DISTRIBUTION OF VIBRIO ORGANISMS IN THE ENVIRONMENT IN BETWEEN EPIDEMICS IN THREE LOCAL GOVERNMENT AREAS OF KADUNA STATE

BY

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DECLARATION

I Egbom C.E., hereby declare that this work presented in this thesis is original. It was carried out by me in the laboratory of the Department of Microbiology Ahmadu Bello University Zaria, Nigeria, under the supervision of Professor (Mrs) Lucy J. Ogbadu and Dr. Musa Galadima. No part of this work has been submitted for any degree or qualification before. The work of other workers were referred to during this work and they were duly acknowledged.

Egbom C.E.

CERTIFICATION

This thesis titled - Distribution of vibrio organisms in the environment in-between epidemics in three local government areas of Kaduna State by Egbom Chukwukere Ephraim meets the regulations governing the award of the degree of master of Science of Ahmadu Bello University Zaria, Nigeria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This piece of work is dedicated to children all over the world (Ages 0 - 5), whose lives are always threatened by cholera and for whose sake this work was initiated and to my family.

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I wish to acknowledge the goodness and mercies of our heavenly Father, who has crowned my efforts with success, during this my "Second Academic journey". In the face of many obstacles He led me through. Therefore, I praise and adore His holy name.

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ABSTRACT

The distribution of vibrio organisms in the environments in Giwa, Zaria and Sabon-Gari local government areas of Kaduna State was investigated. Samples were collected from ponds, wells and stream. One hundred and fifty samples each were collected from these sources. In addition 150 samples each of human stools and duck stools as well as the roots of 5 water-letus were examined for vibrio organisms. A total of 29 vibrio organisms were isolated from 450 water samples while 4 isolates each were obtained from the 150 human stool and 150 Duck Stool samples respectively. No vibrio isolate was recovered from the roots of the water-letus. Out of the total of 37 vibrio isolates, 16 were identified to belong to the vibrio cholerae serogroup 01, while the remaining 21 isolates belong to vibrio cholerae non-01 serogroup. 13 out of the 16 isolates of the 01 serogroup were identified as inaba biotype while 2 could not be conclusively identified. Eleven of the 21 isolates belonging to the non-01 serogroup were identified to be vibrio parahaemolyticus, while the remaining were regarded as other vibio species which could not be identified to species level due to lack of materials. The dependence of the communities to some large extent on pond, stream and well water for agricultural and domestic purposes exposes them to the risk of vibrio cholerae infections.

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CHAPTER ONE

1.0 INTRODUCTION

The genus vibrio is classified in the family vibrionaceae (Farmer **et al**, 1985). They were said to be halophilic because of their requirements for sodium chloride for growth and their presence in salt water environments, such as the sea water, though they have been isolated from several fresh water environments, estuarines and even non-water environments (Smith, 1968; Vernon and Thomas, 1978; Robert and Nathaniel, 1984).

There are a number of vibrio organisms which cause diseases. The disease may be classified as intestinal as in diarrhoea diseases or extraintestinal as in infections of the eye, ear, wounds of leg, and arm, and gallbladder infections (Carol **et al**, 1984; Farmer **et al**, 1985). Vibrio cholerae serogroup 01 is known among the vibrio species for causing cholera in epidemic proportion, though other vibrio organisms such as V parahaemolyticus and most recently vibrio cholera serogroup 0139, cause several sporadic and epidemic diarrhoeal diseases (Judith **et al**, 1994; Popovic **et al** 1995; Dilip **et al**, 1996). Cholera is a severe infection of the intestine (Bernadette **et al**, 1992). It has been described as a lethal secretory diarrhoeal disease caused by the bacterium vibrio cholerae (Dilip **et al, 1996)**. The disease is characterized by abrupt onset with vomiting, extraordinary diarrhoea, muscular

cramps, dehydration, subnormal temperature, fall of blood pressure, suppression of urination and sudden collapse. The organisms produce enterotoxin (CT) which activates the enzymes of the intestine, leading to secretion of large volume of fluid and electrolytes into the lumen of the intestine, consequently leading to the severe continuous diarrhoea and gastroenteritis with vomiting (Vernon and Thomas, 1978; James **et al**, 1992).

