HYGIENIC INVESTIGATION OF FISH IN KENYA WITH SPECIAL EMPHASIS ON VIBRIO PARAHAEMOLYTICUS

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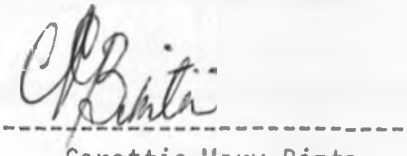
DECLARATION

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



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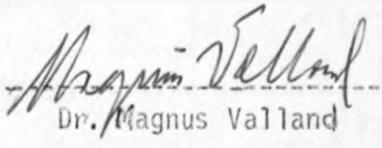

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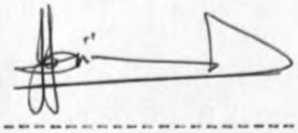

Dr. P.N. Nyaga

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*f I, II, III - Fujino et al., (1974)

*c V, VI, VIII, IX, X - Colwell, (1970).

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ABSTRACT

A survey was undertaken between November 1976 and 1977 to assess the hygienic quality of the fish sold in Kenya. This was accomplished by evaluating the total bacterial count and coliform count per gram of the sample. In addition, the prevalence rate of V. parahaemolyticus a halophilic bacterium causing gastroenteritis in humans, V. Cholerae biotypes and Salmonellae species was determined. The haemolytic properties, growth characteristics, egg pathogenicity and antimicrobial drug sensitivities of the V. parahaemolyticus isolates were studied. Electron microscopy techniques were employed to elucidate the morphology of isolate X_1G_1 later confirmed to be serotype O-10, K-23 of V. parahaemolyticus.

A total of 993 samples collected from the Coast Province and Nairobi area comprised of seafish, shellfish (crustacean and molluscan), sediment and lake fish. Seafish, water and sediment constituted 81.4% (912) of the total number of samples. Lake fish (81) accounted for 8.2%.

The total bacterial and coliform counts per gram of the sample were determined using Total Plate Count agar and Violet Red Bile Salt Dextrose agar medium respectively

Thermophilic coliforms were sought by incubating Violet Red Bile Salt Dextrose agar plates at 44°C instead of 37°C for 24 hours. Enrichment procedures for the isolation of V. parahaemolyticus, V. Cholerae and Salmonellae species were carried out in Glucose Salt Teepol Broth, Peptone water and

Tetrathionate or Selenite broths respectively. Subsequent plating for selective isolation was done on Thiosulphate Bile-Salt-Sucrose (TCBS) for V. parahaemolyticus and V. Cholerae, while Bromothymol Blue agar media were used for Salmonellae. Characteristic colonies thus obtained were subjected to appropriate biochemical tests after their morphologies in the light and electron microscopes, were studied.

Total bacterial counts ranged between 2×10^6 to 2×10^{12} per gram, of fresh sea fish samples. Salted smoke-dried lake fish exhibited bacterial counts ranging from 7×10^3 to 6×10^7 per gram. The coliform counts for both categories of fish appeared to bear a positive relationship to the corresponding total bacterial counts in each sample. The higher or lower the bacterial count, the higher or lower the coliform count.

Neither Salmonellae species nor V. Cholerae were isolated from the samples processed. An overall prevalence rate for V. parahaemolyticus of approximately 8.1% was computed for all the samples tested. Variation in the prevalence rates due to this bacterium existed among the sample types from Nairobi Area and the Coast Province. The prevalence rates differed for the sample types. The ubiquitous V. alginolyticus and Proteus vulgaris were isolated from all the marine samples. However there was a preponderance of Proteus vulgaris in all lake fish samples. Other flora recovered included Pseudomonas species from both marine and lake samples and Alcaligenes faecalis only from marine samples.

The optimal Sodium chloride (NaCl) concentration conducive for the propagation of serotype O-10, K-23 of V. parahaemolyticus was found to be between 1.5% and 3.5%. The growth of this serotype above 9.5% and below 1.5% NaCl was minimal before 24 hours, compared to that at optimal Sodium chloride concentrations. Ability to thrive and propagate below 1.5% Sodium chloride concentration in the medium was thought to be a strain adaptation. None of the V. parahaemolyticus isolated could survive at pH 4.5. The optimal pH for propagation was 8.2. No visible colonies were formed by the isolates when the TCBS agar plates were kept at $0^{o}C$ and $+4^{o}C$ although the viability of these cultures was certain after storage at these low temperatures.

No clear zone of β-haemolysis was observed around the colonies of the 72 V. parahaemolyticus isolates on Wagatsuma Blood Agar (WBA) medium, hence they were termed, Kanagawa negative (K^{-ve}). Similar mortality rates exhibited by the 10 day old chicken embryos after inoculation with serotype O-10, K-23 and the reference K^{-ve} culture was indicative of their relative multiplication rate and perhaps similar virulence.

It is therefore recommended that the Public Health Officials should exercise control over all handling and processing of fish and shellfish for marketing as well as educating the consumers in the hygienic handling and preparing of fish and other seafoods for consumption.

1. INTRODUCTION

Fish is a nutritious and relatively safe protein food. Fish and shell fish are important constituents of the diet in many parts of the world and with increasing shortage of protein due to increasingly high population pressures, fish will assume greater importance as a protein supplement. In 1974, statistics (WHO Technical reports, No.550: 1972) indicate the total world catch of aquatic animals and plants to amount to 65,600,000 tons of which 90% (59,400,000 tons) was fish of marine and fresh water origin and about 8% (5,200,000 tons), molluscan and crustacean shellfish. Extensive utilization of fish as food will increase concomitantly with the world's fishery resources. According to "Kenya's national report to the United Nations on the human environment," the annual fish production had expanded from 23,000 tons in 1965 to 34,000 tons in 1970. Such a boost in the fishing industry will certainly lead to changes in the production systems of fish and shellfish in Kenya. Consequently the use of efficient resource management techniques is of paramount importance, since failure of which will inevitably lead to an increase in fish-borne diseases. Such diseases are shown in table II for Japan. Table I on page 2 shows that many of the food poisoning outbreaks are due to fish, shellfish and their products.

Factors involved in disease causation would be those associated with catching, handling, processing and storage in addition to those special biological and ecological

Table I: Outbreaks of food poisoning and their causative foods, 1963-1971

Year	Number of outbreaks	Causative foods		
		Fishes, shellfishes and their products	Other foods and food-stuffs	Unknown
1963	1,970	1,065	440	465
1964	2,037	1,009	472	556
1965	1,208	579	314	315
1966	1,400	597	404	399
1967	1,565	659	393	513
1968	1,093	428	296	369
1969	1,360	511	366	483
1970	1,133	331	451	351
1971	1,118	517	502	387

Source: International Symposium on Vibrio parahaemolyticus; Edited by Fujino et al., (1974).

factors pertaining to the natural aquatic environment. There has been repeated associations of fish and shellfish with food poisoning outbreaks from all over the world. Not only are bacterial, viral and parasitic agents involved in these food poisoning outbreaks but also biotoxins produced by naturally toxic fishes as well as from chemical pollution of the marine environment. The "Minimata Bay" disease due to mercury poisoning is an example of the latter cause of food poisoning

Food poisoning outbreaks of bacterial origin include those due to Clostridium botulinum (type E and sometimes type B), and Vibrio parahaemolyticus, Vibrio cholerae and Salmonellae species. While the last three bacteria cause food infections, Clostridium botulinum causes an intoxication. The salient features of food poisoning due to V. parahaemolyticus and the above mentioned agents are given in table II on page 4. Unhygienic handling and processing would no doubt introduce secondary bacterial contaminants such as Salmonella and Shigella species, Escherichia coli; Staphylococcus aureus, Clostridium perfringens and many others. Raw oysters, clams and mussels have been implicated as reservoirs of Enteroviruses mainly viral hepatitis virus responsible for the "summer diarrhoea - winter vomiting" syndrome. Consumption of raw or under cooked fresh water fish, crustaceans or molluscan shellfish exposes the human consumer to also parasitic infections namely due to Diphyllobothrium latum, Clonorchis sinesis, Paragonimus species, Opisthorchis species and Heterophyes heterophyes.

Table II: Outbreaks of food poisoning due to bacteria, chemical agents and natural poisons, 1963-1972

Year	Number of outbreaks	total	Bacteria						Chemical agents	Natural poisons
			Salmonella	Staphylococcus	Cl. botulinum	V. parahaemolyticus	Pathogenic E. coli	Others		
1963	955	717	39	98	3	524	27	26	19	219
1964	1,003	738	38	105	1	558	8	28	17	248
1965	659	456	50	97	1	275	15	18	35	168
1966	751	484	59	97	0	284	24	20	20	247
1967	943	666	82	137	3	403	23	18	13	264
1968	663	486	54	109	5	266	27	25	21	156
1969	869	724	115	147	2	410	27	23	12	133
1970	756	576	95	167	4	267	22	21	8	172
1971	744	629	74	220	3	300	20	12	10	105
1972	931	802	74	246	1	436	26	19	9	120

Source: International symposium on Vibrio parahaemolyticus; Edited by Fujino et al., (1974)

Dinoflagellates and photosynthesizing marine micro-organisms such as the Gonyaulax, Gymnodinum and Pyronidinium species found in marine waters, constitute yet another fish and shellfish toxicity hazard. These toxins thus concentrated and harboured by the hostfish feeding on these microorganisms, are innocuous to them, in contrast to the human consumers who upon ingestion of the toxin-harbouring fish manifest neurotoxic symptoms. Scombroid fish poisoning syndrome simulating symptoms observed in humans or animals with a histamine allergy reaction is not uncommon in marine fish-eating communities. However, no such reports have been given for Kenya regarding the significance of the above mentioned hazards as most of the data compiled are from other countries. It is therefore not known whether these cases go undetected or are non-existent. The present study therefore, was undertaken with the following objectives in mind, namely:

(1) To assess the total bacterial count and the degree of faecal contamination in fish and fish products by the total plate count and coliform count per gram of the sample respectively.

(2) To eventually establish the existence of V. parahaemolyticus and to a minor extent, Enterobacteria in fish products using appropriate cultural and other identification techniques.

(3) To study the haemolytic properties and some growth characteristics of the V. parahaemolyticus isolates.

(4) And to test the hypothesis that fish and shellfish under certain circumstances are liable to act as potential foci for disease dissemination in Kenya.

2. LITERATURE REVIEW

2.1.

Ulrich, (1906) indicated the presence of bacteria in the muscles of newly caught fish, although it is generally held that the flesh of fish and the body fluids are sterile upon catching. Subsequent acquisition of the bacteria is therefore due to unhygienic handling, either during transportation or at marketing establishments even before it gets to the consumer (Shewan, 1949). Exposed areas of the fish namely the gills and the surface slime in addition to the intestines of "feedy" fish harbour large quantities of bacteria. Counts of 10^2 to 10^7 (at 20^oC) per cm^2 of skin adhering slime, of 10^3 to 10^8 and 10^3 to 10^6 per milliliter of intestinal fluid and per gram of gill tissue respectively are recorded for temperate sea water fish, (Shewan, 1949). Other investigators later substantiated his findings.

In the cold temperate waters where the temperature range in which fish are caught lies between -2^oC to $+ 12^oC$, the counts at 37^oC rarely exceed 5% of those at 0^oC or 20^oC. The numbers of mesophiles is larger in the warmer seas such as the waters off the African or Indian Coasts whereas a preponderance of Pseudomonas, Coryneforms and Achromobacter species was noted in temperate waters. Of the Pseudomonas group, P. pellicidum accounted for 40-50%. The other species included P. geniculatum, P. pavonaceae, P. nigrefaciens, P. schuylkilliensis and P. fluorescens. Achromobacter alcaligenes featured highly among the Achromobacter species. While these are an inherent innocuous flora, Salmonella species and other Enterobacteria are introduced through

secondary contamination (Shewan, 1949). Catching tends to increase the flora on the fish surface unless accompanied by careful washing (Borgström, 1961). Reduction in the number of bacteria can be of magnitudes ranging from 80-90% but the general consensus is that this has no influence on the generic distribution of the organisms. A proponderance of psychrophiles in the colder seas exists. It has been suggested that the quantitative variation in the flora of the slime and the gills, is seasonal and is a reflection of such variation in the adjacent environment of the fish. Such changes are more evident in the gills since they are not very much tampered with when fish are caught. Seasonal variations in both the quality and quantity of the bacterial which was observed has been attributed to the seasonal blooms of planktons. Some authors consider this to be due to the antagonistic and antibiotic effects exerted by some species of planktons to the bacterial flora.

Consequently, an inverse relationship exists between the growth of the plankton and the proliferation of some bacterial species. The temperature versus bacterial counts has been assessed by Georgala (1957) who reported that the psychrophile bacterial population thriving at $0^{o}C$ reaches its maximum growth when the temperatures are lowest. Furthermore, it is thought that the microenvironments created by the gills and the skin surfaces may account for both the quantity and quality of the bacteria present (Wessler and Werner, 1957). The method employed in catching the fish might also affect the bacterial load, that is whether the fish are trawled or lined.

Reports show heavier loads of bacteria for trawled fish (Borgström, 1961). According to Shewan (1949) trawled fish usually carry loads 10-100 times heavier than lined fish. This could be explained in terms of the fish picking up large numbers of bacteria from the sea bed and also the expression into the the immediate environment of the gut contents when trawled fish are hurled aboard the fishing vessel. Unlike the gills and the slime, the flora of the gut which reflects the nature of the food ingested is not subject to seasonal variation (Liston, 1955 and 1956). Earlier reports indicated that species variations in the total bacterial loads may be due to the constituents of the slime substratum native to each fish species (Wood, 1953). The preponderance of phychrophiles in temperate waters and the mesophiles in the wamer ones supports the idea that the type of aquatic environment determines the inherent flora. As a result of inefficient nomenclature of some of the marine bacteria, characterization of inherent flora, both psychrophiles and mesophiles, at species level, has been difficult. Therefore, most of the qualitative aspects of this subject are incomplete.

Other factors that tend to influence the bacterial flora would include temperature, especially low ones, antimicrobial agents and unhygienic handling. The effect of freezing has been summarized by Georgala (1957) showing that immediately after icing with the ice from the factory, there is a proportional increase in the percentage of Coryneforms and Flavobacterium at $20^{o}C$ and in the Pseudomonas species at $0^{o}C$ while with the trawler ice, the latter species

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greatly predominate both at 0^{o} and $20^{o}C$.

Incorporation of antibiotics in the ice used as a preservative, tends to lower the total bacterial load drastically initially but thereafter, growth proceeds in the normal way (Borgström, 1961). This would imply therefore that the higher the inherent bacterial flora prior to antibiotic incorporation, the higher will be survivors which will then proliferate normally. Exposure of fish in marketing establishments to various surfaces of varying microbiological properties predispose fish to pathogenic flora that is found among human populations. Such has been the case with the finding of Escherichia coli in the fish, a finding suggestive of faecal pollution. Contamination by containers is one of the major extramarine sources of adulteration of the fish or fish products. Wooden containers at some marketing establishments have been shown to carry loads of bacteria in the region of 10-20 x 10^{6} per cm^{2} of surface at $0^{o}C$ and $20^{o}C$ (Spencer, 1959). Other data have shown Aluminium containers to be just as prone to heavy contamination as the wooden ones though the former are easily sanitized. Coupled with a host of other parameters, the bacterial loads of fish are thus greatly influenced by the containers. Spencer (1956) in his survey of 200 fish estimated the average load on the skin to be about 10^{6} (at $20^{o}C$) and about 10^{3} (at $37^{o}C$) per cm^{2}. Such an inverse proportional reduction with temperature increase is attributed to the preponderance of psychrophiles which do not proliferate at a temperature of about $37^{o}C$. The

other three modes of extramarine contamination compiled by Borgström (1961) are:-

(1) The ice used in the transportation boxes may be a pollutant especially when prepared from polluted waters or drawn under unsanitary conditions.

(2) The manual worker as a carrier of microorganisms forms a focus of their dissemination and here special reference is made to chronic Salmonella excretors in marketing establishments.

(3) Poor sanitary conditions in and around the processing facilities may be implicated. Evidence for this has been furnished by Badiali et al., (1957).

