAEMOLYTICUS* AND *AEROMONAS* SPECIES

4.2.1 *VIBRIO PARAHAEMOLYTICUS*

A total of 666 samples were screened for *V. parahaemolyticus*. The number of *V. parahaemolyticus* isolated was 29, representing an isolation rate of about 4%. A high isolation rate was obtained in samples from Mombasa (coastal waters), where out of 62 samples screened, 27 isolates were made, an isolation rate of 44%. Samples from Naivasha and Kisumu yielded one isolate each, representing 1% and 0.3% isolation rates respectively. No isolation was made from the other areas sampled.

TABLE VI Range of Total Bacterial Counts (TBC) in fish and water

Range of TBC	L. Naivasha		L. Nagadi		Masinga Dam	
	Fish *	Water *	Fish	Water	Fish	Water
10^1	-	-	-	1	-	-
10^2	-	-	-	1	-	-
10^3	-	3	-	-	-	-
10^4	-	-	-	-	-	2
10^5	1	-	1	1	1	1
10^6	8	-	1	-	3	-
10^7	1	-	1	-	2	-
10^8	-	-	1	-	1	-
10^9	-	-	-	-	-	-
10^{10}	-	-	-	-	-	-
TOTAL NUMBER of SAMPLES	10	3	4	3	7	3

* Figures in these columns denote the number of samples having TBC corresponding to the range.

4.2.2 AEROMONAS SPECIES

A total of 763 samples (723 fish and 40 water) were screened for Aeromonas species. Three hundred and fifty six isolates were identified representing an isolation rate of 47%. Of these, 345 isolates were from fish and 11 were from water samples, representing isolation rates of 48% and 28% respectively (Table VII).

Table VII Isolation of V. parahaemolyticus and Aeromonas species from fish and water from various areas in Kenya.

Area sampled	Types of samples collected	V. parahaemolyticus		Aeromonas species	
		Isolates/ specimens	Percentage isolation	Isolates/ specimens	Percentage isolation
Coastal water	Fish	25/57	44	22/93	24
	Water	2/5	40	1/5	20
L. Naivasha	Fish	1/94	1	71/107	66
	Water	0/5	0	2/5	40
L. Victoria	Fish	1/310	0.3	136/347	43
	Water	0/15	0	4/15	27
Masinga Dam	Fish	0/74	0	35/74	47
	Water	0/5	0	2/5	40
L. Turkana	Fish	0/87	0	58/98	59
	Water	0/5	0	1/5	20
River Sombeli (Magadi)	Fish	0/4	0	3/4	75
	Water	0/5	0	1/5	20
		29/666	4	356/763	47

In A-Tween 80 medium, Aeromonas species formed halos around the colonies; and colour of the colonies were pale.

Further identification of the Aeromonas isolates to species level showed the following results: Biochemical methods - A. caviae 42% (150/356), A. hydrophila 37% (132/356) and A. sobria 21% (74/356). CAMP-like factor detection - A. caviae 44% (155/356) A. hydrophila 35% (123/356), A. sobria 22% (78/356). (see Table VIII).

Table VIII Differentiation of Aeromonas species using biochemical tests and CAMP-like factor detection.

Place	Total number of Aeromonas isolates	A. hydrophila	A. caviae	A. sobria
		(*)		
Coastal waters	23	9 (7)	10 (13)	4 (3)
L. Naivasha	73	30 (27)	25 (26)	18 (20)
L. Victoria	160	61 (59)	66 (65)	33 (36)
Masinga dam	37	12 (11)	15 (17)	10 (9)
L. Turkana	57	19 (18)	31 (31)	7 (10)
R. Sombeli (Magadi)	4	1 (1)	3 (3)	-
Total	356	132 (123)	150 (135)	74 (78)
Percentages of isolates		37% (35%)	42% (44%)	21% (22%)

(*) The first figures denote the results of biochemical tests while the ones in parentheses denote that of CAMP-like factor detection.

4.2.3 DETERMINATION OF SALT REQUIREMENTS FOR V. PARAHAEMOLYTICUS ISOLATES

Results of growth of V. parahaemolyticus isolates in different concentrations of salt showed that growth can be supported by salt concentrations of 1% to 8%. Maximum growth as indicated by maximum turbidity was

recorded at concentration of 3% for all the isolates except one from L. Naivasha water. Minimum growth occurred at 9% concentration while concentrations of 10% and 13% hardly supported growth. One freshwater isolate from L. Victoria (Vk 60) showed a similar growth pattern as the isolates from coastal waters, although growth at 1% was almost as high as at 3%. The growth pattern for the isolate from Naivasha (Vn 70) varied slightly from that of the other isolates in that maximum growth was recorded at 1%. However, like all the others, growth occurred upto 8% concentration and it exhibited the same biochemical characteristics.

4.2.3 SEROTYPES OF V. PARAHAEMOLYTICUS

Out of twenty isolates only five were serotypable, representing a 20% typability. Three different serotypes were found: O2:K28; O3:K29 and O4:K42 distributed in the ratio of 2:2:1 among the five typable isolates (appendix 1.5).

4.3 PATHOGENICITY ASSAY

4.3.1 KANAGAWA PHENOMENON HAEMOLYSIS FOR V.PARAHAEMOLYTICUS ISOLATES

All the 29 isolates were negative for haemolysis in Wagatsuma medium as tested against the positive and negative reference strain.

4.3.2 TOXIGENICITY ASSAY FOR AEROMONAS SPECIES ISOLATES

4.3.2.1 Enterototoxigenicity assay using baby mice

A total of 166 out of 256 Aeromonas isolates were tested for their enterotoxigenicities in baby mice. In this model, 28% (47/166) of the tested isolates were found to be enterotoxigenic as their IW/RBW ratios were over 0.08. Positive and negative results are illustrated in Figures 1 and 2 respectively.

The occurrence of enterotoxin producing strains among the species varied considerably with the highest rate of these enterotoxigenic strains being found among A. sobria, 51% (20/39), followed by A. hydrophila, 35% (23/65) and least of all is A. caviae 7 (4/62). The results are shown in Table IX.

4.3.2.2 Cytotoxicity assay using vero cells

Results of this test (Table X) showed that 53% (42/80) of these isolates were found to be cytotoxic to vero cells. This percentage of cytotoxic strains as compared to enterotoxicity in baby mouse test is almost twice as much. However, the distribution of cytotoxic

Table IX Results of Enterotoxigenicity testing of Aeromonas isolates using Baby House Test

Place	No. of isolates tested	No of isolates positive	%age positive	A. hydrophila		A. caviae		A. sobria	
				No. tested	No. +ve	No. tested	No. +ve	No. tested	No. +ve
Coastal waters	22	9	40.9	9	5	9	1	4	3
L. Naivasha	32	8	25.0	12	4	10	-	10	4
L. Victoria	35	7	20.0	15	3	10	-	10	3
Masinga Dam	27	10	37.0	10	6	10	-	7	4
L. Turkana	46	12	26.1	18	4	20	2	8	6
River Sombeli (Magadi)	4	1	25.0	1	1	3	-	-	-
Total	116	47	28.3	65	23	62	4	39	20

strains among the different species of Aeromonas showed a similar pattern as the enterotoxic strains in baby mice in that A. sobria had the highest percentage (85%), followed by A. hydrophila (63%), then A. caviae (18%) being the lowest.