Cholera has continuously posed a problem to the developing countries, including Nigeria (World Health Organisation, 1991). It is a communicable disease which has caused high rate of morbidity and mortality in developing countries, with greatest frequency in children (Sack **et al**, 1980; World Health Organisation, 1991; Judith **et al**, 1994). The worldwide spread of cholera began in 1816, and 1823, the first pandemic of cholera was recorded (World Health organisation, 1991). There has been seven pandemics of cholera since the disease was discovered. The spread of the seventh pandemic still continues throughout the world, though the recent discovery of epidemic cholera caused by a new strain of vibrio called - vibrio cholera 0139 - synonym Bengal, is suspected to be the agent of an eighth pandemic of cholera (Swerdlow and Ries, 1993; Mandal, 1993; Albert, 1994).

Cholera is endemic in many countries of the world especially Asian and African subcontinents where socio-economic conditions are poor, sanitary systems and public

hygiene are rudimentary and safe drinking water is not available (Huq **et al**, 1996). In endemic areas, the disease occurs with greatest frequency in children especially after early infancy and young adults, though in recently infected areas, it generally affects all ages including children and adults (Sack **et al**, 1980; W.H.O; 1991).

In Nigeria, infections due to vibrio organisms have continuously been reported since cholera was introduced into the country in 1970 (Nigerian Bulletin, 1991). The epidemic of cholera occurs almost every year in Nigeria and at times twice in a year. In 1991, a devastating epidemic raged through Nigeria and made her the worst hit country in the world (Asagba, 1992). During epidemics, toxigenic vibrios can be isolated from local fresh water and patients. These organisms disappears from the environment after the epidemics. The reservoir or site of survival and multiplication of these pathogenic vibrios during inter-epidemic periods are yet unknown (Islam and Aziz, 1981). There has not been satisfactory explanation as to how the seasonality and endemicity of these vibrios are maintained, though various explanations have been suggested (Khan **et al**, 1984; Cheesbrough, 1985; Lucas and Gilles, 1990; Islam **et al**, 1990). However, vibrio organisms have been found in fresh water estuarines (Robert and Nathaniel, 1984), Marine waters and sediments (Islam **et al**, 1989), aquatic faunas including oysters, Zooplankton (Sarkar **et al**, 1985), and crabs have also

been considered as potential habitat of vibrio cholerae in the aquatic environment (Kaneko and Colwell, 1973). Aquatic plants such as green and blue-green algae have been found associated with the vibrios. They have also been found in association with copepod in estuarine waters, hydrophitic fresh water plants, filamentous green algae and blue - green algae in Bangladesh (Islam and Aziz, 1978; Islam **et al**, 1989 and 1990).

The Federal Ministry of Health and social Services, Nigeria, established an "Emergency task force", on the control of cholera epidemic in 1991. The activities of the task force include - (a) Health education programes (b) Water treatment and (c) Distribution of oral rehydration salt (ORS) to states. Some states spend over 5.2 million Naira annually on purchase of Oral rehydration Salt, antibiotics and Vaccines and yet the epidemics continue in seasonal occurrence (Nigerian Bulletin, 1991).

The epidemiological studies so far, have been valuable to the understanding of the transmissibility of the vibrio Organisms, but the inter-epidemic reservoir of these organisms remain to be identified. Therefore, the aims of this research are (1) to identify the reservoirs of cholera vibrios in parts of Kaduna State. (2) To identify the serogroups and biotypes of vibrio cholerae present in those parts of Kaduna State. (3) It is hoped that information derived from this work will provide better understanding of the transmission of cholera in

Kaduna State and consequently more comprehensive control measures of the disease in this community.

2.0 LITERATURE REVIEW

2.1 THE VIBRIO ORGANISMS

The genus vibrio belongs to the family vibrio-naceae. Members of this genus are small curved or rather helicoidal shaped organisms which vary form 1-3μm in length and 0.4 - 0.6μm in width (Farmer **et al**, 1985). The degree of curvature varies from the short comma-shaped to spiral, with one or two turns. The comma-shaped are common in fresh faecal specimens or in young cultures, while the longer forms are found in older cultures. The organisms assume a distinct bacillary shape after prolonged cultivation on artificial media. They are described as facultative anaerobic gram-negative rods, actively motile with terminal flagella. They are non-sporing, noncapsulated, ferment glucose without gas formation, oxidase positive, non-acid-fast and require sodium chloride for growth (Smith, 1968; Vernom and Thomas, 1978; Greenwood **et al**, 1992).