Other treatments likely to interfere with the flora include smoking. Smoking which could be "cold" or "hot" destroys a considerable portion of bacteria since the techniques incorporate fish preservation techniques such as brining and drying. The effectiveness is dependant upon the degree of penetration of the product by the smoke. It is to be noted that the mesophiles such as Salmonella are sparingly destroyed by hot smoking. Should this technique be carried out below 60^{o}C, it poses a public health hazard, in cases where this bacterium and other mesophiles are present (Borgström, 1961). Crustacean and molluscan shellfish unlike teleosts have in the past been associated with food poisoning outbreaks, by virtue of their filter feeding mechanisms (Dutt et al., 1974). They are more prone to contamination and tend to concentrate bacteria resident in the aquatic environment

in their bodies. Their use in delicacies puts human consumer at risk especially when cultivated in sewage polluted waters. Fortunately, this risk could be reduced since cultivation of the contaminated shellfish in clean water results in spontaneous decontamination hereafter referred to as depuration. Freshly caught whole shrimp has been shown to give bacterial counts as high as 1,600 to 1,200,000 per gram immediately after catching. Qualitatively, the predominant flora comprises of the following genera, namely Bacillus, Micrococcus, Escherichia and Pseudomonas.

2.2 VIBRIO PARAHAEMOLYTICUS IN FISH AND FISH PRODUCTS

2.2.1. History and Epidemiology

The existence of V. parahaemolyticus in Japan was not appreciated until this halophilic bacterium was implicated as causal organism in a food poisoning outbreak which occurred in the southern part of Osaka Prefecture in 1950 (Fujino et al., 1953). The outbreak involved 272 persons including 20 fatal cases. The food suspected to have caused the poisoning was a small semi-dried young sardine Engraulis japonica popularly known as "shirasu" in Japanese. Even after this episode, the significance of V. parahaemolyticus was not realized until the second outbreak of food poisoning, involving 120 persons, 73 of whom were in-patients and 47, hospital employees of Yokohama National Hospital, was described by Takikawa (1956). This time, the implicated foodstuff was salted cucumber known to the Japanese as "asazuke." The reports of Kudoh et al.,(1974) have implicated various foods, most of which being seafoods in food poisoning outbreaks due to this bacterium. Out of 606 food poisoning outbreaks due to V. parahaemolyticus, raw marine fish and shellfish and foods containing marine fish and shellfish accounted for 38.9% each separately. Processed seafood products were implicated in 6.4% of the cases. Vegetables and their products were responsible for only 3.6%. However, the vehicles for 12% of the cases were not identified. The organism was later responsible for

a series of endemic and sporadic cases of food poisoning.

In a decade, between the years 1963 and 1972, V. parahaemolyticus caused 62.2% of all reported cases of bacterial food poisoning in Tokyo and about 1,300 patients with V. parahaemolyticus infections were reported every year, (Kudoh, et al., 1974). Currently it is reckoned that over 70% of the cases of human bacterial gastroenteritis in that country may be caused by this enteropathogenic halophile. Sakazaki (1968) is of the opinion that the real incidence of food poisoning due to this organism is much higher than the numbers reported in the statistics. The organism has been implicated in food poisoning outbreaks in Thailand in 1970 and also in the United States, it has been associated with food poisoning in meals using crab and shrimp meat in delicacies. In Europe, its potential as an intestinal pathogen is disputed although in Britain, cases have been reported among travellers returning from the Far East in 1972 (Peffers et al., 1973). Leistner and Hechelman (1973) reviewed its significance in Europe.

Clinically, certain serotypes of this halophilic organisms cause a gastroenteritis characterized by sudden onset abdominal pain, in contrast to the classical cholera which causes a painless diarrhoea and effortless vomiting (Barrows and Miller, 1972). Other symptoms include bloody stools, chills, headache, mild fever, dyspnoea, tachycardia and cyanosis. Sudden death has also been reported as a result of cardiac arrest. While nausea, vomiting and diarrhoea are consistent in the syndrome, the other symptoms may or may not be manifested.

The incubation period ranges from 2-48 hours with a median of 15-18 hours. The duration of the syndrome is variable depending on the infective dose ingested and perhaps the acidity of the stomach. The infecting dose appears to be in between 10^5 and 10^7 viable cells of the organism, (Sanyal and Sen, 1974). The above authors think that the variation in abdominal discomfort experienced by the infected human patients may be due to the susceptibility of the individual. The illness is self-limiting and its remission may be associated with excretion of the organims in the stools as is the case in cholera. Recovery is usually complete within 2 to 5 days. The case fatality rate is very low and the majority of deaths from this type of food poisoning occurs in old debilitated adults. In West Africa (Togo), V. parahaemolyticus has been identified as an important agent of cholera-like gastroenteritis in humans (Bockemühl et al., 1972; Bockemühl and Trièmer, 1974).

2.2.2. Taxonomic status of the species

In the pioneer days of its study V. parahaemolyticus has appeared under various synonyms, namely Pseudomonas enteritis (Takikawa et al., 1958); Oceanomonas alginolytica (Miyamoto et al., 1961); Oceanomonas parahaemolyticus (Fujino et al., 1961); Oceanomonas enteritis (Takikawa et al., 1961); Vibrio alginolyticus (Miyamoto et al., 1961); Beneckea parahaemolytica (Sakazaki, 1963) and also Beneckea parahaemolytica (Baumann et al., 1971), Bergey's Manual of Determinative Bacteriology, 8th edition, (1974).

The present designation of V. parahaemolyticus was

Table III: Outbreaks of food poisoning due to Vibrio parahaemolyticus - 1958-1972 *

Year	Number of outbreaks	Number of cases	Number of deaths
1958	10	513	0
1959	37	737	0
1960	116	3,994	0
1961	465	20,536	13
1962	273	10,067	3
1963	524	12,968	9
1964	558	14,263	9
1965	275	6,048	3
1966	284	7,509	2
1967	403	8,806	4
1968	266	5,685	3
1969	410	11,235	6
1970	267	7,922	5
1971	300	8,394	1
1972	436	10,011	2

*Source: International symposium on Vibrio parahaemolyticus Saikon Publishing Co. Ltd. Edited by Fujino et al., (1974).

proposed by Sakazaki et al.,(1963). The unreliability of the many attempts to revise the classifiation has been due to the use of a single criterion such as characteristic flagella morphology and curvature of the cell, for confirmatory diagnosis Colwell, (1970), proposed a "polyphasic" taxonomy which according to him, was geared to assemble and assimilate the many levels of information from the molecular to the ecological and incorporates the several distinct and seperable portions of information extractable from a non-homogenous system to a united multidimensional taxonomy. Attempts have been made to revise the taxonomy and nomenclature of the genus Vibrio and hence the species within, without arriving at a concrete taxonomic position.

Despite these attempts, the description of the genus and the intrageneric relationships have been difficult and subject to confusion (Colwell, 1970). Establishment of the correct or appropriate taxonomic position will aid in the epidemiological study of the diseases caused by the members of the genus Vibrio. Furthermore, the species V. parahaemolyticus causes gastroenteritis symptoms in humans similar to those caused by the members of the family Enterobacteriaceae and also cholera-like syndrome simulating food infections due to V. cholerae El-Tor biotype and the classical V. cholerae. Therefore, knowledge of the proper taxonomic status as deduced from morphological, cultural, biochemical or serological reactions will provide us with another entity of differential diagnosis of this syndrome. Apparently, the taxonomic status, of the organism has been and is still a dynamic element and as such, it has been a controversy. According

to Bergey's Manual of Determinative Bacteriology - 8th edition. (1974), V. parahaemolyticus belongs to the Order Eubacteriales; Family Vibrionaceae; genus, Vibrio and species, V. parahaemolyticus. In the older nomenclature two biotypes of the bacterium were proposed by Sakazaki (1968) as biotype 1 and biotype 2. Since these two have ever since been categorized as two separate species, biotype 1 is referred to as the true V. parahaemolyticus and biotype 2 as V. alginolyticus. These two species share a common flagella (H) antigen. Some biochemical characteristics of the genus Vibrio have been compiled by Colwell et al., (1973) as follows:

(1) Fermentative carbohydrate metabolism since glucose fermentation by V. cholerae yields acid but no gas and proceeds via the Embden-Meyerhoff glycolytic pathway.

(2) Sensitivity to the compound 2, 4 diamino 6, 7-di-isopropylpteridine (vibriostatic agent) 0/129.

(3) An overall deoxyribonucleic acid (DNA) base compositional range for the genus Vibrio of 44 to 50% of Guanine plus Cytosine (G + C).

2.2.3. Pathophysiology

The pathophysiology is not exactly known neither is it clear whether or not the cholera-like enterotoxin produced with subsequent multiplication of the organisms in the gut accounts for the clinical features of the syndrome. Therefore, it qualifies as an infectious food poisoning syndrome. The strains which exhibit β- haemolytic activity (Kanagawa phenomenon) otherwise known as Kanagawa

positive type haemolysis (KH), on special blood agar medium commonly known as Wagatsuma agar are associated with pathogenicity (Miyamoto et al., 1969). However, reports of food poisoning due to Kanagawa negative (K^{-ve}) strains are documented (Molenda et al., 1972). While it is an established fact that the diarrhoea producing enterotixin of cholera Vibrios activates adenyl cyclase which causes accumulation of fluid in the gut and hence the development of diarrhoea. The role played by the V. parahaemolyticus toxin in this respect is obscure.. The cytotoxicity of the direct heat stable haemolysin prepared by chromatographic separation has been demonstrated on the cells derived from human amnion (Sakurai et al., 1976). It is the opinion of workers like Honda et al., (1976) that it is the enterotoxic effect of the direct thermostable haemolysin on the gut cells and not to the choleragen-like enterotoxicity. The watery diarrhoea of this syndrome might however be accounted for by yet another unidentified choleragen toxin of V. parahaemolyticus. Other reports of Honda et al.,(1976) indicate the isolation of a factor causing morphological changes of Chinese Hamster Ovary cells (CHO) from the culture filtrate of V. parahaemolyticus. This author thinks that perhaps these changes simulate those in the small instestine in an active infection by the bacterium, since the ability of this factor to induce morphological changes in CHO cells is comparable to that observed with the choleragen. The lethality of this toxin i.e. the animals die immediately after intravenous (i.v.) inoculation of 5 µg of the purified extract has been attributed to its cardio toxicity Honda et al., (1976).

The electrocardiographic changes observed were the depression of the intra atrial and intraventricular excitation and also the rate and rhythm. In response to the cutaneously introduced toxin; erythema, oedema and induration with a peak 6 hours post inoculation were induced (Ghosh et al., 1974). While this may be experimental it must be realised that any foreign material would induce similar inflammatory changes. Therefore, in this case, cytological alteration being a consistent feature is more important. The characteristic response of the choleragen, i.e. diffusive dye permeability was not observed with the toxins of V. parahaemolyticus by the same authors.

Histopathologically dermonecrosis and desquamation of the necrotic mucosa were prominent features. Coupled with neutrophilic and lymphocytic infiltration when stained with Haematoxylin and Eosin, a picture identical to that of autopsied human cadavers who have died of V. parahaemolyticus food poisoning is seen. The pathogenicity of V. parahaemolyticus in relation to the Kanagawa phenomenon β-haemolysis on Wagatsuma Agar medium was studied, using the lapine ligated gut loop by Twedt and Brown (1973). They stated that the ileal loop reaction was strongly associated with Kanagawa haemolysin production by some of the V. parahaemolyticus serotypes. However, the purified Kanagawa haemolysin itself was not found to affect the gut (Sakazaki, 1968). Cellular destruction of HeLa 53 cell monolayer was observed 3 hours after challenge with live Kanagawa positive (K^{+ve}) Vibrio cells and two hours after challenge with culture filtrates regardless of the

Kanagawa reaction of the original culture. On the other hand in the L strain cells also used in the tissue culture method, loss of response of the nuclei to Giemsa stain was recognized on challenge with the vibrios of Kanagawa positive strains and culture filtrates but no cellular destruction was evident. The L cell monolayers showed cellular destruction upon challenge with Kanagawa haemolysin (Sakazaki et al., 1963). Vanderzant and Nickelson, et al., 1974) summarized the main pathological findings in humans as being catarrhal of the stomach, hyperaemia of the mesentery, erosion of the jejunum and ileum and congestion of various organs including the suprarenal gland and interlobal haemorrhages in the lungs. V. parahaemolyticus has been shown to be pathogenic for the brown shrimp, Panaeus aztecus from the Gulf of Mexico. According to this report, high mortalities were observed when the non-infected brown shrimps were fed shrimps that had been infected with V. parahaemolyticus. The organism was then isolated from these dead shrimps. Rapid death which ensued within 24 hours of feeding, therefore points to the presence of an exotoxin. Enteropathogenicity experiments in human beings conducted by some authors attributed this property to per os administration. The haemolytic factors mentioned previously in connection with the Kanagawa type haemolysis, are probably not directly responsible for the virulence (Sakazaki, 1968; Zen-Yoji et al. 1970). Two antigenically related enteropathogenic toxins "a" and "a^1" have been obtained from culture filtrates of V. parahaemolyticus (Zen-Yoji et al., 1965). The molecular weights of both toxins were approximately 45,000. Their

thermostability at 100°C for 18 minutes has been reported (Zen-Yoji et al., 1974). Toxin "a" formerly referred to as the haemolysin exhibits a different electrophoretic morbility pattern in Polyacrylamide gel electrophoresis and immunological aspects. Zen-Yoji, et al., (1971) realised that the highly purified toxin "a" (H.P.T. - a) was identical to the haemolysin which has been an important pathogenic factor though the haemolysin has not been clearly implicated as a cause of enteritis. The HPT - a, induced an inflammatory response identical to those produced by culture filtrates in the skin of guinea pigs and in ligated gut loops. The histopathological picture in these two test systems was similar. This author reports the LD_{50} of HPT - a, toxin obtained by chromatography and sephadex gel filtration as being approximately 1.5 mg for mice.

Futhermore, toxin "a^1" is thought to be a charged isomer of "a", as deduced from comparative physiological and immunological properties in the studies done. Non-haemolytic strains probably do not produce these enteropathogenic toxins (Zen-Yoji et al., 1965). The histopathological changes induced by these toxins simulate those observed in the small intestines of autopsied cadavers. And as such, they are considered to be important entities in the enteropathogenesis. Honda et al., (1976) has attributed the watery diarrhoea, a consistent clinical sign in the syndrome and cardiotoxicity to the lethality of the composite toxin, "a" and "a^1". An Arrhenius type of phenomenon has been demonstrated with the toxin. The implication of this thermostable direct haemolysin as an important entity in the

enteropathogenesis has been further substantiated by the demonstration of the antihaemolysin antibody in the sera of patients suffering from V. parahaemolyticus infection. However, it is hypothesized that the primary role of the toxin (haemolysin) is to cause sudden death in some cases but not the watery diarrhoea. This aspect of the pathophysiology has yet to be elucidated.

2.2.4. Pathogenicity assays

Various methods of pathogenicity assessment have been devised and since none of them show a hundred per cent perfect reliability as sole criterion, it is proposed that these methods should be used in combination. In effect, the virulence of a culture is judged according to its behaviour in thevarious test systems employed. Although this appears to be a subjective type of assessment, chances of arriving at erroneous conclusions are reduced. Such tests include:-

(1) Kanagawa type haemolysis (KH)

(2) Rabbit ileal loop procedure

(3) Per os culture adminstration to infant mice and rabbits and egg pathogenicity bioassy.