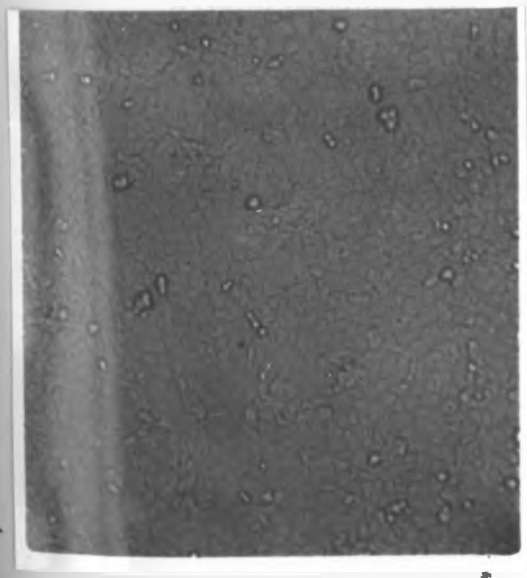

Fig.1 Normal cells 18hrs after inoculation (negative)

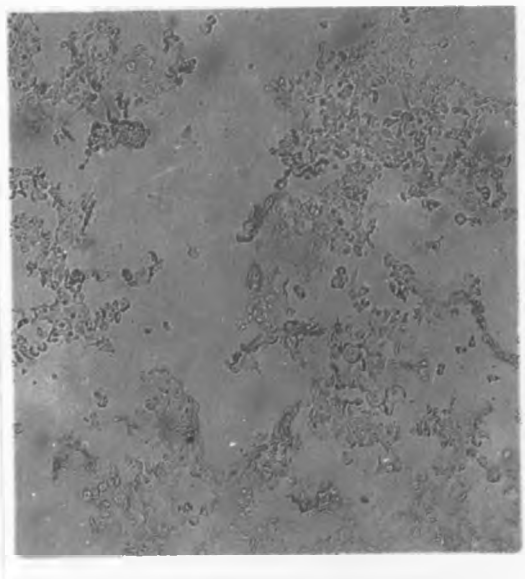

Fig.2 Dead cells 18hrs after inoculation (positive)

Table X Results of cytotoxicity testing of Aeromonas isolates using vero 76 cells

Place	No. of isolates tested	No of isolates positive	%age positive	A. hydrophila		A. caviae		A. sobria	
				No. tested	No. +ve	No. tested	No. +ve	No. tested	No. +ve
Coastal waters	10	7	70	4	3	3	1	3	3
L. Naivasha	12	6	50	4	2	4	1	4	3
L. Victoria	30	15	50	15	9	10	2	5	4
Masinga Dam	9	3	33	3	1	3	0	3	2
Turkana	15	10	67	5	4	5	1	5	5
R. Sombeli	4	1	25	1	1	3	0	0	0
Total	80	42	52.5	32	20	28	5	20	14

4.4 ANTIMICROBIAL DRUG SENSITIVITY TO V. PARAHAEMOLYTICUS AND AEROMONAS ISOLATES

Generally the pattern of antimicrobial drug sensitivity of V. parahaemolytivcus isolates shows that all isolates were sensitive to chloramphenicol, nalidixic acid, streptomycin, sulphonamides, trimethoprim + sulpha, tetracycline and doxycycline; whereas resistance to ampicillin, fucidin, penicillin and oxacillin was shown by all strains tested except the Naivasha isolate (Vn 70) which was sensitive to ampicillin (Table XI).

Table XI Frequency of drug resistance among the 20 isolates of *V. parahaemolyticus*.

Antimicrobial agent tested	Number of resistant isolates	Percentage of resistant isolates
Ampicillin (33 ug)	19	95
Doxycycline (80 ug)	0	0
Chloramphenicol (60 ug)	0	0
Fucidin (400 ug)	20	100
Nalidixic acid (130 ug)	0	0
Neomycin (120 ug)	14	70
Nitrofuran (260 ug)	12	60
Penicillin (low) (5 ug)	20	100
Oxacillin (5 ug)	20	100
Streptomycin (100 ug)	0	0
Trimethoprim (5.2 ug)	1	5
Trim + sulpha (5.2+240 ug)	0	0
Tetracycline (80 ug)	0	0
Sulphonamides (240 ug)	0	0

Intermediate sensitivity was shown to neomycin and nitrofuran with some strains being sensitive while others were resistant. One isolate from coastal waters (Vm 39) showed resistance to trimethoprim to which all the others were sensitive.

The sensitivity patterns of Aeromonas isolates varied from those of V. parahaemolyticus isolates in that all the Aeromonas isolates showed multiple resistance to the antimicrobial agents. However, most strains were also sensitive to chloramphenicol (13/14), tetracycline (13/14), nalidixic acid (12/14) fucidin (11/14) and sulphonamides (11/14). Of the 16 strains tested including the three reference strains, 50% (8/16) were sensitive to streptomycin, 56% (9/16) to trimethoprim. Generally most strains were resistant to ampicillin, bacitracin, neomycin, penicillin, oxacillin and nitrofuran. All the isolates tested exhibited multiple resistance to at least six of the 14 antimicrobial agents used and resistance to all the 14 agents was manifested in one strain (K13) from L. Victoria. This strain was found to have six plasmids including a heavy one. The frequency of drug resistance among the isolates is illustrated in Table XII. Sensitivity of isolates to the antimicrobial agents were determined according to the table of Casals and Pringler (1985).

4.5 PLASMID PROFILES

All the *V. parahaemolyticus* and *Aeromonas* isolates were screened for the presence of plasmids. In case of *V. parahaemolyticus*. Only two isolates of *V. parahaemolyticus* (Vm 18 and Vm 39) were found to bear plasmids.

Table XII Frequency of drug resistance among *Aeromonas* isolates

Antimicrobial agent tested	Percentage resistant Isolates			
	Total number n = 16	*A. hydrophila* n = 6	*A. caviae* n = 4	*A. sobria* n = 6
Ampicillin (33 ug)	100	100	100	100
Bacitracin (40 IU)	100	100	100	100
Chloramphenicol (60 ug)	6	17	0	0
Fucidin (400 ug)	19	17	50	0
Nalidixic acid (130 ug)	13	33	0	0
Neomycin (120 ug)	94	100	100	83
Nitrofuran (260 ug)	63	67	75	50
Penicillin (low) (5 ug)	100	100	100	100
Oxacillin (5 ug)	100	100	100	100
Streptomycin (100 ug)	50	50	25	67
Trimethoprim (5.2 ug)	44	50	75	17
Trim + sulpha (5.2+240 ug)	63	66	75	50
Tetracycline (80 ug)	6	16	0	0
Sulphonamides (240 ug)	25	50	25	0

n = number of isolates tested.

Vm18 carried two light plasmids. Their molecular weights were 4.34 Md and 1 Md. Vm 39 also had two light plasmids with molecular weight of 4.36 and 2.15 Md.

As for Aeromonas species, a total of 32 isolates (15 from L. victoria, 8 from L. Naivasha, 5 from Masinga Dam, 2 from L. Turkana, 1 from coastal waters and the reference strain of A. media) were found to carry plasmids with varying numbers and molecular sizes as shown in Table XIII. Fourteen of these 32 had only one plasmid each, while seven strains had two each, seven others with three and two had four each. The remaining two had five and six each. Molecular weights of these plasmids ranged from very light (1.0 Md) to heavy (60 Md). The majority of these plasmids were light with only ten strains bearing both heavy and light ones. Some of the plasmid bands are illustrated in Fig. 3.

Table XIII Number of plasmids in the *Aeromonas* isolates

No. of plasmids in an isolate	No. of isolates	Total No. of plasmids	No. of light plasmids	No. of heavy plasmids
1	14	14	13	1
2	7	14	11	3
3	7	21	17	4
4	2	8	7	1
5	1	5	3	2
6	1	6	5	1

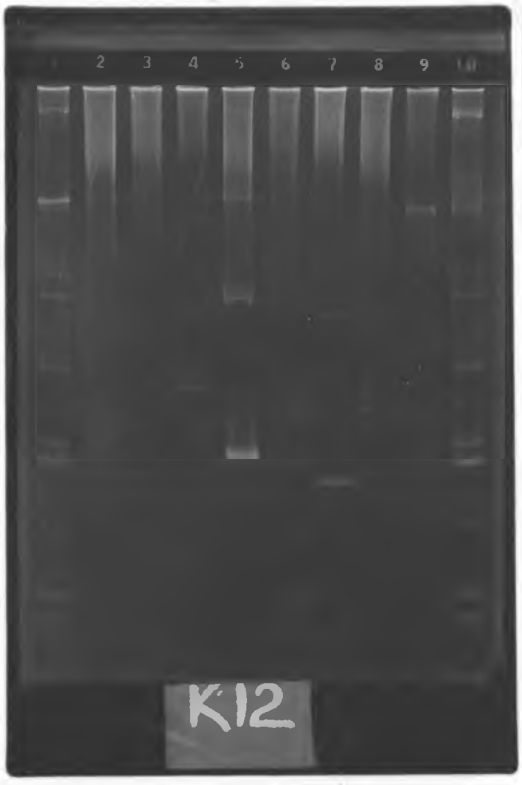

Fig 3. The plasmid bands of some of the *Aeromonas* isolates.

4.6 COAGGLUTINATION TEST FOR THE DETECTION OF *V. PARAHAEMOLYTICUS* AND *AEROMONAS* SOMATIC 'O' ANTIGEN

Ouchterlony immunodiffusion tests showed that strong precipitation lines of identity were given by antisera raised against the somatic 'O' antigens of both *V. parahaemolyticus* and *A. hydrophila* from the third bleedings.