Several members of the genus have been implicated in a number of human infections, which can be classified as intestinal or extraintestinal (Blake **et al**, 1979; Safrin **et al**, 1988). Five of the members have been associated with or found to cause several outbreaks of intestinal infections, such as diarrhoea (Morris **et al**, 1990). Several other strains have been implicated in several extra-intestinal infections, such as the infections of the blood (Safrin **et al**, 1988),

wounds of the arms and legs (Love **et al**, 1981), the ear and the gallbladder (Carol **et al**, 1984). Cases of other infections such as pneumonia and meningitis have also been reported (Blake **et al**, 1979; Kelly and Avery, 1980; Farmer **et al**, 1985).

Vibrio Cholerae is well known above other vibrio species as the causative agent of cholera. There are more than sixty serogroups of vibrio cholerae but only the serogroup 01 occurs as two biotypes - the classical and EL Tor biotypes. Each biotype also occur as two serogroups - Inaba and Ogawa (Farmer **et al**, 1985). The EL TOR biotype, (Named after the EL TOR quarantine station in the sinai, where the organism was first isolated), is known to cause higher infections and survives longer than the classical biotype in environment such as water, waste materials, faeces and sewage (Vernon and Thomas, 1978; Pelczar **et al**, 1982; Farmer **et al**, 1985).

Vibrio Cholerae serogroup 01, has gained a worldwide notoriety for causing epidemic cholera or "Asiatic Cholera" as frequently designated (Vernon and Thomas, 1978; Popovic **et al**, 1995). Isolates from other serogroups were believed to be associated with sporadic diarrhoeal and invasive infections, but they rarely produce cholera toxin (CT) and were believed not to have epidemic potential (Janda **et al**, 1988; Morris **et al**, 1990). The severity of diarrhoea associated with vibrios from other serogroups, were reported to be of lesser magnitude than that associated with strains of vibrio cholerae serogroup

01 (Black **et al**, 1980). However, in October, 1992 an unprecedented change in cholera occurred, with the emergence of massive epidemic of the disease caused by a newly described vibrio cholerae serogroup 0139, Synonym Bengal (Faruque **et al**, 1994; Robert **et al**, 1994; Dilip **et al**, 1996).

The strains of vibrio cholera serogroup 01 are differentiated from other vibrio species because, they agglutinate with group 01 antiserum (Farmer **et al**, 1985). The other strains of vibrio which cannot agglutinate in group 01 antiserum are therefore classified as vibrio cholerae non-01 or non-agglutinating vibrio cholerae (Lennette **et al**, 1978; Farmer **et al**, 1985). The vibrio cholerae non-01 are morphologically and biochemically similar to those of vibrio cholerae serogroup 01, except in the agglutination factors. The vibrio cholerae non-01 include vibrio parahaemolyticus vibrio vulnificus, vibrio mimicus, vibrio damsela and other vibrio species including the most recent, vibrio cholerae 0139 (Farmer, **et al**, 1972; Albert, 1994).

Vibrio parahaemolyticus, is a well documented causative agent of acute gastroenteritis (Philip **et al**, 1989). It is mostly encountered in countries where fish and other seafoods are eaten raw, such as in Asia (Dutt **et al**, 1971), Europe (Aldova **et al**, 1968), Australia (Farmer, **et al**, 1985), North America (Jean **et al**, 1993), and South America (Finch **et al**, 1987).

Vibrio vulnificus is a particular virulent halophilic

vibrio that produces overwhelming primary sepsis, especially among patients with heart diseases and infections of pre-existing wounds (Blake **et al**, 1979); cases of gastroenteritis has also been reported (Morris **et al**, 1990). It has also been implicated in pneumonia (Carol **et al**, 1984), and meningitis (Blake **et al**, 1979; Kelly and Avery, 1980; Farmer **et al**, 1985). In nature vibrio vulnificus lives in coastal waters (Kelly and Avery, 1980). Therefore, patients with wound infections due to vibrio vulnificus are apparently infected directly by contact with brackish or salt water or indirectly by exposure to shellfish harvested from coastal waters (Carol **et al**, 1984; Farmer **et al**, 1985).