2.2.4.1. Kanagawa type Haemolysis (KH)

On a special medium referred to as modified Wagatsuma Blood agar (WBA), some strains of V. parahaemolyticus exhibit a clear zone of β-haemolysis (Sakazaki, 1968). This medium contains, Yeast Extract, 3 g; Peptone (Bacto) 10 gm;

Sodium Chloride, 70 gm; Dipotassium phosphate, 5 gm; Mannitol, 10 gm; Crystal Violet, 10 gm and either 5% washed lapine or human erythrocytes. According to Sakazaki (1977), in the test of human volunteers and rabbit intestinal loop tests, only the Kanagawa positive (K^{+ve}) culture extracts gave a positive reaction manifested as gastroenteritis in the former and dilatation, in the latter while K^{-ve} strains constantly gave negative reactions in these tests. The intriguing question is why the K^{+ve} cultures are rarely isolated from sea foods and sea water although most of the cases of gastroenteritis due to V. parahaemolyticus are caused by eating raw fish products. It is the opinion of Sakazaki (1977), that the K^{+ve} V. parahaemolyticus strains may be present in marine sources but then the population is too small to be detected in routine isolation procedures and that these strains could multiply selectively in the intestines. This ties up with the idea of the cryptic plasmid influencing the resistance and hence the toxicigenicity of the organisms in the gut. The survival of the vibrios in a marine environment is temperature dependent. Their numbers decline below $15^{o}C$ and are rarely isolated from sediment below $0^{o}C$. Both K^{-ve} and K^{+ve} survive in the sea below $25^{o}C$ but at $37^{o}C$ the K^{+ve} strains lose ability to cause β-haemolysis and hence are regarded as K^{-ve}. The haemolysis is better demonstrated when the medium contains certain carbohydrates and Sodium chloride of optimal concentration, 3%. The work of Doki Chun et al., (1975) has indicated the absence of haemolysis on WBA without carbohydrates at $37^{o}C$ or without fermentable Sucrose or Lactose. The results of the Kanagawa haemolyis (KH)

in the absence of a fermentable carbohydrate differ according to the strain and the type of carbohydrate added.

According to some workers, inclusion of Lactose and Trehalose in the media converted more than half of the K^{+ve} strains in the absence of Galactose and Arabinose and they become K^{-ve}. Some of the K^{-ve} strains were positive in Maltose-containing medium. Incorporation of Dextrose in the medium containing 7% Sodium chloride, produced large zones of KH by K^{+ve} and K^{-ve} strains but a brownish zone was frequently noted around the bacterial growth. Reduction of Sodium chloride to 3% resulted in the production of a hazy zone of pseudohaemolysis around all K^{+ve} strains and some K^{-ve} strains while in the medium containing about 5% Sodium chloride, the haemolysis appears like the KH. As suggested by Barrow and Miller (1977), it is possible that the breakdown products of carbohydrate and low pH may influence the production of KH factors but this statement requires careful interpretation. While Kato et al., (1966), succeeded in demonstrating the phenomenon using a medium containing 2-5% Sodium chloride and no carbohydrates, other workers like Doki Chun et al. (1975) could not demonstrate the difference in haemolysis between K^{+ve} and K^{-ve} strains using this medium. For reasons, not yet known, the empirical KH does seem to differentiate the more virulent from the less virulent strains of V. parahaemolyticus (Barrow and Miller, 1977). Vanderzant and Nickelson (1972 a,b) tend to believe that most K^{-ve} strains of V. parahaemolyticus isolated from human gastroenteritis cases are K^{+ve}, whereas almost all strains isolated from marine specimens are negative (Miyamoto et al., 1969 and Sakazaki 1968).

However, the observation is not completely accepted by other workers (Twedt and Brown 1973). Reports of food poisoning associated with K^{-ve} strains are documented (Molenda, et al., 1972). Barrow and Miller (1977) suggest that perhaps the pH is important and that the consumption of a sufficient amount of alkaline sea foods permits the survival of some of the organisms of which the K^{+ve} strains multiply rapidly in the small intestine. Recent isolation of cryptic plasmid deoxyribonucleic acid from K^{+ve} strains of V. parahaemolyticus seems to offer a plausible explanation both for the KH phenomenon and the persistence of some of the strains in the gastrointestinal tract. It was observed that some of the K^{+ve} strains harboured the covalently closed circular (CCC) plasmid DNA. Correlation of this property and KH phenomenon is based on experimental finding that some of the V. parahaemolyticus strains are capable of not only receiving but also maintaining plasmids conjugally transferred from Escherichia coli (Guerry and Colwell, 1977). No perfect positive correlation between the KH and the presence of CCC (DNA) is so far known to exist, however this does not eliminate the possibility of R-plasmid involvement in the haemolysin production. It is the opinion of these authors that the gene for haemolysin production may be chromosomal in some cases and in others, extrachromosomal. Possible acquisition of the R-plasmid in the gut by some K^{+ve} strains from members of the family Enterobacteriaceae such as E. coli is not remote. It is therefore postulated that the R-plasmid promotes longevity of the receptive K^{+ve} strains and in so doing enhances their haemolysin production.

Yanagase et al. (1970) reported a heat stable direct haemolysin factor as being responsible for the KH and that the activity is enhanced by Calcium ions. Most likely, a deficiency of Calcium ions (Ca^{++}) ions in some strains of the bacterium confers to them a weak haemolytic reaction which can be enhanced by the replenishment of Calcium in the WBA medium. The pH could also have a bearing on the degree of haemolysis in WBA. While the pH at 24 hours decreased it was noted to increase after 48 hours in both the K^{-ve} and K^{+ve} strains. The influence of the type of blood used has also been studied (Doki Chun et al., 1975). Erythrocytes of human and lapine origin do not differ in the KH although red blood cells from the latter are more easily lysed than the former by the weakly haemolytic strains on ordinary Blood agar. These workers have observed no difference between washed erythrocytes and defibrinated blood. The thicker the blood in the plates, the more difficult it is to read the haemolysis in WBA. The acid tolerance of the K^{-ve} and K^{+ve} strains was related to the individual strains rather than the Kanagawa phenomenon. Most strains can tolerate a pH of 5.2 and are able to multiply at pH 5.3 (Doki Chun et al., 1975). According to these authors the Kanagawa reaction of V. parahaemolyticus is an expression of strongly haemolytic activity which is distinctly differentiated from the activity of the weakly haemolytic strains by the incorporation of Crystal Violet and a high salt concentration in the WBA. Exclusion of Calcium by incorporating 0.005 EDTA into the medium results in retarded haemolysis of both K^{-ve} and K^{+ve} which is strongly suggestive of interferance

or inhibition of Calcium utilizing or synthesizing system in the K^{-ve} strains.

2.2.4.2. Ligated rabbit ileal loop test

This model as an auxillary test for the pathogenicity of bacterial toxins has been used in the literature for V. cholerae, E. coli, C. perfringens and many others. The initial model to assess the pathogenicity of V. parahaemolyticus was devised by Twedt and Brown (1973). Recent modifications have been improvised by Brown et al., (1977). The latter model for the example has been used to investigate the role played by whole cells, cell fragments, cell free preparations and lypholized media constituents of V. parahaemolyticus in the enteropathogenesis of V. parahaemolyticus.

In both the original models and the modifications, New Zealand rabbits are starved for 48 hours, and deprived of water for 24 hours prior to laparatomy were used. Asceptic procedures are adhered to, during exposure of the ileum. Twedt and Brown (1973), segmented the ileum in such a way that at 30 cm above the ileal-caecal junction, 6-8 12 cm loops were ligated leaving 2 cm of intervening segment. This leaves a 10 cm test loop. Thereafter intraluminal inoculation of the test material is done. Dilatation of the gut is the criterion for the positivity and hence virulence of the test material. This dilatation is due to fluid accumulation in the gut lumen. The index of virulence is a ratio V/A, where V and A denote the volume and the area of the gut

respectively. This model has been used to determine the association between ability to cause (KH) on Wagatsuma Blood Agar and dilate the rabbit ileum. Cell lysates from Kanagawa negative isolates on many occasions have been unreactive both on WBA medium and the rabbit ileum. Brown et al., (1977) think that this model is a reliable aid in assessing virulence using dilatation as sole criterion.

2.2.4.3. Feeding experiments in animals

Initially the experiments were used to assess the pathogenicity of both K^{+ve} and K^{-ve} strains (Doki Chun et al., 1974). Mongrel dogs and cats, in these experiments were fed with broth cultures containing a known amount of colony forming units of both K^{-ve} and K^{+ve} strains. To provide an alkaline medium for the propagation of the bacterium, milk and Sodium bicarbonate were administered per os, along with the cultures. Gelatin was also administered to the test animals to facilitiate the passage of these materials into the gut. In order to inject the material into the jejunum of some animals a laparatomy had to be performed first. According to Aiiso and Matsumo (1961) no difference between strains was exhibited as regards excretion of the pathogenic and non-pathogenic strains. Doki Chun et al., (1974) maintain that there is no difference between these two strains when a certain inoculum of each is introduced into the jejunum. Sakazaki (1977) is of the opinion that only the K^{+ve} strains give rise to symptoms of gastroenteritis although occasionally a K^{-ve} strain is isolated from a

case of gastroenteritis. Human volunteer study in the pathogenicity of V. parahaemolyticus showed that administration of a calculated number of viable V. parahaemolyticus of the K^{-ve} cells did not give rise to any gastroenteritis symptoms regardless of the size of the inoculum or whether the bacterium was excreted by the human volunteer. The egg pathogenicity bioassay performed by Kampelmacher et al., (1972) is not a major evaluating technique.

2.2.5. Cultural characteristics and growth requirements

On the maintenance medium, most of the colonies are smooth, moist, circular and opaque green with a dark raised centre (Sakazaki, 1968). Twedt et al., (1969) have reported mixtures of smooth and rough textured variants having serrated edges, a raspberry centre and a dark green appearance. More complex series of variations of these Vibrio cultures have been compiled Sakazaki et al., (1963). The 18 hour colonies average 3-4 millimetres in diameter. While most of the cultures of V. parahaemolyticus do not swarm on 1% agar those of V. alginolyticus do (Sakazaki, 1968). The colonies of V. alginolyticus are yellow on TCBS, agar a colour that differentiates them from the non-Sucrose fermenting colonies of true V. parahaemolyticus. "Dissociation", otherwise known as withering is exhibited by the cultures of V. parahaemolyticus after 24 hour and this shows up as fringing of the edges. V. parahaemolyticus has a wide range of growth temperature, the lowest being $2^{o}C$ and the highest $42^{o}C$, depending on the environment of origin (Beuchat, 1974).

The optimal temperature is 37°C and the organisms tends to exhibit temperature response of a typical mesophile. The strains of marine origin do not differ markedly from those of human origin. This contradicts the observation by some authors that a variation exists among strains depending on the nature of their Kanagawa type haemolytic reaction, that while the K^{+ve} strains do not thrive and propagate below 25°C, the K^{-ve} ones do.

Most workers have reported the minimum temperatures to be in the range between 9.5-10.5°C (Vanderzant and Nickleson, 1972 a ; Colwell et al., 1973). The organism is able to thrive at temperatures below 5°C depending on the pH of the growth medium (Beuchat, 1974). The reciprocal relationship between these two parameters is such that a decrease in the growth temperatures had to be followed by an increase in the lowest pH permitting growth. At a temperature of 9°C, the minimum pH for growth ranged from pH 7.1-7.7. Although a more alkaline environment approximately pH 8.2 is preferable, V. parahaemolyticus can thrive at acidic pH as low as 4.0.

Some authors refer to the organism as an obligate halophile (Twedt et al., 1969), while others regard it as a facultative halophile, surviving in a medium of Sodium chloride (NaCl) concentration ranging from 1% to 8% with an optimum of 3%. However only V. alginolyticus can thrive in 10% or more of NaCl (Sakazaki, 1968). Brown (1976) estimated the salt tolerance to be 0.2-1.57 M. The oxygen requirement of the two Vibrio species is such that both V. parahaemolyticus

and V. alginolyticus are facultative anaerobes.

On first isolation, V. parahaemolyticus cells appear as Gram negative coccoid or ellipsoid rods, with rounded ends and convex sides, modal size 0.5-1.4 by 0.4-0.6 μ, with slight pleomorphism and occasional filaments, (Bergeys Manual 8th edition, 1974). Pleomorphism is accentuated in older cultures. Tendency towards granular staining is a constant feature of the strains of the organism. Rods with tapering ends, curved spiral bacilli, big twisted sea serpentine forms, clubs, spheroplasts and some rare giant forms have been described. The latter strains are capsulated. All strains of V. parahaemolyticus are motile and flagellated. The type of flagellation ranges from polar monotrichous designated (M) flagellum formed either in liquid or solid medium, to lateral (L) flagellum formed exclusively on solid medium (Sumio and Keinosuke, 1977). The L flagella are randomly distributed on the bacterial soma and has been likened to the peritrichous flagella of the Enterobacteria (Takeda et al., 1974). The degree of flagellation is a direct function of swarming ability and it is thought that the formation of L flagella is an adaptive mechanism for growing on solid media.

2.3 Salmonella species in fish and fish products

Evidence as to the possible role of fish in Salmonella species transmission does exist contrary to some reports. While seafish would be rare vehicles of Salmonella species, Crustacean shellfish namely crabs, lobsters and prawns and molluscan shellfish comprising mainly of oysters, mussels and clams, have frequently in the past been associated with gastroenteritis epidemics (Old, 1940 ; Hart, 1945 ; Gangarosa, 1968). Dismuskes et al., (1969) report that crustacean and molluscan shellfish become a health hazard when cultivated under unsanitary conditions, such as sewage polluted waters.

Association between shellfish and typhoid fever outbreaks are documented (Pivnick 1951 ; Hompesh 1953 and Olitzky et al., 1956). Contamination could be in icing or filleting establishments and possibly also at sea. While the latter may be a possible mode of contamination it has been in the past regarded as a remote one. However, the demonstration of specific bacteriophages against Salmonella in some waters may be significant in the above context (Guélin, 1948 and 1954). Since bacteriophages parasitize specific members of the family Enterobacteriaceae, their presence could be used as an index of faecal pollution (Guélin 1952).

Salmonella species in mussels, oysters and prawns beds areinevitable in cases where waters are sewage polluted. The longevity of the organism in the waters is sufficient to allow for its intake by the aquatic fauna along with water while feeding. Should we assume that the bacterial flora

of fish and shellfish is a reflection of the bacteriologic conditions prevalent in these waters, logically, fish would be used as indicators of the hygienic status of these waters. Considering the rapidity with which fish cover great distances in various waters, the gut flora of the fish is remniscent of the bacteriologic conditions in these waters. Floyd and Jones (1954), found the incidence of Salmonella in fish from River Nile, sold in a market in Cairo to be 11%. Sewage pollution of coastal waters can be a source of Salmonella in oceanfish (Gulasekharam et al., 1956). It is not clear whether fish is the vehicle or carrier of Salmonella in these instances.

Some authors considered that the presence of Salmonella species and other members of the family Enterobacteriaceae is not necessarily indicative of infection in the fish but demonstrates that fish under certain circumstances may serve as temporary hosts of human enteric pathogens. This has been substantiated by the work of Janssen (1968) who demonstrated specific antibodies to several bacterial pathogens to humans in the sera of the White Perch from surface waters adjacent to heavily polluted areas in Cheasapeake bay. In contrast, the White Perch from surface waters adjacent to sparsely polluted areas were free from these antibodies. This piece of evidence is suggestive of the fact that fish may become actively infected with human pathogens by exposure to contaminated water and may constitute a public health hazard if presented as human food. How long Salmonella species may be harboured by fish in nature is not

known. Bruns (1909) has shown that fish artificially infected with Salmonella enteritidis continue to excrete the pathogen for several months and perhaps the whole of the lifetime of the fish. Under natural circumstances this may not be valid since the inoculum of the bacterium may be small. Fortunately, in some cases, decontamination ensues after such fish enter non-polluted waters. This is a long established phenomenon in crustacean shellfish such as prawns and molluscs like oysters and mussels. In cases where the water polluting bacteria gain access to the viscera, they migrate from there into the flesh of the fish soon after the fish are caught (Bruns, 1909). This contradicts the long standing hypothesis that fish muscle is sterile at the time of catching. In view of this, Bruns (1909) envisaged two possible ways in which Salmonella species are acquired, namely:-

(1) Intrinsically, indicating contamination of aquatic origin and

(2) Extrinsically, pertaining to non-aquatic sources.

Furthermore, the inherent bacterial flora may be resistant to treatments such as freezing, cooling, packaging and smoking, which poses a major public health hazard to the fish eating communities.