In the slide coagglutination tests, only four of the 29 (14%) isolates of *V. parahaemolyticus* were able to form a clear carpet of coagglutinated cells.

Among the *Aeromonas* isolates, only 29 of the 356 isolates (8%) showed positive agglutination reactions. Of these 29 strains that agglutinated, two were *A. sobria* while the rest were *A. hydrophila*. No *A. caviae* strains agglutinated with the antiserum.

DISCUSSION

5.1 TOTAL BACTERIAL COUNT

The total bacterial counts of fish in this study ranged from 10^5 to 10^8. Most of the fish samples had total count values of 10^6. These values are within the range obtained from L. Turkana (Gjerstad et al., 1987).

Values obtained by Binta (1978) with market fish from Kenyan coastal waters showed a wider variation and were generally higher, that is between 10^5 and 10^{10}. These differences may possibly be explained by the fact that she was getting her samples from the market where they might have been stored for sometime, giving chance for the organisms to proliferate, as it has been indicated that bacterial multiplication continues to take place even when fish is stored on ice (Hobbs, 1983; Caramello et al., 1986). Another reason for the variation may be the fact that bacterial species and load from freshwater fishes differ from the normal microflora of marine fishes (Trust and Sparrow, 1974). The samples for bacterial counts in this case were all from freshwater fishes while Binta's (1978) samples were from marine environs.

Compared to similar work of Cann et al. (1971) in

temperate regions, the values obtained here were generally higher. These authors reported a range of 10^3-10^6 at 20°C and 10^1-10^8 at 37°C. However, their study was carried out on samples whose microbial flora consisted mainly of psychrophilic organisms and thus their rate of multiplication differs from that of mesophiles found in tropical freshwaters and fishes.

Living fish normally carry few microorganisms, but immediately after catch, they get contaminated from the deck, fish boxes, and other equipments during handling (Huss et al., 1974). The level of contamination can be kept low by strict hygiene. Although most of the samples were obtained directly from landing sites of the fish in order to minimize the chances of contamination, it is quite possible that bacteria from this source contributed to some extent to the values obtained.

The other source of fish bacterial load is their acquatic environment (Cann et al., 1971). Results of total bacterial counts obtained from water samples ranged from 10^1 to 10^5. The two lowest counts were obtained for samples from Lake Magadi, which is extremely salty and thus does not seem to support growth of microorganisms well. However, a tributary of R. Sombeli where the fish samples were obtained had high counts. This was expected as it is the only

source of drinking water for both the people around and their livestock, hence, contamination is high.

The values obtained for total bacterial counts of fish and water shows that fish counts are higher than that of water. This is understandable in view of the fact that during feeding of fish, micro-organisms tend to be filtered from the water and get concentrated on the gills and in the intestine. Another important fact is that some of the commonly occurring microorganisms in fresh water environs such as Enterobacter and Aeromonas species (Cann et al., 1971) produce adhesins which enable them to specifically attach to fish skin cells and the slime produced (Krovacek et al., 1987). Certain genera are known to use active attachment to fish and shellfish as a survival technique during adverse environmental and climatic conditions (Sarkar et al., 1985). This inherent bacterial flora on the fish and contamination during handling are important factors in bringing about spoilage changes and thus lowering the quality of fish.

5.2 VIBRIO PARAHAEMOLYTICUS

Vibrio parahaemolyticus was isolated from 44% of the coastal fish samples and 40% of the marine water samples. These values are in agreement with that obtained by Ijumba (1984) who found an average isolation rate of 47.3%. However, Binta's (1978)

values were generally lower (8.1%). All the samples in this study were obtained from seafish while Binta et al. (1982) and Ijumba (1984) reported a higher isolation rate from shellfish than seafish sampled from the same area and they attributed this to the type of feeding where shellfish, being filter feeders tend to concentrate these organisms during the process. They also reported higher isolation rate for seafish than seawater.

Vibrio parahaemolyticus from the inland freshwater lakes and their fishes was found to be low or non-existent in some cases. An isolation rate of only 1% from L. Naivasha and 0.3% from L. Victoria were found from the fishes while no isolation at all was made from fishes in the other lakes.

Although Waiyaki et al. (1985) reported having recovered the organism from seasonal streams which drain into L. Victoria, the low occurrence in fish and none at all in water could be explained by the dilution effect of the lakes.

It is possible that when the water reaches the lake, there is a very high dilution factor which makes the organisms very rare and difficult to isolate in random sampling of the water. Moreover, since it is a freshwater lake, the organisms do not survive and proliferate freely in it, thus the few that might survive normally adsorb onto fish, crustaceans and

planktons as a survival strategy (Sarkar et al., 1985).

In L. Naivasha, only one isolate of V. parahaemolyticus was made from a fish. This indicates the presence of the organism in the lake but in low numbers. Seasonal occurrence of this organism in freshwater environs and in association with freshwater fishes has been reported (Sarkar et al., 1985). It could, therefore, be possible that the low recovery rate from L. Naivasha was due to seasonal variation as only one sampling trip was made to this area. However, this explanation does not apply to L. Victoria where four trips were made at different seasons over a period of two years.

Another possible explanation for the low isolation rate of V. parahaemolyticus is that this organism is not indigenous to these inland freshwater lakes as no isolation was made in some cases. The two isolates from L. Victoria and L. Naivasha might have been introduced transiently by carriers from places like the coastal waters where they are known to occur. Such a fortuitous occurrence of the organism in freshwater environs after introduction by ambulatory cases has been reported before (Sarkar et al., 1983). In this case it is possible that fish crates might also play ambulatory role as they get exchanged by fishmongers from the coast and other places at the central market of Nairobi.

The results of the salt requirement by the isolates confirmed the fact that this organism is a moderate halophile with optimum growth at 3% salt concentration. The slight variation observed in the pattern of growth in the isolate from L. Naivasha, where maximum growth occurred at 1%, may be due to adaptation to environments with low salt concentration.

Of the twenty isolates only five could be serotyped, representing 25% typability. This is a low rate compared with similar results obtained by Chatterjee and Sen (1974) who reported typability of 32.8% for non-marine fish and water sample isolates, 80.2% for human diarrhoea isolates and 40.7% for marine fish and water isolates, and Nair et al. (1985) who were able to type 50.9% of the 324 environmental isolates of V. parahaemolyticus. However, they all concluded that a high number of untypable strains exists in environmental strains as compared to clinical isolates. Also Nair et al. (1985) found that fresh water isolates have the largest numbers of untypable strains. This may be in agreement with the results obtained here where the two freshwater isolates were both untypable, although the number in this case was too small for any meaningful conclusion to be drawn.

It has been noted that some serotypes appear to be constantly associated with the natural environs while

others are only found in humans (Nair et al., 1985). These authors reported the predominant environmental serotype to be O2:K28. In this study, two of the five typable isolates were found to be O2:K28. Serotype O1:K56, reported to be the most common of the clinical isolates (Chatterjee and Sen, 1974; Sircar et al., 1976), was not found among the tested isolates.

The Kanagawa phenomenon test done on the isolates showed that all the isolates were Kanagawa negative. This result is in agreement with the commonly accepted concept that apparently all environmental isolates are Kanagawa negative as opposed to clinical isolates which are usually Kanagawa positive (Sakazaki et al., 1968; Twedt et al., 1981).

Kanagawa phenomenon positivity has been generally associated with enteropathogenicity (Sakazaki et al., 1968). However, noting that most reported outbreaks of this disease have so far been traced to seafoods (Blake et al., 1980) which harbour the Kanagawa negative strains, the risk of infection by this organism still remains within a population which consume large quantities of fish. In Tanzania, a reported outbreak of cholera-like disease occurred where Kanagawa positive V. parahaemolyticus was incriminated and the source of infection was suspected to be seafish, shellfish and freshwater fish which are the natural reservoirs of the Kanagawa negative strains

(Mhalu et al., 1982; Ijumba, 1984). It is therefore possible that infections due to these strains do occur in Kenya, particularly in areas where fish is consumed in large quantities like Mombasa and Kisumu but is either misdiagnosed or remain undiagnosed as the infection is usually mild and self-limiting.

The general antibiogram of V. parahaemolyticus isolates obtained here agrees with that established for the genus Vibrio (Farmer et al., 1985) although some slight variations of sensitivities exist. Finding one isolate which is sensitive to ampicillin is not surprising as Twedt et al. (1981) reported a number of strains to be sensitive to ampicillin. Resistance to trimethoprim, as observed in some of these isolates, has also been reported before (Arai et al., 1983a). However, the strain tested by Arai et al. (1983a) was also resistant to chloramphenicol, tetracycline, streptomycin and kanamycin unlike strain Vm 39 which was resistant to trimethoprim only among the agents to which the organism normally respond.