Vibrio cholerae serogroup 0139 synonym Bengal, has been described as the most recently defined species of the vibrios (Albert, 1994). The strain was assigned with the synonym Bengal, to indicate its first place of isolation in the coastal areas of the Bay of Bengal (Shimada **et al**, 1993). In late 1992, a massive cholera epidemic was reported from the Southern and eastern parts of India and Bangladesh, associated with the 0139 serogroup (Albert **et al**, 1993; Ramamurthy **et al**, 1993; Shimada **et al**, 1993; Islam **et al**, 1994). The vibrio cholerae 0139 strains have been reported to be closely related to vibrio cholerae serogroup 01 (Popovic **et al**, 1995). The serogroup 0139 are related to vibrio cholerae 01 biotype EL TOR in morphology, culture, fimbrial antigens, cholera toxin (CT), genes for zonula occludens toxin (ZOT), and accessory

cholera enterotoxin (ace) (Johnson **et al**, 1992 Albert, 1994;). Morris and cholera task force (1994) in a separate report confirmed several distinctive features such as capsular expression and structural genes between the serogroup 01 and 0139. Morris and the cholera task force, (1994), working on experimental animals reported of the possible blood stream infection of the serogroup 0139 Bengal. They attributed its pathogenicity to the possession of capsular expression. Rivas **et al**, in (1993), opined the possibility of the 0139 representing either mutation of the vibrio cholerae 01 EL TOR strain or that it acquired the EL TOR virulence gene. Higa **et al**, (1993), reported of strains of vibrio cholerae 0139 harbouring a template bacteriophage related to the "Kappa" family of 01 bacteriophage. Kappa phages are characterized as vibrio phages with Bradley group A morphology and a host range restricted to vibrio cholerae biotype EL TOR, serogroup 01 (Takeya and Shimodiri, 1963; Takeya **et al**, 1965; Bradley, 1967; Ackermann **et al**, 1984).

Other vibrio species of importance include - vibrio damsela, which is associated with wound infections (Vernon and Thomas, 1978; Love **et al**, 1981). It is a Marine bacterium which most often causes skin ulcer on the Damsel fish. Vibrio alginelyticus has been associated with infections of several soft - tissues (Farmer **et al**, 1985). Vibrio Metschnikovii, is an environmental organism, but has been isolated in peritonitis of patients with inflated gallbladder and in

immuno-compromised patients (Vernon and Thomas, 1978; Farmer **et al** 1985).

2.2 CHOLERA DISEASE

The disease cholera, is one of the oldest disease known to man. It was first mentioned by two Greek physicians, between 460 - 377 BC (Sack **et al**, 1980). It is a severe diarrhoeal disease caused by vibrio cholerae serogroup 01. Until late 1992, the vibrio cholerae serogroup 01 was recognised as the only strain of vibrio capable of causing epidemic cholera. But recent findings have confirmed that a new strain of vibrio cholerae designated serogroup 0139, synonym Bengal, is also capable of causing epidemic cholera (Popovic **et al**, 1995).

In severe cholera, there is massive diarrhoea with large volume of "rice water" stool, with fishy odour passed painlessly. It is produced as clear fluid with flakes of mucus which makes the stool appear yellow. The stool contain several vibrio organisms (Cheesbrough, 1985). Amount of fluid passed can be upto one litre per hour. The fluid will amount to over twice the body weight within 4 - 6 days, if unchecked. The watery diarrhoea serves to spread the vibrio cholerae in the environment and eventually to the humans (Gill, 1977; Vernon and Thomas, 1978). There is vomiting, nausea, loss of appetite, abdominal cramp, watery eyes, loss of skin elasticity, anuria (absence of urine excretion), subnormal

temperature, fall of blood pressure and rapid temperature fall (Vernon and Thomas, 1978; W.H.O., 1991). If untreated, there will be severe dehydration, shock and death can follow because of severe dehydration. The mortality rate can be as high as 50-70% (Sack **et al**, 1980; W.H.O, 1991; Judith **et al**, 1994).

To produce the disease, the organism colonizes the gastrointestinal tract and secretes a potent enterotoxin known as the cholera enterotoxin (CT), which activates the enzymes of the intestine (Herington **et al**, 1988). The cholera toxin binds to the GM_1, receptors of the intestine. The binding of the cholera toxin (CT) to the GM_1, ganglioside receptors of the enterocytes microvilli is followed by internalization of the toxin which catalizes the activation of adenylate cyclase causing a rise in intracellular cyclic AMP (CAMP) (Gill and Meren, 1978; Fishman, 1982). This leads to the secretion of large volume of fluid and electrolyte into the lumen of the intestine, leading to the severe continuous diarrhoea and gastroenteritis with vomiting. Sodium and chloride transport into the villus epithelial cells is inhibited and chloride is secreted into the lumen from the crypt cells since water flows passively with electrolytes in response to osmotic gradients, cholera toxin causes the absorbtion of water through the villi to cease and amplifies the secretion of water from the crypt cells (Armstrong, 1987; field **et al**, 1989).