2.4. Coliforms and Enterobacteria in fish and fish products

Evaluation of faecal pollution using Escherichia coli has been in use and was accepted as early as 1899 when E. coli was isolated from the intestines of sea water fish. Surveys carried out by other investigators later, however, make no mention of this organism in non-polluted waters. The relationship between polluted waters and fish contamination was reviewed by Gibbons (1934). This author indicated the presence of Proteus species in a few cases in his survey and did not consider coliforms normal inhabitants of the digestive tracts of sea water fish. Despite the contradictionary reports, the general consensus of opinion is that Enterobacteriaceae members are accidentally introduced in the waters, as pollutants. Variations as to these relative numbers has been thought to be influenced by heat labile substances of antibiotic nature in addition to the salinity and pH which are native inhibitors (Carlucci and Pramer 1959, 1960). Griffiths (1937) has pointed out that coliforms are present in fish as a result of sewage pollution.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Fish

(i) The sea fish species encountered in the survey were as follows:-*a

Scientific family name	English name	Swahili name
1. Lethrenidae	Bareface	Changu
2. Serranidae	Rockcod	Tewa
3. Labraindae	Rainbow fish	Pona
4. Siganidae	Rabbit fish	Taafi
5. Scomberomoridae	Kingfish	Nguru
6. Sphyraenidae	Baracuda	Mzia
7. Pleuronectidae	Sole (Malindi sole)	Ulimi wa Ng'ombe
8. Heinognathidae	Slimys,	Koro koro (kwele kwele)
9. Mugilidae	Mullet	Mkizi
10. Chondrichyes	Shark	Papa
11. Sparidae	Seabream	____*

(ii) Crustacean and Molluscan Shellfish

12. Portudidae	Crabs	Kaa
13. (a) Macrobranium equideus		
(b) Metaphanaeus monoceros	Prawns	Kamba ndogo
(Species names)		
(c) Metaphanaeus stebhingi		

14.	Panulirus ornatus Panulirus versicolor	Spiny lobster	Kamba kubwa (Mkamba)
15.	Ostrea cucullata	Oysters	Chanza

(iii) Of the Lake fish samples, Tilapia species namely, T. nilotica and T. esculenta were predominant. Occasionally, the Nile perch (Lates niloticus) would be encountered in the survey. Whole fish, intestines, swabs of skin and gill surface, whole shellfish (molluscan and crustacean) were collected.

3.1.2. Water

Sea water was collected at different points along the Kenyan coast. Although 100 millilitres were used in the methods, a bag full of it was collected to guard against spilling during transportation to the laboratory. Sterile plastic bags were used in collecting the water after which cotton strings were used to tie them up.

3.1.3. Sediment

This was obtained by weighting a bucket into the sea at different points. The sediment thus collected was poured into 50 gram sampling cups along with two to three millilitres of sterile physiological saline to prevent dehydration of the bacterial flora. The cups were then covered soon after filling.

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*a Source: Borgström, 1961. "Fish as Food". page

__* Corresponding Swahili names not available. Personal Communication with the local fishermen along the Coast was done to obtain the Swahili names.

3.2. Sampling

Samples were collected from the following areas:-

I. Nairobi area

Specific unit and sample quality			No. of samples
(i) City Market			
Sea fish			320
Prawns			130
Lobsters			44
Oysters			20
Lake fish			71
Crabs			20
(ii) Industrial area			
Fresh sea fish			50
(iii) Kariokor Market			
Dry lake fish			10
Total from Nairobi area			665

II. Coast Province

(i) Mombasa			
Magongo Market			
Sea fish	(i)	Fresh	60
	(ii)	Dry	40
(ii) Kilindini	(i)	Sediment	20
	(ii)	Water	53

(iii)	Port Reitz	(i) Sediment	20
		(ii) Water	20
(iv)	Malindi	(i) Fish	35
		(ii) Sediment	40
		(iii) Water	40
		Total from Coast Province	328

Each sampling was always done within two days during which time the fish samples were kept in cool boxes at + 4^{o}C to minimize decomposition. Upon reaching the laboratory the samples were processed immediately.

3.3 Methods

3.3.1. Sterilization of equipments

All materials used in the experiment were sterilized according to the recommendations of the manufacturers. Glassware was washed with water into which was incorporated a detergent "Teepol"*[a]. Addition of Savlon*[b] to this preparation during the actual sanitization conferred anti-microbial properties. Then the glassware was rinsed in clean distilled water prior to drying. Drying was achieved using oven temperatures of 50°C and subsequent sterilization at 160°C for 2 hours.

3.3.2. Bacteriological and biochemical methods

3.3.2.1. Total aerobic plate count and coliform count

Total aerobic plate count: The dehydrated Plate Count Agar (PCA) (Oxoid*[c] - Batch number 019/19280) was reconstituted and subsequently prepared according to the directions of the manufacturers. Petri dishes (Pyrex, size 20 mm by 15 mm) were used in media preparation.

A homogenate of the fish material, either gills or intestines or a mixture of these two, was obtained by blending 50 grams of the material for two minutes with 450 ml of sterile physiological saline in a Waring blender jar. Physiological saline was used to provide

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*[a] Shell product - Anionic detergent.

*[b] I.C.I. Company product.

*[c] Oxoid Limited, Southwark Bridge Road, London SE1 9HF.

a halophilic environment for the V. parahaemolyticus to be sought in the same homogenate. The salinity of the homogenate was also ideal for the isolation of Enterobacteriaceae. Using, sterile pipettes, decimal dilutions of 10^{-1}, 10^{-2} through to 10^{-10} of fish homogenate were prepared.

The decimal dilutions were prepared by transferring 10 ml of the previous dilution into 90 ml of the diluent. Each dilution bottle was agitated to resuspend the material that might have settled out and 1 ml of each dilution was pipetted into each of appropriately marked duplicate petri dishes. Twelve to fifteen millilitres of PCA were poured into the petri dishes after cooling to 44-46^{o}C within 15 minutes after making the dilutions. For each series of sample inoculations agar control plates were poured. The sample inoculations and the agar medium were mixed immediately by rotating and tilting. The agar medium was allowed to solidify after which the petri dishes were inverted and incubated at 35^{o}C for 48 hours.

Following incubation all colonies on plates containing 30-300 colonies were counted using artificial light and the results per dilutions were recorded. Finally the average counts were calculated and reported as aerobic plate count per gram of fish material.

Coliform count: Violet Red Bile Dextrose (VRBD) dehydrated agar (Oxoid Batch number 095, 13598) was prepared according to directions of the manufacturers. Using decimal dilutions used in the total aerobic plate count method 1 millilitre of

dilutions 10^{-1} to 10^{-6} were inoculated in two plates containing the (VRBD) agar. One set of plates from each dilution was incubated aerobically at $37^{o}C$ and the other set at $44^{o}C$ for 24 hours. Then, the coliform colonies were counted and the number registered as coliforms per gram of the sample.

3.3.2.2. Vibrio cholerae

Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar (Oxoid-Batches 98, 193 and 358, 20475) was used as the selective isolation medium. Unless otherwise stated, all incubation temperatures were $37^{o}C$ for 24 hours aerobically. Yellow colonies of average diameter 2.5 mm on TCBS agar were to be considered as being V. cholerae (both the classicial and the El-Tor biotypes) suspects. The biochemical tests described below were used as auxilliary identification tests, which may be consistent but not completely diagnostic. The methods for these tests are after (Blair et al., 1970).

Cholera red test: This reaction which depends on the development of a crimson red colour when 3 or 4 drops of Concentrated Sulphuric acid are added to a 24 hour Peptone water culture was performed. The red colour is due to the formed complexes of Nitrosoindole. This test however, is not specific since non-pathogenic Vibrios and many organisms that produce Indole and reduce Nitrate to Nitrite give this reaction.

Resistance to Polymixin B was done by spreading an 18 hour Peptone broth culture of the suspect colonies on the surface of a Trypticase Soy agar (Oxoid) plate. After 20 minutes, a 50 unit Polymixin B disc (Oxoid) was pressed on to the inoculated surface. The plates were incubated overnight at 37°C. A positive reaction is exhibited as a zone of growth inhibition around the classical V. cholerae biotype while none is present around the El-Tor biotype.

Haemolysis test was performed in such a way that 1 ml of a 24 hour, heart infusion broth culture and 1% saline suspension of washed red blood cells were mixed. As controls, similar mixtures were set up with:

(i) a portion of the culture heated for 30 minutes at 56°C and

(ii) a known haemolytic and non-haemolytic strain.

The mixtures were incubated in the water bath at 37°C for 2 hours and then held overnight at 4°C, then examined for haemolysis. V. cholerae strain El-Tor haemolyses the sheep red blood cells. Since the haemolysin is thermolabile, the heated portion of the culture should show no haemolysis. The classical V. cholera is non-haemolytic, in contrast to the haemolytic El-Tor biotype.

Enzymatic reactions: Unless otherwise indicated, the temperature of incubation was 37°C. Subcultured characteristic colonies were put into Sucrose, Mannose and Arabinose fermentation tubes, incubated at 37°C for 24 hours. Thereafter, fermentation of Sucrose and Mannose with acid but no gas production and

failure to ferment Arabinose are suggestive of cholerae vibrios. Using the standard methods of Blair et al.,(1970), carbohydrate fermentation, Citrate and Malonate utilization, Nitrate reduction; Phenylalanine deaminase, Urease and Indole production, were performed. The other carbohydrates employed in the fermentation tests included, Adonitol, Dulcitol, Fructose, Glucose, Salicin, Sorbitol, Trehalose and Xylose. These were dispensed in concentrations of 1%.

3.3.2.3. Vibrio parahaemolyticus

3.3.2.3.1. Detection

Unless otherwise stated, all culture media contained 3% Sodium chloride. Various media were used as enrichment in the study of growth characteristics and pathogenicity of the organism. In all cases, either Pyrex or Kumax petri dishes of sizes 100 mm by 15 mm were used.

Direct culture medium: The TCBS dehydrated agar (Oxoid Batches 98, 1939 and 358, 30475) were used. Prepation of this medium before dispensation was in accordance with the manufacturers' instructions. On this medium, V. parahaemolyticus grows well, producing characteristics smooth, green, slightly dome shaped colonies of diameters ranging from 2-4 millimetres. All the large or medium sized yellow colonies could be V. cholerae, V. alginolyticus or other non-cholerae vibrios. Table IV on page 45 was used as a provisionally for differential diagnosis in identifying the above mentioned vibrios.

Table IV Differentiation of Vibrio parahaemolyticus from other organisms

Organism	Colonial appearance on TCBS agar	Triple sugar Iron agar Slant/butt.	Lysine decarbo-lase	Voges-Proskauer	Growth in 8% NaCl
Vibrio parahaemolyticus	Large with dark green centre	Alk./Acid	+	-	+
V. cholerae	Yellow, medium size	Acid/Acid	+	d or +	-
V. alginolyticus	Large, yellow	Acid/Acid	+	+	+
Other marine vibrios	Large, green	d/d	d	-	-
Aeromonas	Small; yellow or no growth	Acid/Acid (gas d)	-	d	-
Pseudomonas species	Small; pale green or colourless	Alk./Alk.	-	-	-
Proteus species	Small: variable in colour (yellow, black or green)	Alk./Acid (d)/gas (d)	-	mirabilis (d)	-
Pleisomonas	No growth	Alk./Acid	+	-	-
V. anguillarum	No growth	Acid/Acid	-	+	-

Key: d = variable Source: Barrow, (1974)

Two liquid selective media were used in parallel for V. parahemolyticus isolation. The chemical composition and concentration of the ingredients in the two media is described below as follows:-

I. Salt Colistin broth (SCB)

Composition	Concentration	
	Single Strength	Double Strength
Yeast extract (Oxoid)	3.0 g.	6.0 g.
Tryptone (Oxoid)	10.0 g.	20.0 g.
Sodium chloride	20.0 g.	40.0 g.
Colistin sulphomethate sodium (Colomycin)	500,000 I.U.	1 million I.U.
Sterile distilled water	1 litre	1 litre

The ingredients were dissolved in the refrigerated sterile distilled water. The pH was then adjusted to 7.4 before addition of Colomycin (final concentration, 500 I.U. and 1,000 I.U.). No heat was employed during these procedures. The mixture was then maintained at + 4^{o}C and then dispensed as required. Both single and double strength were used but the latter was used in cases where the expected recovery rate of the organism would be low.

II. Glucose Salt Teepol Broth (GSTB)

Composition	Concentration: Single Strength	Concentration: Double Strength
Beef extract (Oxoid)	3.0 g.	6.0 g.
Tryptone (Oxoid)	10.0 g.	20.0 g.
Sodium chloride	30.0 g.	60.0 g.
Glucose	5.0 g.	10.0 g.
Methyl violet	0.002 g.	0.004 g.
Teepol 610 (BDH)	4.0 ml.	8.0 ml.

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

Fish: The homogenate was prepared as indicated for the total plate count method. The fish material consisted mostly of fish scales and overlying slime plus some of the top-most skin, gut and gills.

The initial dilution was calculated as 10^{-1}. Serial dilutions 1:1,000 and 1:10,000 using appropriate dilution blanks were prepared. Then, three, ten millilitre portions of 1:10 dilution were put in single strength GSTB (This constituted the 1 gm portion). The broth tubes were incubated overnight (18 hours) at 37^{o}C. On the second day after incubation a loopful of the 18 hour culture was streaked from the highest dilution of GSTB

showing growth, on to TCBS agar for 18-24 hours at 37°C. The colonies resembling V. parahaemolyticus on TCBS were identified biochemically as described on page 45.

Sediment: Loopfuls of the material were streaked on to TCBS agar plates which were then incubated at 37°C for 24 hours. The rest of the material was by means of sterile spatulas removed from the original container and poured into Pyrex 200 ml Conical flasks (No. 4980). The flasks were sealed off soon after the samples had been mixed with enough GSTB - to make a suspension. The mixtures were incubated at 37°C for 24 hours. Thereafter, loopfuls of the material from the flasks were streaked onto TCBS agar plates which were later incubated at 37°C for 24 hours to get typical colonies.

Water: A membrane filtration procedure for V. parahaemolyticus (MVP) was used. In this method both a selective medium and a differential recovery medium were employed. A hundred millilitres of sea water were treated with 1 millilitre of 0.05% Copper sulphate. This was to inhibit the growth of bacteria except V. parahaemolyticus thus enhance the isolation of the latter. From the same plastic bag was removed another 100 ml of sea water which was mixed with 100 ml of GSTB. The GSTB suppresses the growth of V. alginolyticus. The bags were then tied up. Both bags thus treated were incubated at 37°C aerobically for 48 hours after which loopfuls of the material from each bag were directly plated on to TCBS. The inoculated TCBS agar plates were incubated aerobically at 37°C for 24 hours after

which the plates were examined for typical V. parahaemolyticus colonies. The remaining water from each bag was filtered through membrane discs of size 47 millimetres (grade 45 mm code NA7/45 - Batch 3557, Oxoid). The samples treated with Copper sulphate and GTSB were also filtered in the sameway. The membrane filters were asceptically placed onto TCBS agar plates for incubation at 37°C for 18-24 hours. The typical colonies of V. parahaemolyticus were checked and then inoculated on fresh TCBS to get pure cultures for the confirmatory biochemical tests.

To minimize the swarming tendencies of Proteus vulgaris

Proteus vulgaris had a tendency to overgrow V. parahaemolyticus on TCBS agar plates. This was minimized by pouring 5 ml of Ethanol on the TCBS agar plates and spreading it evenly on the agar plates. Gradual evaporation of the Ethanol dried up the surface so that P. vulgaris colonies would be localized.

Kanagawa type haemolysis (KH)

Cultures studied: The test strains were the 72 isolates of V. parahaemolyticus. The reference Kanagawa negative (K^{-ve}) strain of V. parahaemolyticus was supplied by R. Sakazaki, National Insitute of Health, Japan.

Culture media

Special blood agar used for the observation of KH was modified Wagatsuma Blood Agar (WBA) of Sakazaki (1968), whose composition is described in section 3.2.4.1, on page 22.

Unless otherwise stated, 3% Sodium chloride was always incorporated in the other media used.

Determination of haemolytic activity

The KH was determined according to the description of Miyamoto et al., (1969). Cultures of the test strains in Nutrient broth were streaked linearly on WBA plates and the result was read after incubation for 24 hours at 37°C.

Well defined, clear β. haemolysis around the bacterial growth was recorded as positive, no haemolysis as negative and a very narrow zone of haemolysis as doubtful.