Conjugal R-plasmids coding for multiple drug-resistance have been reported in V. parahaemolyticus (Arai et al., 1985). In this study, only two strains (Vm 39 and Vm18) were found to bear light plasmids. The antibiograms of these isolates were like the other non-plasmid bearing isolates except that Vm 39 was the only strain resistant to trimethoprim. Arai et al. (1985)

indicated that trimethoprim resistant plasmid so far isolated was a heavy, stably transmissible plasmid which codes for multiple-drug resistance. Thus the observed resistance to trimethoprim by Vm 39 may be due to factors other than the two light plasmids. Therefore, these plasmids in the two isolates may be considered to be cryptic.

Cryptic plasmids in V. parahaemolyticus have been reported (Guerry and Colwell, 1977; Arai et al., 1983b) but their plasmids ranged in molecular weights from 6.2-7.5 md and 5.8-22.0 md respectively None of the plasmids found here fall within their range. Thus these may be cryptic plasmids which stably exist in some strains of this organism not yet reported before or acquired from other organisms through mobilization.

Only two of the twenty nine V. parahaemolyticus isolates contained plasmids. Compared to previous studies of Guerry and Colwell (1977) and Twedt et al. (1981), this is a low plasmid carriage. This variation in occurrence could be explained in part by the difference in source and place of the isolates were the frequency of use of antibiotics which are likely to induce acquisition of plasmids also vary. Due to the low occurrence of plasmids amongst the isolates in this study, it was not possible to establish any specific pattern hence plasmid profiling as an epidemiological tool has limited value here.

The results of slide coagglutination test showed that only 14% of the isolates were identifiable by the antiserum. As the antigen for raising the antiserum was prepared in a way as to leave only the thermostable 'O' antigen active, it could be assumed that the four isolates that coagglutinated have homologous 'O' antigens. Among the typable isolates, only one strain has the same serotype as the reference strain (O4:K42) used for raising the antiserum. This serotype and three others were the ones which were picked by the antiserum.

Shinoda et al. (1983) stated that 'O' antisera of V. parahaemolyticus are not very specific for species as they react with some strains of V. alginolyticus and V. harveji. Therefore, although coagglutination increases the sensitivity of the 'O' antiserum, the lack of specificity limits the use of this test in epidemiological survey and as a rapid identification tool.

5.3 AEROMONAS HYDROPHILA GROUP

The results of isolation of motile Aeromonas species showed variation in prevalence of this organism in fish from different areas and that a relatively higher frequency of isolation was recorded from fish (48%) than water (28%). This could be due to the fact that this organism, being an indicator of pollution

(Rippey and Cabelli, 1980; Kaper et al., 1981), has higher occurrence in polluted waters. This could, therefore, imply that high rate of this species in some areas such as R. Sombeli, is an indication of high rate of pollution of these areas. The higher isolation rate from fish may be attributed to the fact that the organisms tend to be concentrated on fish during feeding, particularly on the gills and intestine (Kaper et al., 1981; Krovacek et al., 1987).

During the primary isolation using A. Tween 80, Aeromonas species were identified easily from the other species because of the haloes formed around the colonies. The medium has also the advantage of distinguishing Aeromonas species from lactose positive E. coli as the colour of Aeromonas colonies were pale rather than pink.

Differentiation of the Aeromonas isolates into species was done by using two methods, i.e. biochemical methods as recommended by Barer et al. (1986a) and detecting CAMP-like factor according to Figura and Guglielmetti (1987). Results of the two methods differ as can be seen in Table III. The biochemical method in this case was considered to be more accurate and reliable because it gave consistent results when the test was repeated. Also there was no variation of results with the reference strains which were always included in the tests. On the other hand, it was

noticed that the results obtained by using CAMP-like factor method varied when the test was repeated. This method consistently gave higher values of A. caviae and lower A. hydrophila than that obtained in biochemical tests. This could be attributed to the fact that haemolysis produced by Aeromonas species is not as complete as that produced by positive control thus the results were sometimes inconclusive, moreover, there was always a zone of haemolysis inhibition around the clear zone which could give false results. This test was considered to be unreliable. However, because of its quick results (only 18 to 24 hrs) the test becomes a very useful method for preliminary screening of Aeromonas isolates which should be confirmed by biochemical means.

Despite differences in results of the two methods, the general trend for both was that the predominant species among the isolates was A. caviae, 42% for biochemical methods and 44% for CAMP-like detection, followed by A. hydrophila, 37% and 35% and A. sobria 21% and 22% respectively. These findings agree with those of previous workers (Daily et al., 1981; Pittock and Donovan, 1986) who reported predominance of A. caviae in their isolates of Aeromonas species from the environment. In clinical isolates, A. sobria predominates followed by A. hydrophila while A. caviae is rarely recovered (Daily et al., 1981; Barer et al., 1986a).

Of the various systems that have been developed for assaying toxin production by motile Aeromonas species, the suckling mouse test and cell cultures using vero cells were employed because of their reproductivity, comparatively high sensitivity and ease of performance (Burke et al., 1981a; Barer et al., 1986a; Notermans et al., 1986). Although the figures for the two systems vary, the general trend was that the highest frequency of toxigenic strains occur among A. sobria (51%) by suckling mouse test and 85% by vero cells followed by A. hydrophila (35%) in suckling mouse test and 63% in vero cells followed by A. caviae (6% in mouse test and 18% in vero cells). This kind of trend had been noted earlier (Janda et al., 1985; Gosling, 1986; Janda and Brendsen, 1987).

The variation in results of the two methods can be explained by the fact that cell cultures are highly sensitive to toxins as compared to animal models (Asao et al., 1986; Morgan et al., 1985; Bunning et al., 1986). Another explanation for this variation could be the fact that suckling mouse test detects enterotoxin while cell culture system detects cytotoxin production (Kindschuh et al., 1987; Potomski et al., 1987a). This contention was however disputed by other workers (Asao et al., 1984; Asao et al., 1986; Millership et al., 1986) who claimed that the two systems detect the same toxin.

Some variations in drug susceptibilities of Aeromonas isolates were found with multiple resistance being manifested by all the isolates with varying degree. One strain (K43) from L. Victoria was resistant to all the antimicrobial agents tested. Species difference in drug susceptibility has been reported where A. hydrophila is found to be more resistant than A. sobria to antimicrobial agents especially the penicillins and cephalosporins (Motyl et al., 1985; Chang and Bolton, 1987). Such a relationship could not be established here because the frequency of resistance among the species vary from one drug to another. But it is worth noting that the only isolate resistant to all tested antimicrobial agent was A. hydrophila and out of four isolates resistant to nine agents, two were A. hydrophila while the other two were A. sobria and A. caviae.

Resistance to beta-lactams as shown here by all the tested isolates have been reported before (Richardson et al., 1982). This is due to the fact that the majority of Aeromonas species produce beta-lactamase. There are, however, some strains that have been reported to be beta-lactamase negative, hence ampicillin sensitive (McNicol et al., 1980; Richardson et al., 1982) although none of such strains was found in these isolates.

It has been reported that freshwater bacteria

such as Aeromonas and Edwardsiella share common resistance-plasmids and the predominantly occuring among this group are those that code for sulphonamides and tetracyclines (Aoki et al., 1977). In this study, it was not possible to determine whether any of the plasmids detected specifically codes for the multiple resistance observed among the isolates. However, one isolate (K43) which was resistant to all tested antimicrobial agents also carried six plasmids including a heavy one. It might be possible that one of them, especially the heavy one, might be encoding the observed multiple resistance.

The plasmids detected in these isolates could not be associated with enterotoxin production by these organisms as the assay for enteropathogenicity showed that some of the plasmid carrying isolates as well as some non-plasmid bearers were enterotoxin producers. A Similar conclusion was drawn by Cumberbatch et al. (1979) who reported that half of their toxigenic strains did not carry plasmids. This conclusion is further supported by the demonstration that different genes located on different chromosomal segments code for production of various toxins by A. hydrophila (Chakraborty et al., 1986; Howard et al., 1987).

Screening all the isolates for presence of plasmids revealed that only 9% carried plasmids having molecular weights between 1 Md to 60 Md. This is a low

plasmid carriage frequency as compared with the results of Chang and Bolton (1987). They observed a frequency rate of 26.7% from their wild-type of Aeromonas isolates. This difference could be attributed to differences in origin and source of the two groups of isolates as the use of antibiotics, and the level of environmental pollution which induce acquistion of plasmids (Toranzo et al., 1983; Chang and Bolton, 1987) vary from one geographical zone to another.