The cholera enterotoxin (CT) is composed of a catalytically active A subunit which stimulates adenylate

cyclase activity (Cyclic AMP) and five identical B subunits which are responsible for the binding of the cholera toxin to the GM_1, ganglioside receptor of the intestinal mucosa (Holmgren, 1981).

Fasano **et al** (1991), in an attempt to identify the basis for the different clinical responses observed with two strains of vibrios, discovered a new toxin factor produced by vibrio cholerae known as "Zot" (Zonula occludens toxin). This toxin increases, the permeability of the mucosa of the small intestine. In addition, vibrio cholerae strains produces a neuraminidase which catalyses the conversion of higher-order gangliosides to GM_1, (Holmgreen **et al**, 1975). The vibrio cholerae neuraminidase is hypothesized to act synergistically with cholera toxin (CT) and increases the severity of a secretory response, thereby enhancing the binding and penetration of the cholera toxin resulting in greater efflux of water and electrolytes (Holmgreen **et al**, 1975). James **et al**, (1992), confirmed the activity of the vibrio cholerae neuraminidase. They observed that the neuraminidase plays a subtle but significant role in the binding and uptake of cholera toxin by susceptible cells under defined conditions such as temperature and other environmental conditions. Miller and Mekalanos, (1988) had earlier shown that the production of the cholera toxin as well as the expression of the vibrio cholerae toxin coregulated pillus (TCP), required for colonization are regulated by diverse environmental

signals including osmolarity, pH, temperature and the presence of certain amino acids. Therefore, the result of the work of James and his colleagues, (1992), suggests that when environmental conditions do not promote optimal expression of cholera toxin or toxin coregulated pillus (TCP), the ability of neuraminidase to enhance the binding of the cholera toxin could contribute to the pathogenic process. However, deletion of neuraminidase activity does not necessarily decrease the biological effect of cholera toxin elaborated by mutant strains relative to the effect of cholera toxin produced by wild-type vibrio cholerae strains (James **et al**, 1992; Bernadette **et al**, 1992).

2.3 EPIDEMIOLOGY

Cholera has probably afflicted man since prehistoric times, but was not clearly distinguished from other diarrhoeal diseases (Vernon and Thomas, 1978). As a result, the ancestral home of cholera vibrio is unclear. Although description by Portuguese explorers of diarrhoeal epidemics in India from the late fifteenth century suggests that the Bengal region of India and Bangladesh has been one continuous epidemic region of cholera (Smith, 1968; Vernon and Thomas, 1978; Sack **et al**, 1980; Farmer **et al**, 1985). It was previously thought that only vibrio cholerae serogroup 01 was the only one of the enteric pathogens which has the potential of producing epidemic disease. But recently, the etiologic

significance of non-01 vibrio cholerae as cause of cholera diseases was demonstrated by studies in human volunteers. After oral administration of a strain of vibrio cholerae non-01, six of eight volunteers developed symptoms of cholera (Morris **et al**, 1990). Epidemiological evidence indicated that strains of vibrio cholerae in the other serogroups are associated with cholera in Asia (Dutt **et al**, 1971; Islam **et al** 1992). Europe (Adolva **et al**, 1968; Bark **et al**, 1974; Wistrom, 1989), Australia (Farmer **et al**, 1985), North America (Morris **et al**, 1981; Bonner **et al** 1983; Philip **et al**, 1989; Jean **et al**, 1993), South America (Finch **et al**, 1987). They have been implicated in foodborne diseases and severe sporadic cholera epidemic outbreaks (Philip **et al**, 1989; Charles **et al**, 1989; Jean **et al**, 1993). In late 1992, an unprecedented outbreak of cholera caused by non-01 vibrio known as vibrio cholerae serogroup 0139 synonym Bengal ravaged the eastern and southern regions of India and Bangladesh ending the earlier belief that only vibrio cholerae in the serogroup 01 have the exclusive potential of causing epidemic cholera (Albert **et al**, 1993; David **et al**, 1993; Robert **et al**, 1994; Islam **et al**, 1994).