Egg pathogenicity:

Before the eggs were used, it was confirmed by candling that the ten-day old embryos were alive. Twenty such eggs were divided into two batches with another two eggs as controls (one control egg per batch). Asceptically, 1 ml of an aliquot containing 10^3 cells of V. parahaemolyticus (K^{-ve}) estimated using the pour plate method were inoculated into the 10 eggs, constituting one batch, using an 18 G needle. The conventional yolk sac route was used for the inoculation. Similarly, an equal number of cells of isolate X_1G_1 (0-10, K-23) were introduced into the other 10 eggs. The two control eggs were not inoculated. Thereafter, the sites of inoculation were sealed with paraffin wax after disinfection with Lugol's iodine solution. All the twenty eggs were incubated in an egg incubator at 37°C. At intervals of 12 hours, the eggs were candled to check for the death of the embryo. The mortality rate in each batch at these intervals was evaluated

and then tabulated. To ensure that it was the inoculum experimentally introduced into the embryos and not any other inherent flora responsible for the mortality of the embryos, yolk sac material was aspirated after 48 hours from each egg, asceptically and streaked onto Nutrient agar. The agar plates were incubated for 24 hours at 37°C after which all the possibly different colonies were examined microscopically after gram staining. Some of the material from the same colonies were subcultured onto TCBS, VRBD and MacConkey agars. This provided a rough guide as to the bacterial quality of the yolk sacs harvested.

Characterization of the 72 V. parahaemolyticus isolates

Morphological, physiological and biochemical properties were used as criteria for classifying the isolates as members of the genus Vibrio. To distinguish the isolates and then differentiate them from the ubiquitous marine halophilic bacterium, V. alginolyticus, the tables compiled by Colwell (1970) in his numeric taxonomy of the genus Vibrio were used. These are shown on page 59 in table IX.

The frequency of occurrence of the characteristic is shown by the percentages with the superscript a. This also indicates the degree of reliability of these characteristics as criteria for differentiation.

Professor Sakazaki provided us with a list of the biochemical tests in appendix I done to characterize X_1G_1 as serotype O-10, K-23.

Table V: Morphological and cultural characteristics for Vibrio parahaemolyticus

Character	Fequency of occurrence[a] in % V. parahaemolyticus
Cell morphology	
Rods	+ 100
Curved rods	± 63
Filaments	± 38
Spiral (rods)	- 0
Refractile	- 0
Single cells	+ 100
Paired cells	+ 100
Chains of cells	± 38
Short length (0.2 to 0.6 µm)	- 13
Medium length (0.6 to 1.2 µm)	- 9
Long (1.0 to ≥ 3 µm)	+ 93
Slender (0.2 to 0.6 µm)	- 21
Stout (0.6 to 1.0 µm)	+ 78
Round end	± 41
Tapered (lanceolate) end	± 59
Motile	+ 100
Polar flagella	+ 100
Monotrichous	+ 100
Lophotrichous	- 0
Gram-negative	+ 100
Gram variable	- 0
Colony morphology	
Entire edge	+ 91
Convex	+ 100
Translucent	+ 78
Opaque	- 25
Rough	- 0
Small colony (1 to 2 mm)	+ 69
Medium colony (2 to 5 mm)	- 9
Spreading growth on agar	- 0

Table V Cont'.

Growth in liquid medium	
Even turbidity	+ 84
Granular turbidity	- 15
Slight turbidity	- 3
Moderate turbidity	± 34
Heavy turbidity	± 63
Pellicle	+ 72
Ring	- 28
Slime formation	- 0
Pigmentation	
Fluorescent under ultraviolet light	- 0
Visible insoluble pigment	- 0
Diffusible pigment	- 0
Pyocyanin produced	- 0
Pyorubin produced	- 0
Fluorescein produced	- 0
Oxychlororaphine produced	- 0
Pigment produced on Sabauraud agar	- 0
White	- 3
Off-white	+ 66
Gray	- 31

[a]Frequency of occurrence of characteristic in sample set in percentage. Total number of V. parahaemolyticus strains = 32.

Source: Colwell (1970)

Table VI: General physiological, biochemical, and nutritional characteristics of Vibrio parahaemolyticus

Character	Frequency of occurrence[a] in % V. parahaemolyticus
Temp. (C) of growth	
0-5	- 0
15-41	+ 100
42	+ 100
Sodium chloride tolerance (%)	
0	- 25
0.5-5.0	+ 100
7	+ 100
10	+ 100
Seawater required for growth	+ 88
pH tolerance	
pH 4.0	- 0
pH 4.5	- 0
pH 5.0	± 63
pH 5.5-10.0	+ 100
Oxidase (Kovac's)	+ 100
Catalase	+ 100
Urease	+ 97
Nitrate reduced	+ 100
Nitrite reduced	- 0
Methyl red reaction	+ 88
Voges-Proskauer reaction	- 3
Indole produced	+ 94
Trimethylamine oxide → Trimethylamine	+ 88
Hemolysis (sheep RBC)	+ 100
Lipase (Tween 20, 40, 60, 80)	+ 100
Lipase (Spirit Blue Agar + Olive oil)	NT
Calcium lactate-acetate-carbonate	- 0
Citrate utilization	
Koser's method	+ 100
Simmons' method	+ 72
Cristensen's method	+ 100

Table VI Cont'.

Lecithinase	+ 97
Skim milk agar-growth	+ 100
Skim milk agar-casein hydrolysis	+ 91
Litmus milk peptonized	+ 97
Litmus milk surface peptonized	- 0
Litmus milk acid	± 34
Litmus milk alkaline	- 22
Litmus milk reduced	+ 97
Gelatin liquefied	+ 100
Lysine decarboxylase	+ 100
Ornithine decarboxylase	+ 97
Phenylalanine deaminase	NT
Phenylalanine → phenylpyruvic acid	- 13
Arginine dihydrolase (Thornley)	- 6
Arginine decarboxylase (Moeller)	- 0
L-Tyrosine → melanin	+ 94
Pepton → H_2S (lead acetate agar)	+ 75
Cystein → H_2S	+ 97
Cystine → H_2S	NT
Sodium thiosulfate → H_2S	+ 100
Peptone → NH_3	+ 100
Acetamide reaction	- 0
Growth in vitamin-free Casamino Acids	+ 100
Growth with the following as C and N source	
0.1 % L-Proline	+ 100
0.1 % L-Arginine	+ 97
0.1 % DL-Alanine	+ 97
0.1 % L-Lysine	100
0.1 % L-Phenylalanine	+ 97
0.1 % β-Alanine	- 0
Utilization of $NH_4H_2PO_4$ as source of N	+ 78
Growth with Sodium acetate as C source	+ 97
Growth with Sodium formate as C source	+ 100
Growth in 0.3% Sodium malonate broth	+ 97

Total number of V. parahaemolyticus strains = 32.
NT = Not tested

Source: Colwell (1970).

Table VII: Differentiation between V. parahaemolyticus and V. alginolyticus using certain biochemical reactions

	Biotype 1 (parahae-molyticus)	Biotype 2 (algino-lyticus)
Growth in 10 % NaCl	-	+
Voges-Proskauer	-	+
Methyl red	+	-
Acid from Arabinose	d	-
Acid from Sucrose	-	+

Key d = doubtful; - ≡ negative; + ≡ positive

Source: Bergey's Manual of Determinative Bacteriology 8th Edition, (1974).

Table VIII: Carbohydrate reactions of the Vibrio parahaemolyticus strains studied

Character	Frequency of occurrence[a] in % V. parahaemolyticus
Acid production	
Glucose aerobic	+ 100
Glucose anaerobic	+ 100
Glucose	+ 100
Adonitol	- 0
Arabinose	+ 84
Cellobiose	+ 97
Dextrin	+ 97
Dulcitol	- 3
Fructose	+ 100
Galactose	+ 97
Glycerol	+ 100
Inositol	- 0
Inulin	- 0
Lactose	- 3
Maltose	+ 97
Mannitol	+ 100
Mannose	+ 100
Melezitose	- 0
Melibiose	- 13
Raffinose	- 0
Rhamnose	- 13
Salicin	- 3
Sucrose	- 6
Sorbitol	- 0
Trehalose	+ 97
Xylose	- 0
Gas production from carbohydrates	- 0
Glucose + iodoacetate → growth	± 47
Glucose + iodoacetate → acid production	- 22
Starch hydrolyzed	+ 100

Table VIII Cont'.

Levan produced	+ 94
Ethanol → growth	+ 100
Ethanol → Acetic acid	- 0
Glycerol → Dihydroxyacetone	+ 97
Aesculin hydrolyzed	+ 88
Gluconate → 2-keto-gluconate (7)	- 3
Gluconate → 2-keto-gluconate (48)	- 0
Gluconate oxidized	- 3
Cellulose digested	NT
Agar digested	- 0

[a]Values in parentheses = frequency of occurrence of characteristics. Total number of V. parahaemolyticus strains = 32. NT = not tested.

Source: Colwell,(1970).

Table IX: Differences between Vibrio species examined in this study

	Frequency of occurence[a] in percent	
	V. parahaemolyticus	V. alginolyticus
Dihydrostreptomycin	- 69	+ NT
Penicillin	- 6	- 0
0% NaCl	- 25	+ 100
7% NaCl	+ 100	+ 100
10% NaCl	+ 100	+ 100
Seawater required for growth	+ 100	- 0
Voges-Proskauer	- 3	- 0
L-Tyrosine → melanin	+ 94	+ 100
Peptone → H_2S (Lead acetate agar)	+ 75	+ 100
Acetamide reaction	- 0	- 0
0/129	+ 75	- 0
4 to 7 C	- 0	+ 100
Gluconate → 2 ketogluconate	- 3	+ 100
Xylose	- 0	+ 100
Salicin	- 3	+ 100
L-Phenylalanine → Phenylpyruvic acid	- 0	+ 100
Spreading on agar	- 0	± 33
Starch hydrolyzed	+ 100	- 0
Erythromycin	+ 66	- 0
Tetracyclin	± 59	- 0
Aureomycin	+ 66	- 0
Terramycin	± 50	- 0
Arabinose	+ 84	- 0
Sucrose	- 6	+ 100
Glycerol → Dihydrocyacetone	+ 99	± 33
Aesculin hydrolyzed	+ 80	+ 100
Mannitol	+ 100	- 0
Mannose	+ 100	- 0
Oxidase	+ 100	- 0

Table IX Cont'.

Lecithinase	+ 97	- 0
Lipase (Tween 40, 60, 80)	+ 100	- 0
Lysine decarboxylase	+ 100	- 0
Ornithine decarboxylase	+ 97	- 0
β-Alanine → growth	- 0	- 0
Dextrin	+ 97	- 0

[a]Values in parentheses denote frequency of occurrence.
NT = not tested

Source: Colwell, (1970).

Table X: Antibacterial and antibiotic sensitivity *Vibrio parahaemolyticus*

	Frequency of occurrence [a] in percentage
	V. parahaemolyticus reaction
Chloromycetin (Chloramphenicol), 30 µg	+ 94
Dihydrostreptomycin, 10 µg	- 31
Erythromycin (Erythrocin) 15 µg	+ 66
Kanamycin (Kantrex), 30 µg	+ 69
Novobiocin (Albamycin), 30 µg	+ 91
Penicillin, 10 units	- 6
Polymyxin B (Aerosporin), 300 units	- 19
Tetracyclin (Achromycin), 30 µg	± 59
Oxytetracycline (Terramycin), 30 µg	± 50
Chlortetracycline (Aureomycin) 30 µg	+ 66
2, 4-Diamino-6, 7-diisopropyl-pteridine (Vibriostat 0/129), saturated	+ 75
Sodium lauryl sulfate, 0.01%	NT

[a] Values in parentheses = frequency of occurrence of characteristic, in percentage. Total number *V. parahaemolyticus* strains = 32

Symbols: + = sensitive, ± = variable, - = insensitive, NT = not tested.

Source: Colwell (1970).

Determination of growth characteristics

Optimal Salt Concentration determination was done by inoculating 10^9 cells of serotype O-10, K-23 of V. parahaemolyticus into Peptone Water* tubes of different Sodium chloride concentration. The final salt concentrations used, were 0.5%, 1.5%, 3.5%, 8.5%, 9.5%, 10.5% and 13.5%. These broth tubes were then incubated at 37°C for 6 hours after which growth was monitored in terms of turbidity using spectronic 20 at wavelength (λ) 400 nm. The results were recorded as shown in table XXIII on page 83.

Optimal pH evaluation was done using Peptone Water into which incorporated 3% Sodium chloride, at various pH, namely 4.5, 5, 5.5, 6, 7.0, 7.5, 8.2, 8.5 and 9.0. Each tube was inoculated with 10^9 cells of serotype O-10, K-23, then incubated for 24 hours at 37°C. Growth was determined qualitatively by visual inspection of the tubes.

Optimal temperature determinations, done in the course of the study involved incubating 24 hour cultures of serotype O-10, K-23 on TCBS agar plates at various temperatures, namely 37°C, 23°C, + 4°C, 0°C and -20°C, for 1 week. The colonies before and after incubation were determined as a measure of growth and propagation. Their viabilities were determined by subculturing the colonies on TCBS agar plates and reincubation at 37°C for 24 hours.

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*Peptone Water (Oxoid) (5 g Sodium chloride in 1 litre).

Electron Microscropy (EM)

This technique a was used to demonstrate the morphology of the vegetative cells of serotype O-10, K-23. The flagellation pattern was also studied. Typical colonies of the V. parahaemolyticus serotype were streaked on TCBS agar. The agar plates were incubated for 24 hours at 37°C, after which thin sections 4 x 4 millimetres of the colony together with the underlying agar were cut asceptically. The agar sections thus cut were then processed both for primary and secondary fixation in Formaldehyde-glutaldehyde fixatives using the method of Ito and Karnovsky (1968). Thereafter routine sectioning for electron microscopy was done, and the material coated on the grids of mesh size 200. The grids were then stained initially with Uranyl acetate for 6 minutes followed by washing in distilled water. The final staining was done using lead acetate for 2 minutes which was washed off too, with distilled water, after which the dried grids were examined in an EM 9A CarlZeiss unit, and photographs taken at various magnifications.

3.3.2.3.2. Antimicrobial drug sensitivity of the isolates

The cultures used were as follows: (1)(X_1G_1) identified as O-10, K-23 strain of V. parahaemolyticus

isolated from prawns and lobsters. (2) (X_1Y_1) conforming biochemically to the species, V. alginolyticus (3) a pure culture of V. parahaemolyticus used as reference supplied by Barrow and Miller of Truro Cornwall Hospital, Britain. The aim was to establish the relative antimicrobial drug resistance of X_1G_1 and X_1Y_1 with the known reference culture of V. parahaemolyticus. Mueller - Hinton agar (Oxoid-Batch number 310, 16067) with and without 3% Sodium chloride incorporated was used as a solid culture medium. Four plates were prepared one for each bacterium, the fourth being the control. Using a hockey stick type of glass rod, pure colonies from 18 hour cultures placed on the Mueller-Hinton agar surfcae were generously spread to cover the entire petri dish surface. Multidiscs (Oxoid Batch number 13384) impregnated with the following antimicrobial agents listed below were placed on the agar surface namely:- Chloramphenicol, 10 µg; Erythromycin, 10 µg Sulfafurazole, 100 mg; Penicillin G, 1.5 units; Streptomycin 10 µg; and Tetracycline, 10 µg . The plates were then incubated aerobically at 37°C for 24 hours after which the sizes of the inhibition zones of bacterial growth around the discs were calculated as shown in Figure I on page 66 and recorded in millimetres as shown in Table XXII on page 82,

3.3.2.3.5. Reference culture maintenance

The reference cultures used for comparison in the biochemical reactions were supplied by Drs. Barrow and Miller of Truro Laboratory, United Kingdom and

Professor Sakazaki of the National Institute of Health, Japan. They were received either in the lypholized state or on agar slants. Immediately upon receipt, all the culture were inoculated on TCBS and then examined for purity. Thereafter, they were maintained on semi-solid *Nutrient agar slants from which they were subcultured onto new Nutrient agar slants every two weeks to ensure purity and viability.

3.3.2.4. Salmonella

Samples for Salmonella isolation were cultured on: Bromothymol Blue agar (BB) MacConkey agar (MA); Desoxycholate agar (DCA), and Salmonella-Shigella agar (SS).

Biochemical characteristics

Unless otherwise stated, the temperature of incubation was 37°C. The following biochemical reactions were employed in attempting to identify the Salmonella suspected colonies;- Indole, Urease and Gelatinase, production; Nitrate reduction; Citrate utilization; Glucose and Sucrose fermentation in the Triple Sugar Iron agar (TSI) reaction.