The use of plasmid profiling as an epidemiological tool for tracing transmission of pathogenic organism from animals to humans, differentiating organisms into species and diagnosis has been advocated (Tacket et al., 1984; Olsvik et al., 1985; Kvello et al., 1987). Genetic comparison of different isolates can show whether the isolates are from the same clone or not. Results of the plasmid profiles here indicated that most of the strains did not fall into specific plasmid pattern groups as their plasmid numbers and sizes varied widely. This could mean that either these strains do not have a common origin or they were exposed to different conditions that could induce plasmids and have acquired plasmids from different sources. There were however few isolates from either the same area (N53 and N54 both from L. Naivasha) or different places (No 1 from L. Victoria and M 17 from Masinga Dam) having the same numbers and sizes of

plasmids. These could have come from a common origin even when they were isolated from different lakes as there is a possiblity of transferring them from one place to another by running streams and rivers.

The results of testing all the isolates for coagglutination showed that only 8% of the isolates shared common 'O' antigen with the reference strain used for raising the antiserum. The low percentage of serologically homologous strains to the antiserum indicates that this genus possesses many different antigenic groups. So far, about 44 antigenically different groups have been identified (Sakazaki, 1986). This large number of antigenic groups amongst this genus makes this method ineffective as rapid means of identification of either the species or the genus itself because of the difficulties involved in raising a useful bank of sera which would incorporate all the groups.

Two isolates of A. sobria also agglutinated with the antiserum. This means that some strains of this species share common 'O' antigens with A. hydrophila. It has been reported that most virulent strains of A. hydrophila and A. sobria share 'O' serogroup antigens (Dooley et al., 1986; Janda et al., 1987). However, this relationship was not established here.

CONCLUSIONS

From this study, the following conclusions could be drawn:

1. Bacterial counts of the fish and water samples from L. Naivasha, Masinga Dam and L. Magadi fall within the same range as those of two previous studies from other parts of Kenya.
2. Vibrio parahaemolyticus and motile Aeromonas species were isolated in varying frequencies from Kenyan waters and fish.
3. All the V. parahaemolyticus isolates were Kanagawa phenomenon negative.
4. The V. parahaemolyticus isolates showed a normal sensitivity pattern to antibiotics tested which could mean that these isolates have not been exposed to antibiotics.
5. The isolates had low plasmid carriage hence no plasmid pattern could be established.
6. Coagglutination technique using somatic 'O' antigen was found to have limited use as a method for rapid identification of V. parahaemolyticus.
7. Motile Aeromonas species were found to occur in both freshwater and marine environs of Kenya. Of the three species identified, A. caviae was found to be most prevalent, followed by A. hydrophila and A. sobria.

8. Some of the isolates were found to produce enterotoxins and cytotoxins.

9. The isolates showed variations in their susceptibility patterns to antibiotic with some having multiple resistance.

10. The usefulness of plasmid profiling in epidemiological studies and diagnosis in this organism could not be assessed properly because of the low plasmid carriage amongst the isolates, and that the few isolates that bear plasmids did not fall into specific pattern.

11. The isolation of toxigenic and resistant strains of motile Aeromonas species from fish and water indicates a potential reservoir and possible public health hazard for both fish handlers and consumers.

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APPENDICES

Appendix 1.1 TOTAL BACTERIAL COUNT FOR LAKE NAIVASHA

SAMPLE NUMBER	DILUTIONS					BACT. COUNT/ ML OF SAMPLE
	-1	-2	-3	-4	-5	
NF1	-	-	-	288	30	3×10^{4}
NF2	-	-	-	380	32	3.2×10^{4}
NF3	-	-	-	625	102	1.02×10^{5}
NF4	-	-	-	400	50	5.0×10^{4}
NF5	-	-	-	114	20	2.0×10^{4}
NF6	-	-	-	360	29	2.9×10^{4}
NF7	-	-	-	250	12	1.2×10^{4}
NF8	-	-	-	444	41	4.1×10^{4}
NF9	-	-	-	660	70	7.0×10^{4}
NF10	-	-	-	171	9	9.0×10^{3}
NW1	-	80	-	-	-	8.0×10^{3}
NW2	118	19	-	-	-	1.9×10^{3}
NW3	161	57	-	-	-	5.7×10^{3}

Ten fish samples, all tilapias, and three water samples were processed for total bacterial count.

Appendix 1.2 Total bacterial count for Lake Magadi samples.

SAMPLE NUMBER	DILUTIONS						BACT.COUNT/ ML OF SAMPLE
	-1	-2	-3	-4	-5	-6	
MF1	-	-	113	53	37	13	1.3×10^{3}
MF2	-	-	520	21	3	-	3.0×10^{5}
MF3	-	-	160	122	26	9	9.0×10^{4}
MF4	-	-	-	553	225	113	1.3×10^{5}
MW1	-	140	95	26	-	-	2.6×10^{5}
MW2	-	3	-	-	-	-	3.0×10^{4}
MW3	-	25	5	-	-	-	3.0×10^{4}

Four fish samples (Catfish) MF1 - MF4 and three water samples MW1 - MW3 were tested

NB: MW1 was a sample from Sombeli River.

MW2 and MW3 were from Lake Magadi at different points.

All four fish samples were from Sombeli River.

Appenndix 1.3 TOTAL BACTERIAL COUNT FOR MASINGA DAM

SAMPLE NUMBER	DILUTIONS						BACT. COUNT/ ML OF SAMPLE
	-1	-2	-3	-4	-5	-6	
MDF1	-	-	350	200	117	87	8.7×10^7
MDF2	-	-	-	415	120	112	1.2×10^8
MDF3	-	-	105	37	13	7	7.0×10^6
MDF4	-	-	300	48	10	4	4.0×10^6
MDF5	-	-	30	17	3	-	3.0×10^5
MDF6	-	-	-	250	31	2	2.0×10^6
MDF7	-	-	-	375	112	61	6.1×10^7
MDW1	200	30	5	2	-	-	2.0×10^3
MDW2	250	42	9	3	3	-	3.0×10^5
MDW3	325	39	7	1	-	-	1.0×10^4

Seven fish samples MDF1 - MDF7 and three water samples MDW1 - MDW3 were used in determining the count from Masinga Dam.

Appendix 1.4 DETERMINATION OF SALT REQUIREMENTS OF THE VIBRIO PARAHAEMOLYTICUS ISOLATES.

Isolates	Optical densities at different Nacl concentrations							
	0	1	3	6	8	9	10	13
VM1	0.08	0.41	0.55	0.45	0.21	0.10	0.04	0.003
VM2	0.10	0.39	0.51	0.36	0.15	0.13	0.08	0.009
VM3	0.05	0.28	0.47	0.22	0.19	0.12	0.07	0.010
VM5	0.07	0.30	0.41	0.24	0.13	0.12	0.09	0.015
VM7	0.07	0.35	0.47	0.29	0.20	0.10	0.06	0.007
VM10	0.05	0.34	0.48	0.23	0.17	0.11	0.03	0.009
VM13	0.09	0.32	0.50	0.30	0.16	0.12	0.08	0.01
VM15	0.08	0.37	0.49	0.40	0.25	0.17	0.10	0.009
VM16	0.10	0.41	0.49	0.37	0.24	0.14	0.09	0.05
VM17	0.10	0.45	0.61	0.42	0.21	0.10	0.03	0.009
VM18	0.09	0.41	0.50	0.31	0.22	0.13	0.02	0.008
VM20	0.08	0.39	0.45	0.22	0.13	0.13	0.10	0.018
VM21	0.05	0.34	0.50	0.34	0.17	0.10	0.03	0.004
VM36	0.10	0.33	0.49	0.29	0.19	0.11	0.07	0.008
VM38	0.12	0.39	0.52	0.24	0.12	0.09	0.07	0.010
VM39	0.08	0.38	0.44	0.29	0.17	0.09	0.007	0.003
VM41	0.14	0.44	0.56	0.40	0.22	0.13	0.09	0.009
VM42	0.08	0.40	0.51	0.34	0.16	0.12	0.09	0.010
VM45	0.10	0.37	0.49	0.37	0.20	0.14	0.08	0.009
VM46	0.09	0.30	0.49	0.31	0.16	0.05	0.01	0.003
VM48	0.05	0.30	0.42	0.16	0.10	0.08	0.02	0.007
VM51	0.11	0.41	0.53	0.32	0.17	0.12	0.06	0.009
VM52	0.08	0.28	0.47	0.28	0.16	0.14	0.08	0.01

Appendix 1.4 (cont.)