Food and water - borne cholera outbreaks due to non-01 vibrio cholerae have been reported in several parts of the world. These includes Czechoslovakia (Adolva **et al**, 1968), Iraq (EL-Shawi and Thewani, 1969), Sudan (Kamal, 1971), India and Bangladesh (Islam **et al**, 1992; Robert **et al**, 1994). Food

borne cholera outbreaks has also been reported in the United States of America (Jean **et al**, 1993). In most of the epidemic outbreaks vibrio parahaemolyticus have always been implicated as the causative agent. This includes the epidemic outbreak in Louisiana in 1986 and the outbreak in the Chesapeake Bay Community in 1989 (Charles **et al**, 1989; Robert **et al**, 1992).

Cholera epidemics have occurred since 1816 (Vernon and Thomas, 1978). The worldwide spread of cholera began in 1817 and in 1823 the first pandemic of cholera was recorded. There were six periods of pandemic spread between 1900 and 1923. Until recently, the classical biogroup had been the only cause of pandemic cholera. However, the seventh pandemic ravaging the world was started by the "EL TOR" biogroup, although, it has been replaced by the "classical" biogroup in some areas such as India, Bangladesh and other countries of Asia (Vernon and Thomas, 1978; W.H.O., 1991).

The seventh pandemic started in 1937, as a localized epidemic in the Celebes, Indonesia. By 1961, it has spread to other parts of Asia including Bangladesh and reached India between 1963 and 1964. It spread to several mediterranean countries and into Europe between 1965 and 1966 (Vernon and Thomas, 1978).

The history of cholera entered a dramatic new phase in 1970, when the pandemic spread through Africa including the south, west, central and north coasts of Africa (Vernon and Thomas, 1978; W.H.O., 1991). The epidemic came into west

coast of Africa through Conakry in Guinea in August, 1970. From Guinea, the disease spread along the coast into Sierra-Leone, Liberia, Cote D'ivoire, Ghana, Togo, Benin Republic (Dahomey), Nigeria and Southern Cameroon. The disease was first reported in Nigeria on the 26th December 1970, in a village near Lagos (W.H.O, 1991). The epidemic suddenly spread northward into Mali, Niger, Upper Volta (Burkina Faso), Northern Nigeria and Chad. By 1971, the epidemic has spread to join with countries of North Africa which had been infected by that originating from the middle East Countries. By the end of 1971, twenty five African Countries had experienced the cholera epidemic (W.H.O., 1991).

The only region of the world spared by cholera during this pandemic was the American continents. However, since 1973, several sporadic cases have been experienced in many parts of the American continents, such as in Mexico and Texas in the United States of America. There were two separate outbreaks in 1982 in the United States of America including one on an oil-drilling platform on a Bayau. There were also two other sporadic cases in Louisiana in 1978 and 1986. Another outbreak of cholera associated with the consumption of imported commercial frozen fresh coconut milk from Thailand occurred in Maryland in 1991 (Sack **et al**, 1980; Philip **et al**, 1989; Jean **et al**, 1993).

As the seventh pandemic of cholera continues to spread throughout the world, it becomes endemic in countries where

socio-economic conditions are poor, sanitary systems and public hygiene are rudimentary and safe drinking water is not available (Huq **et al**, 1996). It has become endemic in Asia, Latin America and Africa and the epidemic occurs repeatedly with regular seasonal pattern, as long as environmental and seasonal conditions permit the survival of vibrio cholerae (Martin **et al**, 1969; MacCorkMark **et al**, 1969; Glass **et al**, 1982; Samadi **et al**, 1983; Asagba, 1992).

The World Health Organisation (W.H.O) reported of the world distribution of cholera in 1982. Their report showed that 54,856 cases were reported from 37 countries. The majority of the cases were from Africa, with 37,429 cases, Asia had 15,191 cases and the Pacific Ocean countries had 2,217 cases (W.H.O., 1991). Peru experienced a major cholera epidemic in 1991. The cholera epidemic in Peru was the first manifestation of the seventh pandemic of cholera in the Americas. Peru was the worst affected country of the Americas by the epidemics with 300,000 cases. From Peru, the epidemic spread to seven other countries of Latin America including Mexico (Glass **et al.**, 1982; Carillo **et al**, 1994). In Africa about 102,000 cases and 10,391 deaths (a case fatality rate of 10.2%) was recorded by September of 1991. It was estimated that the total world record of the 1991 epidemic was 568,546 cases with unestimated number of deaths (W.H.O., 1991).