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*Nutrient agar (Oxoid - Batch number 057, 16605)

Fig. I: The method used to measure the size of the bacterial growth inhibition zones (D), in Table XXII on page 82*

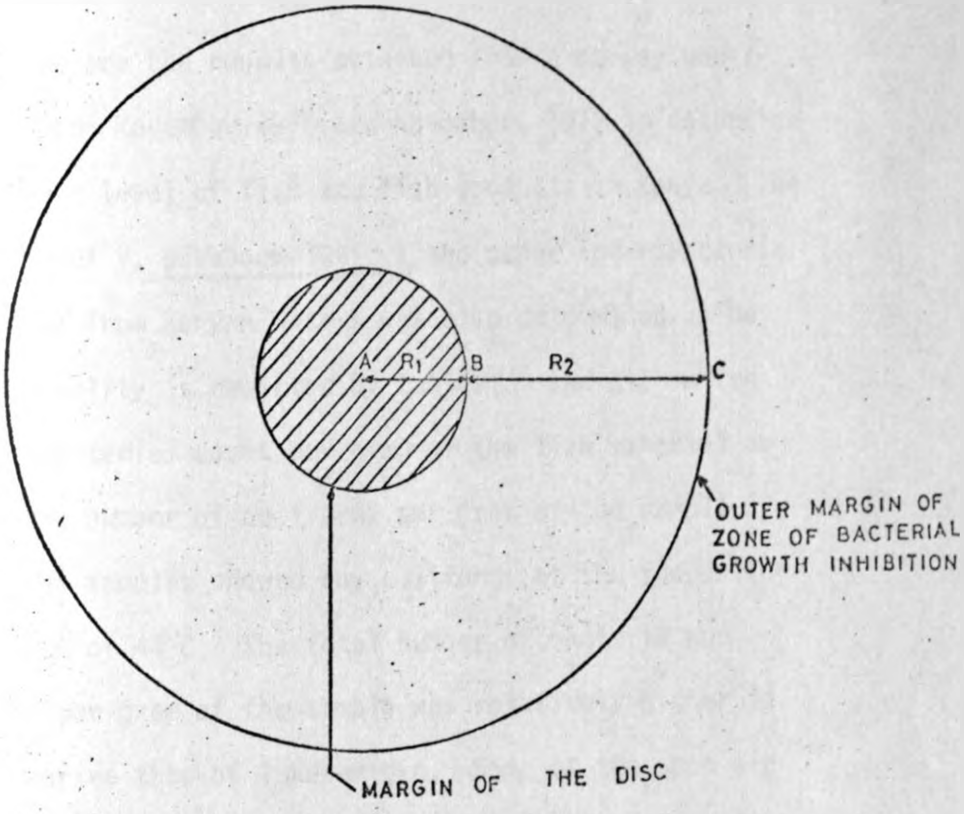

*Figure not drawn to scale.

Let AC represent the distance from the centre of the disc impregnated with the antimicrobial agent to the outer edge of the zone of inhibition. Suppose A is the centre of the disc, B the edge of the disc and C the outer edge of the zone of bacterial growth inhibition, then R_2 is given by (AC) - (AB) or AC - R_1 in millimetres, R_2 represents the zone of bacterial growth inhibition measured in millimetres. The diameter (D) of the zone of inhibition is given by the formula $D = (2R_2 + 6)$, where 6 is the diameter of the disc in millimetres.

4. RESULTS

4.1. Hygiene

Below are the results obtained from a survey undertaken between November 1976 and November, 1977 to determine the hygienic level of fish and fish products in Kenya. The prevalence of V. parahaemolyticus and other Enterobacteria in the fish from Kenyan waters was also determined. The hygienic quality is depicted by tables XI and XII which show the bacterial count per gram of the fish material as TPC and the number of coliforms per gram of the sample. None of the samples showed any coliforms at the incubation temperature of $44^{o}C$. The total number of bacteria and coliforms per gram of the sample was relatively higher in fish of marine than of lake origin. Some of the open air marketing units were located in the vicinity of unhygienic conditions which were potential breeding areas for the common housefly, (Musca domestica). It has to be stressed that the initial pilot experiments using intestines of fish gave total counts as high as 2×10^{15}. Since these initial results were too high for enumeration, it was decided that gills be used instead. Most of the agar plates showed overgrowth. The results obtained from the gill samples were reasonable and easy to evaluate. A positive relationship between the total plate count and the number of coliforms in a particular sample was observed. The higher the TPC per gram, the higher was the coliform count. This trend was observed in data from

both marine and lake fish. Of the 993 samples collected, approximately 8% were fish from Lakes Victoria and Turkana. The bigger portion of 912 samples (approximately 92%) were collected from the Kenyan Coastal waters namely, seafish, shellfish, sediment and water. Out of the 912 samples, 584 (approximately 64%) were seafood samples consisting of seafish, crustacean (prawns, lobsters and crabs) and molluscan (Oysters) shellfish collected from the City Market and Industrial area in Nairobi.

4.2 Vibrio parahaemolyticus, V. cholerae and Enterobacteria

V. parahaemolyticus was present in approximately 9.1% of the total number of samples in the survey as shown in tables XIV and XX on pages 74 and 80 respectively.

It appears that seafish from the Coast Province exhibits a higher prevalence of V. parahaemolyticus than either sediment or water, regardless of the area of origin as illustrated in Tables XV to XX. Table VI shows an overall V. parahaemolyticus prevalence rate of 6.4%. Apparently seafish contributed more to this prevalence rate than either sea water or sea sediment. However a difference in the rate of incidence is shown by sea water and sea sediment from Nairobi area. The significance of an incidence of 8% of V. parahaemolyticus contamination in marine fish in Kenya will be discussed later.

The membrane filtration technique for V. parahaemolyticus proved satisfactory in the recovery of V. parahaemolyticus from the sea water samples. The arbitrary concentration of 0.05% of Copper sulphate used was high enough

to inhibit the excessive growth of V. alginolyticus, thus enhancing the recovery rate of V. parahaemolyticus. Enumeration of the latter was not done because of the possible inhibitory effect exerted by the Copper sulphate. Rather, the qualitative aspect of the colonies was more emphasized. The species V. alginolyticus (formerly known as V. parahaemolyticus biotype 2) was not isolated from samples of lake fish. Other prominent bacterial flora encountered in some of the marine samples included coliforms, Alcaligenes faecalis and Pseudomonas species. Proteus vulgaris was constantly encountered in the 81 samples of lake fish processed in an attempt to isolate Salmonella species. Overgrowth of the TCBS agar surface with Proteus was overcome by spreading alcohol over the agar surface. This technique helped dry up the surface of the agar which minimized the swarming of Proteus.

Isolate X_1G_1 was confirmed to be serotype O-10, K-23 by Prof. Sakazaki. An arbitrary marking system was improvised to identify the isolates, ranging from X_1G_1 to $X_{74}G_{74}$. The X subscript refers to the number of the isolate, which in turn could be traced to a particular unit and G stands for the non-Sucrose fermenting property on TCBS agar. The isolate X_2G_2 was sent to Messrs. Miller and Barrow of Truro Cornwall Hospital who confirmed it to be serotype O-10, K-52 of V. parahaemolyticus. The X_1Y_1 designation was adopted for V. alginolyticus.

Attempts to isolate Salmonella species from the 81 samples of lake fish origin yielded negative results. None of the 993 samples processed contained V. cholerae biotypes.

4.3. Kanagawa haemolysis

Using WBA medium, the 72 V. parahaemolyticus isolates exhibited a negative KH phenomenon. The haemolytic properties of these isolates could not be fully evaluated when the cultures were point inoculated on ordinary human Blood agar.

4.4. Egg pathogenicity biassay

The relative virulence of X_1G_1 - confirmed to be O-10, K-23 was comparable to that of the K^{-ve} reference culture as shown by the mortality rates of the chicken egg embryos, in table XXI on page 81.

4.5. Antimicrobial sensitivity

The general trend was that all the cultures were very sensitive to Chloramphenicol, sparing inhibitory activity was demonstrated by Streptomycin, Tetracycline and Erythromycin. None or very little inhibitory activity was exerted by Penicillin G and Sulfafurazole. This is illustrated in table XXII on page 82.

4.6. Growth characteristics in table XXIII show that although salt concentrations ranging from 1.5% to 8.5% were tolerated by O-10, K-23 the optimal salt concentration conducive to the propagation was between 1.5% and 3.5%. Beyond 8.5% very little growth as indicated by the turbidity of the broth culture, was manifested. The latter observation also applied to salt concentrations less than 1.5%.

Table XI: Total aerobic plate and coliform count per gram as indices of the hygienic quality of dry lake fish sold in Kariokor Market in Nairobi

Sample number	Total aerobic plate count per gm.	Coliforms/gm at 37^{0} C
1	1.5×10^{4}	-*
2	1×10^{5}	-*
3	7×10^{3}	-*
4	2×10^{6}	-*
5	2.6×10^{3}	-*
6	5×10^{5}	-*
7	6×10^{6}	1×10^{1}
8	6×10^{7}	1×10^{2}
9	6.7×10^{6}	2×10^{1}
10	1.5×10^{7}	2×10^{1}
11	1×10^{5}	4×10^{1}

-* Indicates absence of coliform colonies on the Violet Red Bile Salt Dextrose agar plates.

Table XII: Total aerobic plate count and coliform count per gram as indices of the hygienic quality of fresh sea fish sold at the City Market in Nairobi

Sample number	Total aerobic plate count/gm	Coliforms/gm at $37^{o}C$
1	2×10^{12}	2×10^{11}
2	1×10^{12}	2×10^{4}
3	3×10^{11}	1×10^{4}
4	4×10^{11}	1×10^{4}
5	10.5×10^{6}	2×10^{4}
6	4×10^{6}	3×10^{4}
7	5×10^{11}	6×10^{4}
8	3.5×10^{6}	7×10^{4}
9	6×10^{6}	5×10^{4}
10	16.5×10^{7}	1×10^{4}
11	5.7×10^{11}	1×10^{4}
12	8×10^{11}	3×10^{4}
13	7.5×10^{11}	-*
14	9.6×10^{11}	-*
15	5.5×10^{11}	-*
16	5.5×10^{11}	1×10^{3}
17	7.4×10^{11}	1×10^{8}
18	7.7×10^{9}	-*
19	1×10^{12}	4×10^{4}
20	6.3×10^{9}	-*

-* Indicates absence of coliform colonies on the Violet Red Bile Salt Dextrose agar plates.

Table XIII: Total aerobic plate and coliform Counts per gram recovered from gills of various sea fish sold at the City Market in Nairobi

Sample* number	Species of fish	Total aerobic plate count/gm	Coliforms/gm
1	Rock cod	1.2×10^{12}	1.3×10^{11}
2	Rock cod	1.8×10^{11}	8×10^{10}
3	Rock cod	1×10^{12}	1×10^{11}
4	Sea brim	2×10^{6}	1×10^{4}
5	Sea brim	2×10^{6}	1×10^{4}
6	Rock cod	1.3×10^{9}	1×10^{3}
7	Rock cod	1.5×10^{9}	4×10^{3}
8	Changu	1×10^{11}	1×10^{8}
9	Rainbow	2×10^{11}	4×10^{8}
10	Rainbow	6×10^{6}	5×10^{3}
11	Rainbow	3×10^{4}	2×10^{3}
12	Rainbow	11.5×10^{8}	1×10^{4}
13	Sea brim	6×10^{6}	1.2×10^{6}
14	King fish	1.2×10^{7}	1×10^{6}
15	King fish	3.6×10^{9}	1×10^{3}
16	King fish	3.4×10^{11}	9×10^{5}
17	Rock cod	3.2×10^{11}	2×10^{3}
18	Rock cod	8×10^{11}	8×10^{10}
19	Rock cod	2×10^{9}	2×10^{7}
20	Mullet	1.1×10^{12}	2×10^{7}

*V. parahaemolyticus was not isolated from any of these samples however, V. alginolyticus and Proteus vulgaris were recovered from all the samples.

Table XIV Recovery of V. parahaemolyticus from sea fish and various shellfish sold in the Nairobi area subunits

Type of samples	Number positive	Number negative	Total number of samples	Percent positive
Sea fish	5	365	370	1.4
Prawns	29	101	130	22.3
Lobsters	13	31	44	29.5
Crabs	5	15	20	25.0
Oysters	1	19	20	5.0
Total	53	531	584	9.1

Table XV: The distribution of V. parahaemolyticus in sea fish, water and sediment obtained from all the subunits within the Coast Province

Type of sample	Number positive	Number negative	Total number of samples	Percent positive
Sea fish	13	122	135	9.6
Sea water	4	109	113	3.5
Sea sediment	4	76	80	5.0
Total	21	307	328	6.4

Table XVI: The distribution of V. parahaemolyticus in fish, water and sediment samples obtained from Magongo Market, Kilindini, Port Reitz and Malindi in the Coast Province

Source	Number positive	Number negative	Total number of samples	Percent positive
Magongo Market	2	98	100	2.0
Kilindini	2	71	73	2.7
Port Reitz	1	39	40	2.5
Malindi	16	99	115	13.9
Total	21	307	328	6.4

Table XVII: The distribution of V. parahaemolyticus in the sediment obtained from three areas in the Coast Province namely, Malindi, Port Reitz and Kilindini

Source	Number positive	Number negative	Total number of samples	Percent positive
Malindi	3	37	40	7.5
Port Reitz	1	19	20	5.0
Kilindini	0	20	20	0
Total	4	76	80	5.0

Table XVIII: The distribution of V. parahaemolyticus in the water samples collected from three subunits within the Coast Province (Malindi, Port Reitz and Kilindini)

Source	Number positive	Number negative	Total number of samples	Percent positive
Malindi	2	38	40	5.0
Port Reitz	0	20	20	0
Kilindini	2	51	53	3.8
Total	4	109	113	3.5

Table XIX: The distribution of V. parahaemolyticus in sea fish, sediment and water collected from Malindi in the Coast Province

Type of sample	Number positive	Number negative	Total number of samples	Percent positive
Sea fish	11	24	35	31.0
Sea sediment	3	37	40	7.5
Sea water	2	38	40	5.0
Total	16	99	115	13.9

Table XX: The distribution of V. parahaemolyticus in sea fish and various shellfish from subunits within both Nairobi area and the Coast Province

Type of sample	Number positive	Number negative	Total number of samples	Percent positive
Sea fish	5	365	370	1.4
Shellfish	48	166	214	22.4
Total	53	531	584	9.1

Table XXI: Results of the Egg Pathogenicity bioassay *a

Incubation time in hours	12	24	36	48
V. parahaemolyticus (K^{-ve}) Reference culture *b	30%	90%	95%	100%
Isolate from prawns X_1G_1 - (0-10, K-23) (K^{-ve})	25%	80%	96%	100%

*a Shows the mortality rate in percent at 12 hour intervals of incubation of the eggs at 37°C.

*b This reference culture was provided by Doctors Barrow and Miller of Truro Cornwall Hospital, United Kingdom.

Table XXII — Antimicrobial drug sensitivity expressed as diameters (D) of bacterial growth inhibition zones in millimetres

Antimicrobial drug	Dosage	Strain 0-10, K-23	*V. alginolyticus* X_1G_1	Strain 0-10, K-52	*V. parahaemolyticus* (K^{-ve}) *
Chloramphenicol	10 µg	26	20	20	20
Erythromycin	10 µg	16	14	14	16
Sulfafurazole	100 µg	-	-	10	-
Penicillin G	1.5 units	-	-	-	-
Streptomycin	10 µg	7	7	7	-
Tetracycline	10 µg	11	8	8	11

*This was a reference culture provided by Doctors Barrow and Miller of Truro, Cornwall Hospital, United Kingdom.

Table XXIII: The growth of X_1G_1 (serotype 0-10, K-23) in Peptone Water containing different Sodium chloride concentrations, at an incubation temperature of 37°C. The tabulated figures indicate absorbance as an index of growth at wavelength λ 400 nm.

Minutes	Sodium chloride concentration % [b*]							
	0	1	3	5	7	8	11	13
0 [a*]	.090	.430	.410	.190	.045	.100	.085	.095
30	.060	.440	.390	.250	.045	.100	.085	.095
60	.070	.420	.400	.210	.035	.090	.075	.080
90	.090	.440	.410	.230	.050	.120	.095	.120
120	.095	.440	.420	2.40	.075	.120	.095	.100
150	.095	.450	.460	.290	.080	.120	.095	.095
180	.095	.490	.520	.300	.160	.120	.110	.100
210	.330	.520	.520	.390	.160	.125	.120	.100
240	.250	.525	.530	.395	.165	.120	.120	.100
270	.250	.525	.535	.395	.165	.130	.125	.100
300	.300	.550	.535	.400	.165	.135	.125	.100
330	.300	.551	.540	.410	.170	.135	.130	.100
360	.350	.560	.560	.440	.170	.140	.140	.100

[a*] It is to be noted that the 0 minutes initial time is 6 hours after inoculation of the broth tubes and incubation at 37°C.