Isolates	Optical densities at different Nacl concentrations							
	0	1	3	6	8	9	10	13
VM54	0.06	0.31	0.42	0.24	0.14	0.10	0.09	0.09
VM55	0.09	0.37	0.41	0.20	0.12	0.06	0.01	0.09
VM56	0.11	0.44	0.51	0.40	0.19	0.18	0.10	0.006
VM58	0.06	0.30	0.45	0.24	0.14	0.14	0.10	0.008
VK60	0.14	0.30	0.32	0.22	0.12	0.08	0.06	0.004
VK70	0.125	0.23	0.145	0.135	0.115	0.06	0.09	0.005
NICE D 42	0.04	0.29	0.41	0.36	0.17	0.13	0.05	0.007
NICE D 82	0.09	0.33	0.50	0.39	0.14	0.12	0.09	0.015
NICE D 134	0.11	0.37	0.62	0.51	0.29	0.18	0.07	0.008

Appendix 1.5 SEROTYPES OF VIBRIO PARAHAEMOLYTICUS STRAINS

SERIAL No.	STRAIN No.	TCBS	SEROLOGY
1	VM1	T	UT
2	VM2	T	02:K28
3	VM3	T	UT
4	VM5	T	UT
5	VM7	T	UT
6	VM10	T	03:K29
7	VM13	T	UT
8	VM15	T	UT
9	VM16	T	03:K29
10	VM17	T	UT
11	VM18	T	UT
12	VM20	T	UT
13	VM21	T	UT
14	VM36	T	02:K28
15	VM38	T	UT
16	VM39	T	UT
17	VM41	T	04:K42
18	VM42	T	UT
19	VK60	T	UT
20	VN70	T	UT

No. of strains = 20

Untypable (UT) = 15

Serotyped = 5

Kindly serotyped by Dr. S.C Pal of National Institute of Cholera and Enteric Diseases, WHO Collaborating Centre for Diarrhoeal Research and Training, Calcutta, India

Appendix 1.6 Determination of enterotoxigenicity of Aeromonas strains from Lake Turkana using suckling mice.

STRAINS	1W	RBW	1W/RBW	RESULT
T12	0.18	2.20	0.082	-ve
T68		0.77	0.067	-ve
T78	0.16	1.79	0.089	+ve
T94	0.18	2.16	0.083	+ve
T93	0.13	1.98	0.066	-ve
T89	0.20	2.14	0.093	+ve
T86	0.23	2.41	0.095	+ve
T61	0.18	1.79	0.100	+ve
T75	0.11	1.72	0.064	-ve
T65	0.19	2.07	0.092	+ve
T98	0.17	1.73	0.098	+ve
T82	0.21	1.95	0.108	+ve
T92	0.15	1.85	0.081	-ve
T60	0.16	1.77	0.090	+ve
T80	0.12	1.68	0.071	-ve
T36	0.15	2.13	0.070	-ve
T67	0.14	1.86	0.075	-ve
T97	0.11	1.69	0.065	-ve
T92	0.16	1.99	0.080	-ve
T63	0.13	1.81	0.072	-ve
T95	0.15	1.92	0.078	-ve
T73	0.12	2.10	0.057	-ve
T77	0.10	1.78	0.056	-ve

Appendix 1.6 (cont.)

STRAINS	1W	RBW	1W/RBW	RESULT
T71	0.15	2.24	0.067	-ve
T74	0.16	2.12	0.075	-ve
T90	0.13	1.83	0.071	-ve
T69	0.14	1.81	0.077	-ve
T85	0.11	1.75	0.063	-ve
T96	0.14	1.62	0.086	+ve
T70	0.13	1.63	0.079	-ve
T9	0.13	2.01	0.065	-ve
T55	0.15	2.10	0.071	-ve
T88	0.10	1.60	0.063	-ve
T79	0.13	1.69	0.077	-ve
T72	0.16	2.02	0.079	-ve
T84	0.18	2.43	0.074	-ve
T34	0.14	1.75	0.080	-ve
T87	0.12	1.88	0.064	-ve
T10	0.30	2.12	0.142	+ve
T8	0.15	2.07	0.072	-ve
T3	0.15	1.99	0.075	-ve
T30	0.19	1.89	0.100	+ve
T2	0.13	1.98	0.066	-ve
T42	0.16	2.10	0.076	-ve
T27	0.14	1.89	0.074	-ve
T76	0.17	2.26	0.075	-ve

NOTE: 1W = Intestinal weight

RBW = Rest of body weight

Appendix 1.7 Determination of enterotoxigenicities of 22 Aeromonas strains from coastal waters using suckling mice.

STRAIN	IW	RBW	IW/RBW	RESULT
C63	0.17	1.83	0.093	+ve
C51	0.10	1.69	0.059	-ve
C54	0.15	1.77	0.085	+ve
C20	0.12	1.81	0.066	-ve
C32	0.21	1.68	0.125	+ve
C61	0.17	2.25	0.076	-ve
C47	0.11	1.72	0.064	-ve
C49	0.20	1.59	0.125	+ve
C45	0.20	2.07	0.096	+ve
C10	0.29	2.69	0.107	+ve
C50	0.18	2.16	0.083	+ve
C46	0.18	3.08	0.058	-ve
C60	0.11	1.80	0.061	-ve
C68	0.15	1.99	0.075	-ve
C65	0.12	1.97	0.061	-ve
C44	0.17	2.0	0.085	+ve
C7	0.19	1.82	0.104	+ve
C55	0.13	2.05	0.063	-ve
C58	0.16	2.40	0.067	-ve
C42	0.11	2.12	0.051	-ve
C48	0.08	1.95	0.041	-ve
C24	0.18	3.22	0.056	-ve

appendix 1.8 Determination of enterotoxigenicities of 35 Aeromonas strains from Lake Victoria using suckling mice.

STRAIN	IW	RBW	IW/RBW	RESULT
K43	0.18	1.96	0.092	+ve
K46	0.17	2.12	0.080	-ve
K40	0.16	1.69	0.095	+ve
K31	0.13	1.77	0.073	-ve
K51	0.15	1.98	0.076	-ve
K41	0.10	1.69	0.059	-ve
K410	0.12	1.80	0.067	-ve
K36	0.16	2.00	0.080	-ve
K37	0.14	2.10	0.067	-ve
K56	0.15	1.76	0.085	+ve
K18	0.11	1.83	0.060	-ve
K16	0.15	2.54	0.059	-ve
K27	0.14	1.99	0.070	-ve
K39	0.17	2.25	0.075	-ve
K30	0.12	1.78	0.067	-ve
K47	0.20	1.99	0.101	+ve
K34	0.11	2.00	0.055	-ve
K58	0.16	1.98	0.080	-ve
K44	0.16	2.11	0.076	-ve
K38	0.13	1.73	0.075	-ve
K25	0.14	1.91	0.073	-ve

Appendix 1.8 (cont.)

STRAIN	IW	RBW	IW/RBW	RESULT
K23	0.17	2.32	0.073	-ve
K45	0.21	2.25	0.093	+ve
K42	0.15	1.89	0.074	-ve
K50	0.17	1.82	0.093	+ve
K90	0.12	1.77	0.068	-ve
K64	0.14	1.78	0.079	-ve
K67	0.12	1.65	0.072	-ve
K75	0.16	2.25	0.071	-ve
K76	0.13	1.88	0.069	-ve
K60	0.10	1.90	0.053	-ve
K87	0.15	1.98	0.076	-ve
K68	0.19	1.83	0.104	+ve
K53	0.15	1.76	0.085	+ve
K38	0.12	1.65	0.072	-ve

Appendix 1.9 Determination of enterotoxigenicities of *Aeromonas* strains from Lake Naivasha using suckling mice.