In Nigeria, 838 cases and 41 deaths, was reported in 1988 (a case fatality rate of 4.9%). A sharp increase was however

recorded in 1989, when 1059 cases and 81 deaths (a case fatality rate of 7.6%) were recorded from 10 states, out of 31 states and Abuja. 1990 witnessed yet another increase in cases and deaths and the number of affected areas. 4101 cases and 61 deaths (a case fatality rate of 1.2%) were recorded from twelve states of the federation (Federal Ministry of Health, Nigerian unpublished data). The 1991 cholera outbreak was the worst both in Magnitude and intensity since cholera was first reported in Nigeria in 1970. Between January and August 1991, 36,196 cases and 4,293 deaths were recorded from 18 states in Nigeria (a case fatality rate of 11.8/%), and by the end of 1991, 59,474 cases and 7,654 deaths were recorded (a case fatality rate of 13.0%). Borno state recorded the highest number of cases, with 16,596 cases and 1,829 deaths (a case fatality rate of 11.0%). This was followed by Kano state with 7,736 cases and 792 deaths (a case fatality rate of 10.2%). Other states were Bauchi, Akwa-Ibom, Kaduna, Gongola, Oyo, Cross River, Niger, rivers, Imo, Lagos, Ondo and Plateau states. The least affected state was Ogun state with 6 cases and no death (Nig. Bull., 1991; W.H.O., 1991; .Alabi **et al**, 1992;).

Currently, many parts of the world especially the Indian subcontinent, is experiencing epidemic of cholera caused by a strain of vibrio cholerae different from the strain of the 7th pandemic (Islam **et al**, 1994). The epidemic strains was not related to the known serogroup 01 and therefore was assigned

a new serogroup 0139 with the synonym Bengal (Shimada **et al**, 1993). To indicate its first place of isolation (from the coastal areas of the Bay of Bengal). It is believed that vibrio cholerae 0139 might be the agent of the eight pandemic of cholera (Mandal, 1993; Swedlow and Rose 1993). The vibrio cholerae 0139 infections have been reported from India, Bangladesh, Nepal, Burma, Thailand, Malaysia, Saudi Arabia, China and Pakistan (Albert, 1993; Chongsa **et al** 1993; Fisher-Hoch **et al**, 1993). There were also reported cases in the United Kingdom (Cheasty **et al**, 1993), and the United states of America (CDC, 1993). From early January to the end of March 1993, about 107,297 people in Bangladesh have been affect by this strain of vibrio cholerae with an estimated death of 1,473. (Albert **et al**, 1993).

2.4 SEASONALITY OF CHOLERA EPIDEMICS

Cholera epidemics have been found to have a regular seasonal pattern in the cholera endemic areas of the world (Sarkar **et al**, 1985; Bockemuin **et al**, 1986; Venkateswaran **et al** 1989;Islam **et al**, 1989 and 1990). Studies in Bangladesh have shown that cholera epidemics occur twice a year in that country, one in April and the other in August/September (Islam **et al**, 1992). This corresponds with the bloom of algae in their natural environment which have been implicated and found in association with vibrio cholerae (Islam **et al**, 1990). This invites speculations that symbiosis .

between vibrio cholerae and algae might possibly provide basis for the seasonality of cholera. In another development, venkateswaran **et al**, (1989), and Kasthuri **et al**, (1989), confirmed the speculation of the seasonality of cholera in Japan. In several states of the United States of America, vibrio cholerae infections increase during the warmer months of Summer (Carol **et al**, 1984). This seasonality may be explained by the increase in sea water during the summer and fall months as recorded in Louisiana Gulf Coast (Philip **et al**, 1989). In the same vain the seasonality of cholera epidemic in Nigeria have been identified (Nig. Bull 1991; Fed Min. of Health unpublished report 1991). The seasonal trend coincides with the pattern of the rainy seasons in different geographical locations of the country. The epidemics sometimes occurs twice a year as in 1991 (Nig Bull. 1991). The epidemics starts in the month of January and progresses as the rainy season progresses. It often reach its peak between the month of April and July and subsides towards the month of December. The Southern region of the country experiences the disease from January, while the northern part of the country experiences the epidemics from the month of March and it reaches its peak between the months of May and June. This progression is in accordance with the coming of the rains (Nig. Bull. 1991; W.H.O., 1991). There are no satisfactory explanation as to how the seasonality and endemicity of cholera are maintained, though several explanations have been

put forward, (Miller **et al**, 1984 and 1985).