[b*] 0.5% has to be added to the tabulated salt concentrations since Peptone Water, the medium used, contains 0.5% NaCl.

Table XXIV The biochemical properties of the studied Vibrio parahaemolyticus isolates

	Characteristic	Positive	Negative	Atypical isolates
Formation of	Oxidase	72	0	
	Catalase	72	0	
	Indole	72	0	
	Acetylmethylcarbinol	0	72	
	Methyl red test positive	72	0	
	Growth in 7% NaCl broth	72	0	
	Growth in 10% NaCl broth	3	69	$X_{20}G_{20}$;$X_{23}G_{23}$;$X_{26}G_{26}$
	Gelatin liquefaction	72	0	
	Nitrate reduction	72	0	
	Citrate assimilation (Christensen)	72	0	
	Arginine desamination	0	72	
	Lysine decarboxylation	72	0	
	Malonate assimilation	0	72	
	Urea dissimilation	0	72	
	H_2S formation in peptone agar	0	72	
	Growth at pH 9.0	72	0	
Dissimilation of	Glucose, acid	72	0	
	Glucose gas	0	72	
	Fructose	72	0	
	Arabinose	72	0	
	Rhamnose	2	72	$X_{15}G_{15}$;$X_{17}G_{17}$
	Xylose	1	73	X_6G_6
	Maltose	72	0	
	Cellobiose	68	4	X_7G_7;X_9G_9;$X_{10}G_{10}$;$X_{12}G_{12}$
	Sucrose	0	72	
	Lactose	0	72	
	Trehalose	72	0	
	Salicin	0	72	
	Mannitol	72	0	
	Glycerol	72	0	
	Adonitol	0	72	
	Inositol	1	71	$X_{18}G_{18}$

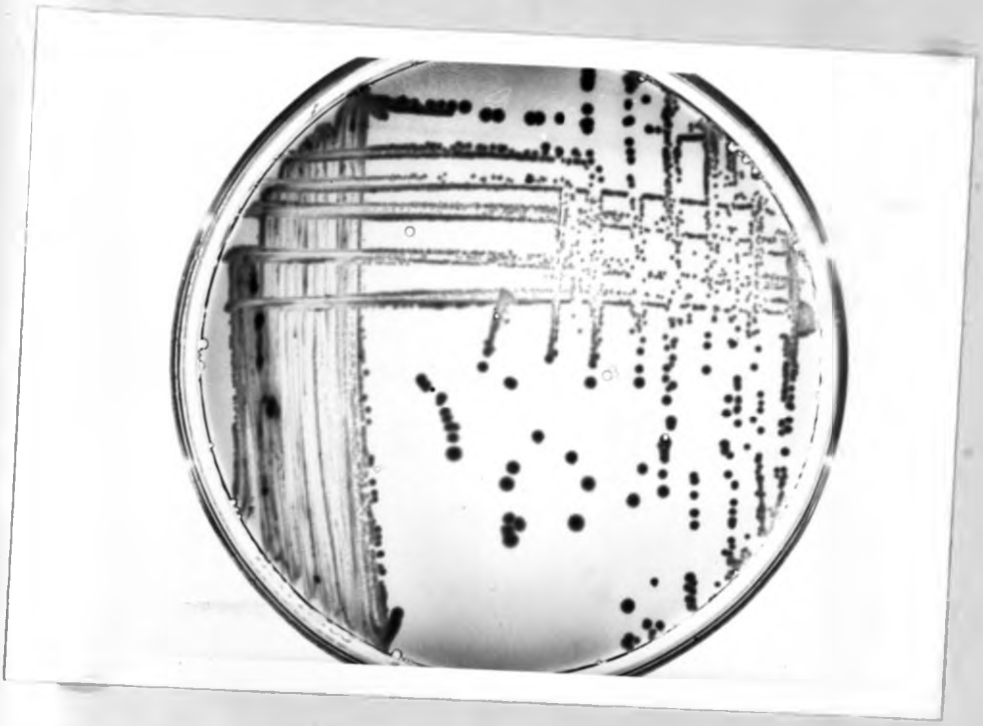

Fig. II: An 18 hour culture of V. parahaemolyticus serotype O-10, K-23 growing on TCBS agar, after incubation, aerobically at 37°C. (x 10).

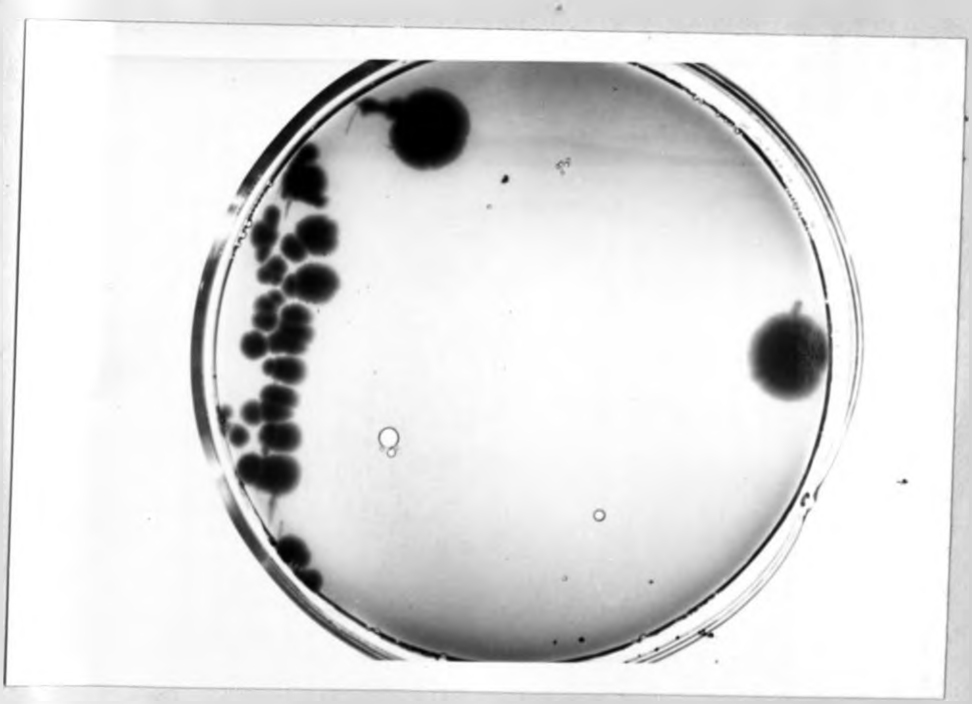

Fig. III. A 48 hour culture of serotype O-10, K-23 illustrating the swarming characteristic of V. parahaemolyticus on TCBS agar. (x 10).

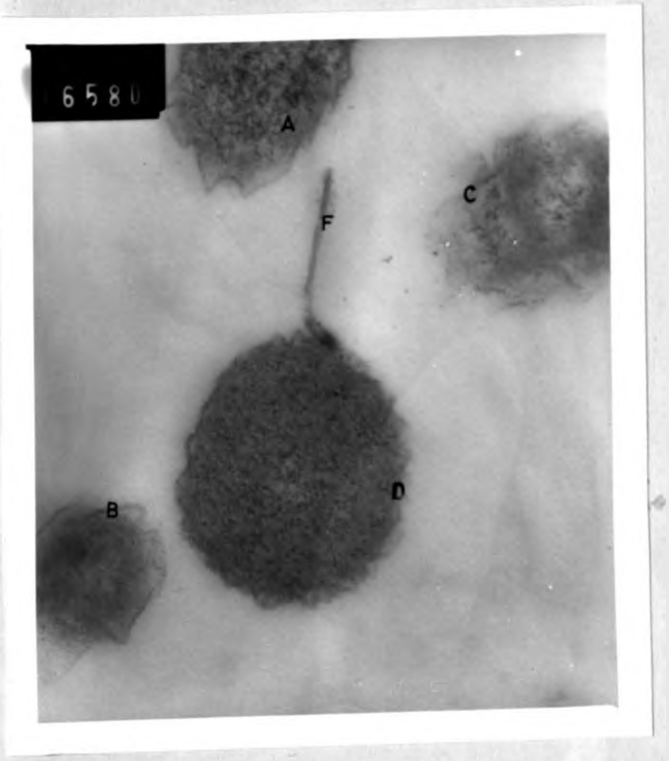

Fig. IV: Electron micrograph showing cells of serotype O-10, K-23. (x 43.000).

The central cell (D) with a single polar flagellum (F) was not sectioned, while the other cells, A, B, and C, were.

Stain: Uranyl acetate and Lead acetate.

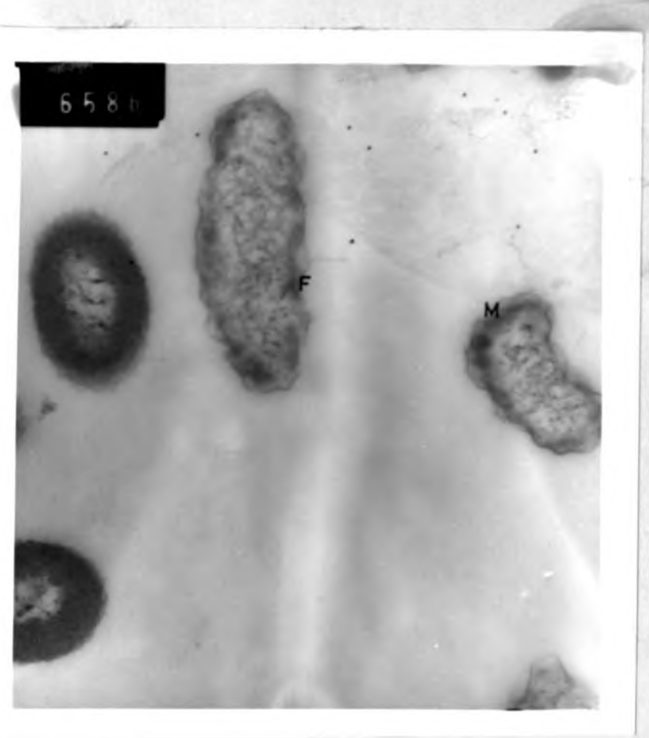

Fig. V: Electron micrograph showing a cross section of a cell of serotype O-10, K-23 with a short lateral flagellum (F). (x 19.000).

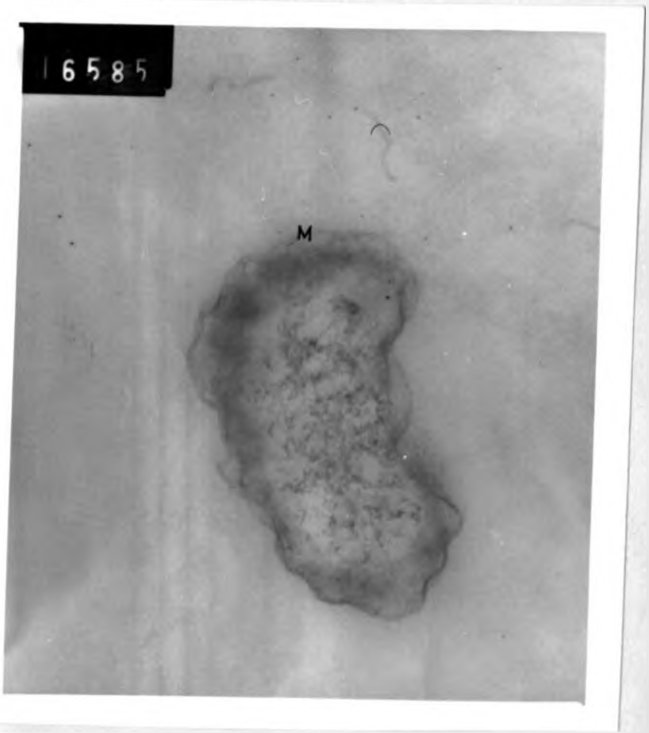

Fig. VI: Electron micrograph of one of the cells (M) from Figure V at a higher magnification, showing the curved form of serotype O-10, K-23. (x 43.000).

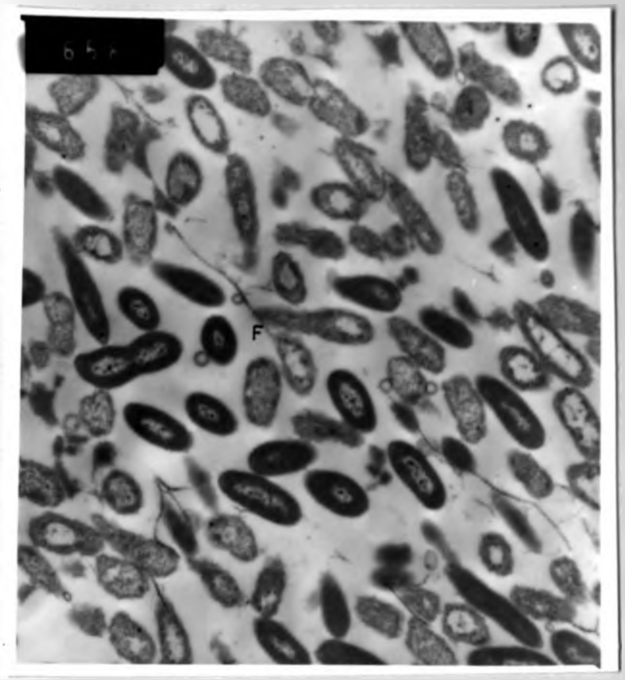

Fig. VII: Electron micrograph showing various sections of cells of serotype O-10, K-23. (x 7.000)
The central area is shown in higher magnification in Figure VIII.

Stain: Uranyl acetate and Lead acetate.

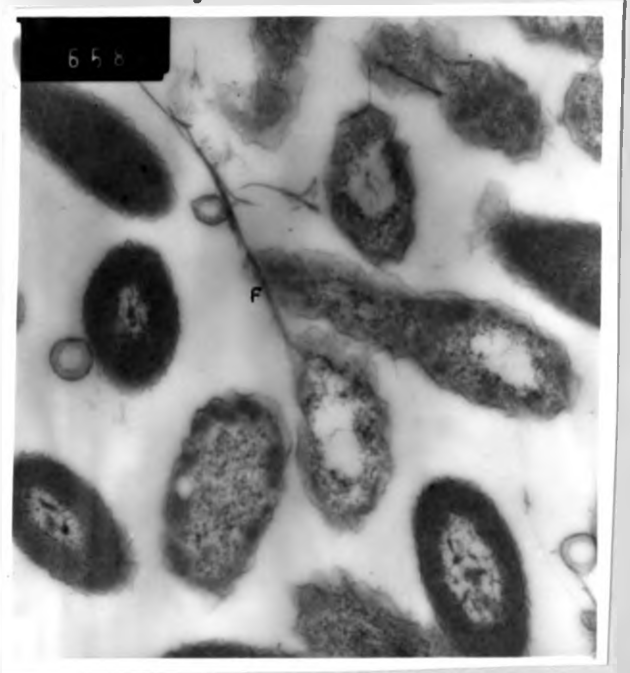

Fig. VIII: Electron micrograph showing sections of cells of serotype O-10, K-23 with a single polar flagellum (F). (x 43.000).

Stain: Uranyl acetate and Lead acetate.

5. DISCUSSION

The food hygienic investigation shows that the total bacterial load is relatively higher in fresh seafish than in dry lake fish as shown in tables XI, XII and XIII on page 72, 73 and 74 respectively. However, compared to those observed by Shewan (1949), the counts obtained both for lake fish and sea fish would be considered to be very high.

High counts could be due to inherent flora or flora acquired through subsequent handling, as secondary contaminants. The lake fish samples processed by fish preservation techniques namely drying, salting and smoking appear to harbour a smaller population of microflora, than fresh sea fish. The salinity of the sea water from which the fresh sea fish is harvested has selected for a population of halophile, which form the inherent flora of the fish. The bactericidal action of sea water reported by ZoBell (1936) is exerted on the non-halophilic bacterial population. Therefore the processed fish should give lower counts since salting, smoking and drying lower the water activity (a_w) of the fish and hence are bacteriostatic.