STRAINS	IW	RBW	IW/RBW	RESULT
N66	0.12	2.12	0.057	-ve
N45	0.16	2.55	0.058	-ve
N54	0.19	2.67	0.071	-ve
N51	0.11	1.62	0.068	-ve
N53	0.25	1.78	0.140	+ve
N78	0.23	2.56	0.090	+ve
N65	0.13	1.96	0.66	-ve
N41	0.15	2.54	0.059	-ve
N98	0.12	2.11	0.057	-ve
N67	0.14	2.22	0.063	-ve
N56	0.16	2.66	0.060	-ve
N90	0.18	3.22	0.057	-ve
N82	0.17	1.59	0.107	+ve
N105	0.15	1.90	0.079	-ve
N52	0.16	2.10	0.076	-ve
N87	0.19	1.92	0.098	+ve
N88	0.13	1.81	0.072	-ve
N97	0.16	1.80	0.088	+ve
N92	0.18	2.00	0.090	+ve
N103	0.17	1.83	0.093	+ve
N61	0.10	1.79	0.056	-ve
N101	0.13	2.11	0.062	-ve
N102	0.11	1.89	0.058	-ve
N70	0.16	2.43	0.066	-ve

Appendix 1.9 (cont.)

STRAINS	IW	RBW	IW/RBW	RESULT
N68	0.12	1.91	0.063	-ve
N42	0.15	2.22	0.067	-ve
N64	0.10	1.79	0.056	-ve
N55	0.19	1.89	0.101	+ve
N85	0.14	2.11	0.066	-ve
N44	0.14	2.00	0.070	-ve
N49	0.11	1.74	0.063	-ve
N100	0.12	1.96	0.061	-ve

Appendix 1.10 Determination of enterotoxigenicities of *Aeromonas* strains from Masinga Dam using suckling mice.

STRAINS	IW	RBW	IW/RBW	RESULT
M45	0.16	2.35	0.068	-ve
M30	0.12	1.79	0.067	-ve
M32	0.11	1.85	0.060	-ve
M9	0.20	2.03	0.098	+ve
M29	0.15	1.78	0.084	+ve
M46	0.11	2.49	0.045	-ve
M54	0.29	2.85	0.102	+ve
M34	0.29	2.75	0.105	+ve
M17	0.18	2.14	0.084	+ve
M33	0.14	1.87	0.075	-ve
M51	0.12	1.98	0.060	-ve
M39	0.12	2.52	0.047	-ve
M11	0.12	2.27	0.053	-ve
M35	0.16	1.84	0.087	+ve
M37	0.08	1.95	0.041	-ve
M44	0.13	2.55	0.051	-ve
M38	0.10	2.00	0.050	-ve
M5	0.18	2.22	0.081	-ve
M20	0.14	1.90	0.074	-ve
M49	0.18	1.75	0.103	+ve
M6	0.15	1.78	0.084	+ve
M41	0.10	1.69	0.059	-ve
M3	0.12	2.10	0.057	-ve
M19	0.17	2.20	0.077	-ve
M15	0.16	1.81	0.088	+ve
M1	0.13	1.74	0.075	-ve

Appendix 1.11 Determination of enterotoxigenicities of *Aeromonas* strains from River Sombeli using suckling mice.

STRAINS	IW	RBW	IW/RBW	RESULT
S2	0.13	2.01	0.065	-ve
S3	0.14	1.62	0.086	+ve
S5	0.14	1.81	0.077	-ve
S7	0.16	1.99	0.080	-ve

Appendix 1.12 Determination of cytotoxicities of *Aeromonas* species from Lake Kisumu.

SERIAL NO.	STRAIN NO.	SPECIES	RESULTS
1	K43	A. hydrophila	+ve
2	K46	A. caviae	-ve
3	K40	A. caviae	-ve
4	K31	A. hydrophila	+ve
5	K51	A. hydrophila	+ve
6	K41	A. sobria	+ve
7	K410	A. hydrophila	-ve
8	K36	A. caviae	-ve
9	K37	A. hydrophila	+ve
10	K56	A. sobria	+ve
11	K18	A. caviae	-ve
12	K16	A. hydrophila	-ve
13	K27	A. caviae	+ve
14	K39	A. sobria	+ve
15	K30	A. hydrophila	+ve
16	K47	A. hydrophila	-ve
17	K34	A. hydrophila	+ve
18	K58	A. caviae	+ve
19	K44	A. hydrophila	+ve
20	K38	A. sobria	-ve
21	K25	a. hydrophila	-ve
22	K23	A. hydrophila	+ve
23	K45	A. caviae	-ve

Appendix 1.12 (Cont.)

24	K42	A. hydrophila	-ve
25	K50	A. caviae	-ve
26	K90	A. hydrophila	+ve
27	K64	A. caviae	-ve
28	K67	A. hydrophila	+ve
29	K75	A. sobria	+ve
30	K76	A. caviae	-ve

Appendix 1.13 Determination of cytotoxicity of Aeromonas species from Lake Turkana.

SERIAL NO	STRAIN NO	SPECIES	RESULTS
1	T12	A. hydrophila	+ve
2	T93	A. hydrophila	+ve
3	T65	A. sobria	+ve
4	T80	A. caviae	+ve
5	T36	A. caviae	-ve
6	T98	A. sobria	+ve
7	T92	A. hydrophila	+ve
8	T96	A. sobria	+ve
9	T82	A. sobria	+ve
10	T97	A. hydrophila	-ve
11	T60	A. caviae	-ve
12	T8	A. caviae	-ve
13	T3	A. caviae	-ve
14	T30	A. hydrophila	+ve
15	T10	A. sobria	+ve

Appendix 1.14 Determination of cytotoxicity of Aeromonas species from coastal waters.

SERIAL NO	STRAIN NO	SPECIES	RESULTS
1	C63	A. hydrophila	+ve
2	C54	A. caviae	+ve
3	C32	A. sobria	+ve
4	C61	A. caviae	-ve
5	C42	A. caviae	-ve
6	C49	A. sobria	+ve
7	C45	A. hydrophila	-ve
8	C10	A. sobria	+ve
9	C20	A. hydrophila	+ve
10	C7	A. hydrophila	+ve

Appendix 1.15 Determination of cytotoxicity of Aeromonas species from Lake Naivasha.

SERIAL NO	STRAIN NO	SPECIES	RESULTS
1	N54	A. hydrophila	-ve
2	N56	A. caviae	+ve
3	N92	A. sobria	+ve
4	N94	A. sobria	-ve
5	N55	A. sobria	+ve
6	N42	A. caviae	-ve
7	N65	A. hydrophila	+ve
8	N88	A. hydrophila	-ve
9	N70	A. caviae	-ve
10	N78	A. sobria	+ve
12	N103	A. hydrophila	+ve

Appendix 1.16 Determination of cytotoxicity of Aeromonas species from Masinga Dam.

SERIAL NO	STRAIN NO	SPECIES	RESULTS
1	M1	A. hydrophila	-ve
2	M23	A. hydrophila	+ve
3	M6	A. sobria	+ve
4	M20	A. hydrophila	-ve
5	M11	A. caviae	-ve
6	M29	A. caviae	-ve
7	M37	A. caviae	-ve
8	M17	A. sobria	+ve
9	M41	A. sobria	-ve

Appendix 1.17 Determination of cytotoxicity of Aeromonas species from River Sombeli (Magadi)

SERIAL NO	STRAIN NO	SPECIES	RESULTS
1	S3	A. hydrophila	+ve
2	S2	A. caviae	-ve
3	S5	A. caviae	-ve
4	S7	A. caviae	+ve

Appendix I.18 Plasmid bearing Aeromonas and their molecular weights.

SERIAL NO	STRAIN NO	PLACE OF ISOLATION	SPECIES OF ISOLATES	NO. OF PLASMIDS	MOLECULAR WEIGHTS OF PLASMIDS IN MEGADALTONS
1	K43	L. Victoria	A. hydrophila	6	33.8 5.44 4.8 3.08 2.7o 2.o
2	CK26	L. Victoria	A. hydrophila	5	37 18 4.3 4.0 2.1
3	4K81	L. Victoria	A. sobria	4	36 3.0 3.0
4	C68	Coast	A. hydrophila	4	o.o 3.04 2.44
5	BK1	L. Victoria	A. caviae	3	60 30 3.9
6	M9	Masinga Dam	A. hydrophila	3	46 4.22 3.04
7	NO1B	L. Victoria	A. caviae	3	30 o.0 4.8
8	4K77	L. Victoria	A. hydrophila	3	8 2.8 2.o
9	N41	L. Naivasha	A. caviae	3	5.4 4.5 2.15
10	K54	L. Victoria	A. sobria	3	3.2 1.7 1.o
11	K36(a)	L. Victoria	A. sobria	3	3.0 1.o 1.5
12	4K50	L. Victoria	A. hydrophila	2	30 3.9
13	AK18	L. Victoria	A. caviae	2	33.8 3.4
14	K25	L. Victoria	A. caviae	2	18 3.0
15	K36	L. Victoria	A. hydrophila	2	4.8 3.5
16	446	L. Victoria	A. hydrophila	2	4.2 2.2
17	M45	Masinga Dam	A. caviae	2	3.7 2.15
18	CK27	L. Victoria	A. sobria	2	3.7 2.0
19	M46	Masinga Dam	A. hydrophila	1	18
20	No1(S)	L. Victoria	A. caviae	1	o.o
21	M17	Masinga Dam	A. sobria	1	o.o
22	M34	Masinga Dam	A. caviae	1	o.0
23	A. media	Ref. strain	AICC 33907	1	4.o
24	N45	L. Naivasha	A. caviae	1	4.47

Appendix 1.1B (cont.)