These processes not only reduce the microflora but also alter it qualitatively. Only some mesophilic bacteria resistant to the detrimental action of these preservation techniques survive in the thus treated fish products. The microflora observed in both sea fish and lake fish is either inherent or acquired subsequent to unsanitary handling and processing. The latter mode of extramarine contamination

has been discussed in the literature review. The inherent flora in the sea fish is remniscent of the bacteriologic status of the adjacent marine environment. The significance of sea water as the environment of origin of the fish and hence a major contributing factor to the quantity and quality of the inherent microflora in the fish has to be determined. No such data was compiled as it was thought to be irrelevant to this survey. The number of coliforms appeared to bear a positive correlation to the total bacterial counts in the particular sample. The higher, the coliform count, the higher the bacterial load. No such association has been pointed out in the literature. However, the presence of coliforms, specifically, Escherichia coli in sea water fish is documented (Carlucci and Pramer 1959; 1960). Varga and Anderson (1968) have reviewed their significance in fish products.

Since no thermophilic coliforms were isolated in this study, this should indicate that either the numbers are so low that they were missed in the routine isolation techniques or they were absent. Their presence would indicate faecal pollution. The containers used for storage and transportation of the fish must not be overlooked as an important source of extramarine contamination as reviewed in the literature.

In some subunits fish was sold in open air markets in the vicinity of which were flies. Exposure of such fish to warm ambient temperatures and the presence of the flies tend to favour proliferation of not only fish spoilage microflora but also mesophilic pathogens.

Under such conditions, the proliferation of the inherent flora whether high or low ensues, even when fish are kept at chilling temperatures in ice boxes.

The prevalence rate of V. parahaemolyticus in the 912 samples of marine origin was computed as 8.1%. Shellfish both from the Coast Province and Nairobi area gave the highest prevalence rates of this Vibrio. It appears that lobsters collected from Nairobi area subunits as shown in Table XIV on page 75 had the highest prevalence rates compared to seafish from the same area. This could be explained in terms of the capacity of shellfish to concentrate bacteria during their feeding processes. However, data from the same table does not support this idea because oysters in contrast to either prawns, lobsters or crabs gave a relatively low prevalence of the bacterium. The discrepancy within the shellfish sample types might be considered insignificant since the size of the oyster samples was relatively small.

According to Clark (1977), one would consider all shellfish as presenting a major health hazard and sea fish from Nairobi area as presenting none. Seafish from Malindi subunit of the Coast Province gave the highest prevalence rates of the Vibrio as shown in tables XVI, XVII, XVIII and XIX. The reason for this is not clear but it could be due to the fact that this particular fish market is situated on the sea. It is therefore, likely that the samples were taken at the time when the V. parahaemolyticus die-off rates due to removal from the protective sea water were minimal.

The relationship between the sample type and the source is not consistent. However, tables XVI, XVII, XVIII and XIX seem to indicate that marine samples from Malindi have higher prevalence rates for the organism. Variations in the salinity due to the rough sea conditions prevalent at time when the samples were collected in September 1977, might partly explain this.

As a result of the rough sea, the turn over rate of the waters becomes high and inevitably, the bacteria therein.

This hypothesis might account for the higher prevalent rates observed in the sediment from Malindi, than the water sampled from the same area. One would assume that the environment adjacent to the sediment is more or less stable compared to the constantly changing waters.

The importance of this halophile as an agent of food-borne illness is confined to special cases. Whether it represents a "practical" or a potential hazard would depend on a number of factors. The most important factors here would be the eating habits of the people i.e. whether seafoods are eaten raw, undercooked or fermented and the hygienic practices of the fish eating communities when preparing seafoods for consumption. Contamination rates of 22% for frozen shellfish and 33% for non-frozen shellfish are documented (Thomson and Thacker 1974). Chilling of the sea products in ice appears to reduce the contamination rate (Johnson et al., 1973 ; Johnson and Liston 1973). This would be on a long term basis because V. parahaemolyticus has been reported to survive at $0^{o}C$ for a period of 30 days. Other reports Clark (1977) consider a

prevalence rate of 1.4% as presenting no major health hazard. In view of the fact that hygiene becomes the most important limiting factor here in Kenya, along the seafood production chain, V. parahaemolyticus poses a 'potential' hazard as a food-borne pathogen. Hygiene sometimes breaks down along this chain as a result of inefficiency.

Generally shellfish appeared to be more frequently contaminated with V.parahaemolyticus than the sea fish from the same subunits. This could be explained in terms of the filter feeding mechanisms of both crustacean (prawns,lobsters and crabs) and molluscan (oysters) shellfish. Within a short time, they filter alot of water from which they extract food along with the bacteria.

Kampelmacher, et al., (1972) reported 0.25% of 407 samples of fish and crustaceans originating from the North Sea to be contaminated with V. parahaemolyticus, a frequently occurring bacterium in molluscs such as oysters. These two pieces of evidence substantiate their mode of feeding as contributing to their adulteration by the halophilic bacterium. Perhaps the incidence rate of V. parahaemolyticus is higher than is otherwise stressed, in this survey. The warm coastal waters are conducive to the propagation of the organism since the bacterium is a mesophile. The temperature effect coupled with cross contamination from the seafood aggravates the situation. Cross contamination would stem from unsanitary handling of the fish by the dealers and consumers. Vegetables and other non-marine foods have been known to be thus contaminated in kitchens as discussed in the literature.

Technical problems in the survey cannot be overlooked as they might have contributed to the results obtained. Enrichment and isolation procedures used in the survey have been reported in the literature as being efficient. However, they cannot be regarded as perfect. To circumvent such a drawback, these procedures would have to be combined, since none of them is outstandingly superior. The inhibitory substances namely Colistin and Teepol used in the enrichment broths not only reduced V. alginolyticus but also V. parahaemolyticus. Hence, this might have contributed to the low contamination rates in some cases. Similar effects may have been exerted by Copper sulphate 0.05% w/w and Sodium cholate used in the membrane filter technique for sea water screening for V. parahaemolyticus.

Because of this shortcoming the membrane filter technique was not used as an enumeration technique but rather, a qualitative one (Watkins et al., 1976).

All media used for selective isolation depends on a high pH, high salt content and temperature. Currently, it is suggested that Starch, Chitin or a fish-based medium should be added to enhance this selectivity.

The preliminary screening tests involving morphologic and biochemical properties were conducted to identify not only the genus but also the species V. parahaemolyticus. Currently, sophisticated studies involving electron microscopy, DNA base ratio, recombinations and fatty acid profiles coupled with intensive phenotypic analysis as done by Colwell et al., (1973) are being done. Citarella (1970), reported the polynucleotide relationship among the Vibrio species. Unfortunately, this

tends to make the nomenclature within the genus Vibrio more uncertain. In this survey such sophisticated methods were not applied due to lack of facilities. This however did not hinder us from identifying the 72 unconfirmed isolates, in the survey samples, as V. parahaemolyticus. The vibriostatic agent 0/129 (2, 4-diamino-6, 7-diisopropIteridine) recommended for identification of the genus Vibrio was used. The use of this agent as a sole diagnostic criterion is questioned, however Colwell (1970), appraised the value of phage typing as an identification procedure for V. parahaemolyticus. Availability of the specific phage to perform to this test was a limiting factor in this survey. Since most identification deductions were based on biochemical reactions of the isolates, the shortcomings of these tests are worth discussing. Vanderzant and Nickelson (1972 b) question the use of 10% Sodium chloride as a differentiating technique of V. parahaemolyticus and V. alginolyticus. The ionic salt concentration of the medium has to be taken into account.

Variations among the V. parahaemolyticus strains as regards tolerance to 12% sodium chloride exists, although generally, V. parahaemolyticus in contrast to V. alginolyticus will not thrive in media containing such concentrations. The isolate X_1G_1 (O-10, K-23) did thrive at an even higher concentration considering the fact that Peptone water media contains a salt concentration of 0.5%, as shown in table XXIII on page 83.

The Tripple Sugar Iron medium reaction used in preliminary screening tests may be consistent but is in no way diagnostic

because Aeromonas species and some other Enterobacteriaceae produce similar reactions. Failure to ferment Sucrose does not always differentiate V. alginolyticus from V. parahaemolyticus because Sucrose positive variants of the latter have been reported. In fact some of the isolates including O-10, K,52 and O-10, K-23 showed a slight tendency towards this.

The TCBS medium used for selective isolation has been recommended as being the best. However, in this study, Pseudomonas and Proteus species and V. alginolyticus were common as background flora on this medium. Differentiation between V. alginolyticus and V. parahaemolyticus was primarily based on the yellow colour of the colonies of the former due to Sucrose fermentation. Such a distinction is not absolute as one gets Sucrose fermenting V. parahaemolyticus variants. In fact, serotype O-10, K-23 showed slight yellow colouring which progressed with incubation period.

The criteria used for identifying the isolates was not concrete since according to Colwell,(1970), the taxonomic status of this species is still uncertain. Kampelmacher, et al., (1972) attributes such discrepancies in some biochemical reactions partly to degenerative changes in the strains, stemming from collections due to strains prolonged storage. Intrinsic variation within these strains may also play a part. Urease positive, Indole negative variants (atypical) of the organism have been described(Zen-Yoji et al., 1973). Ecological and numerical taxonomic studies undertaken by Colwell (1970) are suggestive of exchange of genetic material among marine Vibrios and perhaps other bacteria. This is possible through

conjugation and transduction of bacteriophages, as reported by Colwell et al., (1970); Baross et al., (1974).

Failure to isolate V. cholerae and Salmonella species from the sample is not suggestive of total absence. It is possible that the enrichment procedures were not efficient to pick out the cold stressed cells, although recommended procedures were employed. Therefore, it is recommended that more surveys of this nature be carried out to determine the prevalence of these pathogens in the fish in Kenya.

Until such extensive surveys are done, the role of fish in disease dissemination in this context cannot be underestimated.

Whether the Kanagawa haemolysis can be used as a sole pathogenicity determining criterion is still a controversy. Moleda et al., (1972),have reported food poisoning due to K^{-ve} strains of V. parahaemolyticus. While the egg pathogenicity bioassy, would indicate the relative multiplication or propagation rate of O-10, K-23 as compared to the K^{-ve} reference V. parahaemolyticus strain, it is no measure for pathogenicity. Per os administration of these K^{-ve} strains and perhaps the lapine ileal loop test systems would have been better indices, of their pathogenicity. Their deliberate omission from this survey was due to the fact that their value as pathogenicity evaluating techniques is doubtful. The genetic relationship between the cultures, O-10, K-23; O-10, K-52, V. alginolyticus and the V. parahaemolyticus reference culture was partly reflected in their sensitivities to antimicrobial drugs. Chloramphenical would have been the drug of choice

in Vibrio infections, with Erythromycin as the alternative should infections due to these organisms occur. The reliability of these sensitivities is shown in table XXII. The comparisons have to be carefully interpreted since, the concentrations of the agents in the latter table are higher than those used. Apart from the hazard posed by V. parahaemolyticus as a fish-borne pathogen, its association with leg gangrene and subsequent endotoxic shock have been described (Rowland, 1970). Swimmers, in the Kenya Coastal waters are likely to acquire the organism through broken skin, in which case, it becomes an "opportunist". Hollis et al., (1976) have re ported halophilic Vibrio species in blood cultures. Another report by Zide and Ehrenkranz, (1974), point to its potential as a casual agent of some human septicaemias.

6. CONCLUSION

The halophilic bacterium V. parahaemolyticus; a human enteropathogen was isolated from sea fish, shellfish (crustacean and molluscan), water and sediment samples obtained from Nairobi area and the Coast Province of Kenya.

Neither V. Cholerae biotypes (Classical V. Cholerae and the El-Tor) nor Salmonellae species were isolated in the survey. Fish spoilage microflora namely Alcaligenes faecalis Pseudomonas and Proteus species were recovered from the marine samples. Taking into account the prevalence rate of V. parahaemolyticus in seafoods, water and sediment and the unsanitary fish handling practices along the production system, V. parahaemolyticus poses a "potential" health hazard.

The most likely population at risk would be these communities who eat raw, undercooked or fermented sea foods customarily. The one single important mode of transmission of the organism to the non-fish eating individuals is through cross contamination, either at marketing establishments or in the kitchen. Kitchen tables have in the past been implicated as important formites in disseminating this pathogen in cases of gastroenteritis. It seems that the potential of this organism as an important fish-borne agent is aggravated by its tolerance to chilling temperatures and to high salinity.

It appears that the exposure to high ambient temperatures in fish markets subsequent to the chilling on transit allows a rapid proliferation of the organisms on the seafoods.

To date there are no data on this organism in the medical literature in Kenya. The isolation of this organism from the marine fish and other seafoods, water and sediment, coupled with a follow up of acute human gastroentiritis cases might elucidate the real significance, in the Kenyan Coastal waters. In the light of this, therefore, it is recommended that:

(1) Public Health Officials responsible for fish Inspection programmes in fish marketing establishments should screen for this V. parahaemolyticus in marine seafoods.

(2) V. alginolyticus, the ubiquitous marine halophile should be used to monitor the effectiveness of thermal treatments given to seafoods in hotels.

(3) Proper refrigeration facilities should be installed in fish marketing units responsible for delivering large quantities of fish to consumers. This should be done at $0^{o}C$ for a period of not less than two weeks, so as to reduce or arrest bacterial growth.

(4) Public Health Personnel should exercise control over the cultivation of the shellfish (crustacean and molluscan) especially prohibiting the use of sewage polluted waters used for this purpose. And should sewage gain access to the cultivation areas, depuration in non-polluted, clean waters should be carried out.

(5) The fish eating communities should be educated on the repercussions of handling fish unhygienically. For those who customarily consume raw, partially cooked or fermented seafoods, persuasion to change their eating habits might be the only long term solution.

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Appendix 1: Confirmation of the cultures*

Tests performed for the confirmation of the culture X_1G_1, as serotype O-10, K-23.

Test	Reaction	Test	Reaction
Gram negative rod	+	Lysine decarboxylase	+
Growth in air	+	Arginine dihydrolase	-
Growth anaerobically	+	Urease: Christensen	-
Catalase	+	Gelatinase	+
Oxidase	+	Carbohydrate fermentation	+
OF test	F	Glucose: acid	+
Motility at 37°C	+	gas	-
Growth at 22°C	+	Arabinose	+
" 37°C	+	Melezitose	+
" 42°C	+	Rhamnose	-
Growth in 0% NaCl	-	Sucrose	-
" 1% NaCl	+	Mannitol	+
" 3% NaCl	+	Sorbitol	-
" 6% NaCl	+	Amygdalin	+
" 8% NaCl	+	Inositol	-
" 10% NaCl	-	ONPG	-
Nitrate reduction	+	Growth on TCBS agar	+
Indole +	+	Pteridin O/129	+
Voges-Proskauer at 37°C	-	Kanagawa type	
Citrate (Simmons)	+	type haemolysis	-
Malonate	-		
Hydrogen sulphide	-		
Phenylalanine deaminase	-		

*The tests were performed by Professor Sakazaki, (Japan).

Appendix II: The relationships between the sources of particular samples and the recovered V. parahaemolyticus isolates

Source	Type of sample	Name of Isolate(s)
City Market	Prawns	X_1G_1 - $X_{29}G_{29}$
" "	Oysters	$X_{30}G_{30}$ - $X_{35}G_{35}$
" "	Crabs	$X_{31}G_{31}$ - $X_{35}G$
" "	Sea fish	$X_{36}G_{36}$ - $X_{41}G_{41}$
" "	Lobsters	$X_{42}G_{42}$ - $X_{54}G_{54}$
Magongo market	Sea fish	$X_{55}G_{55}$ - $X_{56}G_{56}$
Kilindini	Sea water	$X_{57}G_{57}$ - $X_{58}G_{58}$
Malindi	Sea water	$X_{59}G_{59}$ - $X_{60}G_{60}$
Port Reitz	Sediment	$X_{61}G_{61}$
Kilindini	Sediment	$X_{62}G_{62}$ - $X_{63}G_{63}$
Malindi	Sediment	$X_{64}G_{64}$ - $X_{66}G_{66}$
"	Sea fish	$X_{67}G_{67}$ - $X_{74}G_{74}$