SERIAL NO	STRAIN NO	PLACE OF	SPECIES	NO. OF	MOLECULAR WEIGHTS OF PLASMIDS IN MEGADALTONS
25	N54(i)	L. Naivasha	A. hydrophila	1	4.36
26	N53	L. Naivasha	A. sobria	1	4.36
27	N54(ii)	L. Naivasha	A. caviae	1	4.36
28	N65	L. Naivasha	A. caviae	1	3.7
29	N66	L. Naivasha	A. hydrophila	1	3.7
30	N51	L. Naivasha	A. hydrophila	1	1.85
31	T39	L. Turkana	A. caviae	1	1.55
32	T68	L. Turkana	A. hydrophila	1	1.0

Appendix 1.19 Number of plasmids of the isolates and their sensitivities to 14 antimicrobial agents.

ISOLATES OF *AEROMONAS* TESTED	SPECIES OF ISOLATES	NO. OF PLASMIDS	NO. OF AGENTS RESISTANT TO	NO. OF AGENTS SENSITIVE TO
K43	A. hydrophila	6	14	0
M9	A. hydrophila	3	9	5
M17	A. sobria	1	9	5
M45	A. caviae	1	9	5
M66	A. hydrophila	1	9	5
M65	A. caviae	1	8	6
M53	A. sobria	1	7	7
M45	A. sobria	2	6	8
M54(i)	A. hydrophila	1	6	8
M54(ii)	A. sobria	1	6	8
41KEM	A. sobria	0	8	6
65KEM	A. hydrophila	0	7	7
M29	A. caviae	0	6	8
A. caviae	Reference strain ATCC 15468	0	10	4
A. hydrophila	Reference strain ATCC 7966	0	7	7
A. sobria	Reference strain ATCC 9071	0	6	8

Appendix 2.1 Glucose Salt Teepol Broth (GSTB)

Composition

Beef stract (Bacto Difco Laboratories)	3.0g
Tryptone (Lab M, England)	10.0g
Sodium chlorine	30.0g
Glucose	5.0g
Methylviolet	0.002g
Teepol (Shell product)	4.0ml
Distilled water	1000 ml

The ingredients were dissolved in the cold and the pH adjusted to 9.4. Subsequent sterilization was done at 121°C for 15 minutes after which the mixture was asceptically dispensed into universal bottles as required.

Appendix 2.2 TCBS Cholera Medium (Oxoid, England).

88g of medium was weighed and suspended in 1000 mls distilled water. The medium was boiled to dissolve completely and poured into sterile plates without autoclaving. The agar was allowed to dry before use.

Appendix 2.3 Triphenyltetrazolium chloride soya tryptone agar (TSAT) (Kourany, 1983)

Composition

Trypticase soy agar (Gibco, Europe)	40g
Sodium chloride	25g
Sucrose	20g
Bile salts No.3 (Difco Laboratories, USA)	0.5g
1% Triphenyltetrazolium chloride solution	3ml
Distilled water	1000ml

The ingredients were boiled to dissolve completely and sterilised by autoclaving at 121°C for 15 minutes. The mixture was poured into sterile plates and used after drying.

Appendix 2.4 Wagatsuma Blood Agar (WBA)

Composition

Yeast Extract (Gibco Bio-cult Diagnostics)	3g
Peptone (Oxoid, England)	10g
Sodium chloride	70g
Dipotassium phosphate	5g
Mannitol	10g
Crystal violet	10g
Distilled water	1000ml

Appendix 2.4 (cont.)

The basal medium was dissolved by heating and cooled to 50°C before adding washed fresh human erythrocytes to 5% concentration. It was then dispensed into sterile plates.

Appendix 2.5 Tryptose Soya Agar (TSA) (Oxoid, England).

40g of medium was weighed and dissolved in 1000 ml of distilled water by boiling. It was sterilized by autoclaving at 121°C for 15 minutes and distrubuted into plates.

Appendix 2.6 Tryptose Soya Broth - Yeast Extract (TSB-YE)

composition

Tryptose soya broth (Gibco, Europe)	30g
Yeast extract (Gibco-Cult Diagnostics)	6g
Distilled water	1000ml

The ingredients were dissolved and distributed into screw-capped tubes before autoclaving at 121°C for 15 minutes.

Appendix 2.7 A-Tween 80

composition

MacConkey agar without crystal violet (lab M, London)	52g
Tween-80	10ml
Calcium chloride	0.2g
Distilled water	1000ml

1000ml of the distilled water was heated and 10ml of Tween 80 was added to it to facilitate the Tween 80 to go into solution. All the ingredients were combined together and autoclaved at 121°C for 25 minutes.

Ampicillin	100mg
Sterile 0.1M phosphate buffer	2ml

The ampicillin was dissolved into 2 ml of PB and added directly to the cooled A-Tween 80 and mixed thoroughly before pouring into plates. The plates were stored at 4°C and used within one week.

Appendix 2.8 O/F Basal medium

composition

Peptone (lab M, London)	2.0g
Sodium chloride	5.0g
Dipotassium phosphate	0.3g
Bromothymol blue	0.03g
Agar No. 1 (Oxoid)	3.0g
Distilled water	1000ml

The ingredients were dissolved completely by boiling and distri-buted into screw capped tubes before autoclaving at 121°C for 15 minutes. Glucose solution was sterilised by filtration and added to the medium to a 1% concentration asceptically.

Appendix 2.9 Stuart Transport Medium (lab M, London)

13g of powder was weighed and mixed with 1000 ml of distilled water. It was dissolved by boiling and dispensed into 1/4 oz bijou bottles, filling to the brim. The bottles were securely closed and sterilized for 15 minutes at 121°C.

Appendix 2.10 TSI agar (lab M, London)

63g of powder was weighed and mixed with 1000ml of distilled water. It was brought to boil, with frequent swirling to dissolve completely. It was distributed into tubes and sterilised for 15 minutes at 121°C. The tubes wew allowed to set in a slopped position so that the slant was over a deep but approximately 3 cm in depth.

Appendix 3.1 Solution 1

50mM glucose

25mM Tris-Hcl (pH 8.0)

10mM EDTA

Add lyzozyne to a concentration of 2 mg/ml when you use.

Preparation

stock lyzozyme solution 10 mg/ml in distilled water kept at -20°C

Solution a: store at 4°C

50% glucose 1.8ml

250 mM EDTA 4 ml

1M Tris-Hcl (pH 8.0) 25ml

Deionized water 51.7ml

Solution 1 = Sol. a (1.5 ml) + stock lysozyme (1.0 ml)

Appendix 3.2 Solution II

0.2M NaOH

1% SDS

Preparation

0.8g NaOH

1g SDS

Dissolve in 100 ml deionized water. Use within one week.

Appendix 3.3 Solution III

3M NaAC (pH 4.8)

Preparation

Dissolve 24.6g Na/ac in 50 ml deionized water. Add at least 10 ml glacial acetic acid. Continue adding till the pH is 4.8. Fill to 100 ml with deionized water.

Appendix 3.4 Solution IV

10mM Tris-Hcl (pH 8.0)

Preparation

1mM Na_2EDTA

1.21g Tris

0.37g EDTA

Dissolve in 500 ml of deionized water adjust pH with Hcl to 8.0 and fill upto 1 litre.

Appendix 3.5 Running buffer (Tris Acetate EDTA-/TAE)

40mM Trio acetate

2mM EDTA

pH = 7.4-8.0

Preparation

Stock 50 x TAE

100ml of 0.5 Na_2EDTA

2 42g of Tris base

Appendix 3.5 (cont.)

Dissolve in 500ml of deionized water. Add 50 ml of glacial acetic acid and continue adding till pH is about 8.0.

Running buffer = 20ml stock 50 x TAE
+ 980 ml of deionized water.
To be used only in one chamber.

Appendix 3.6 Loading buffer

0.25% bromothymol blue

0.25% xylene cyanol

30% glycerol