2.5 SOURCES AND MODE OF TRANSMISSION OF CHOLERA VIBRIOS

Vibrio cholerae serogroup 01 is well adapted to the human intestinal tract. Therefore, the vast majority of strains are found in human faeces. They have also been isolated from the environment and from other animals that have come in contact with faeces of cholera patients (Farmer **et al**, 1985).

Vibrio species were considered to be restricted to saline environments. They require sodium ions (Na^+) for survival and growth (Vernon and Thomas 1978; Greenwood **et al**, 1992). Recent studies have shown that they also occur in non-saline environments. Several of them have been isolated from fresh water environments, such as in Hiroshima in Japan (Kasthuri **et al**, 1989).

The several outbreaks of cholera caused by vibrio cholerae serogroup 01 and other non-01 species, have been associated with several vehicles of transmission. The outbreak of cholera due to vibrio cholerae serogroup 01, biotype EL Tor, Inaba, resistant to several antibiotics at the pediatric ward of a hospital in Thailand in 1989 was traced to water used for bathing, in a reservoir, at the pediatric hospital ward (Ramon **et al**, 1989). In earlier studies, water used for bathing, stored in clay jars in home of patients with diarrhoea was identified as potential source of diarrhoea pathogens (Echeverria **et al**, 1985). However, Colwell (1984)

emphasised that the continued occurrence of clinical cholera in a region may not necessarily depend on repeated contamination of water by patients or carriers. She also believed that the survival of the organism outside the human intestine is not brief as had formerly been thought, rather the vibrio cholerae may survive for prolonged period in the environment (Robert and Nathaniel 1984).

In another development, an outbreak of gastroenteritis caused by vibrio parahaemolyticus occurred among several attendees of the 1986 interscience conference on antimicrobial agents and chemotherapy in New Orlean, Louisiana, U.S.A. The outbreak was associated with eating of raw seafoods including shellfish and oysters (Philip **et al**, 1989). The 1991 outbreak of cholera epidemic in Maryland, U.S.A, among attendees of a private picnic, was associated with the consumption of commercial imported frozen fresh coconut milk from Thailand (Jean **et al**, 1993).

During cholera epidemics in Nigeria, vibrio organisms were isolated from local fresh water as well as from patients (Alabi **et al**, 1992). However, they were known to have disappeared from the environment after the epidemics had subsided. The reservoir or sites of survival and multiplication of these organisms in between epidemics have not been elucidated. There is no satisfactory explanation on how the seasonality and endemicity of the cholera vibrios are maintained in the country, although four alternatives have

been suggested. These include (a) carrier status in man (b) carrier status in animals (c) continuous transmission in man and (d) environmental reservoirs (Khan **et al**, 1984; Cheesbrough, 1985; Miller **et al**, 1985; Lucas and Gilles, 1990; Rodman, 1992). The situation of carrier status in man may occur in areas where cholera is endemic. Many individuals in such areas may ingest the vibrio organisms without the symptoms of the disease. The organisms are frequently recovered from formed stools of asymptomatic individuals (Islam **et al**, 1990; Miller **et al**, 1994). These individuals may have developed immunity against the organism. There is increase in vibrio immunoglobulin A (IgA), produced locally in the intestine and immunoglobulin G (IgG), in the blood of patients who have recovered from cholera for at least 3 - 4 years (Ann - Marri **et al**, 1984; Yamamoto and Yakoto, 1988; Perce **et al**, 1988). Clinical findings suggests an 89 - 90% rate of protection as a result of first episode of cholera in the individuals (Woodward, 1971; Levin **et al**, 1981; Glass **et al**, 1982; Ann-Marri **et al**, 1984).

Environmental reservoirs have been attributed to locally grown melon and water-melon which were often irrigated with untreated water in cholera endemic areas of Bangladesh (Islam **et al**, 1990). The practice of slicing these fruits when they may not be sold for several hours is highly conducive for the growth and transmission of vibrio organisms (Miller **et al**, 1984; W.H.O., 1991).

Vibrios have been isolated from various fresh estuarine and marine waters and sediments, They have also been isolated from various components of pond ecosystems in Dhaka, Bangladesh (Baross and Liston, 1970; Kaneko and colwell, 1975; Islam **et al**, 1990). At Chancay, U.S.A., cholera vibrios were isolated from sea waters 500 metres from the shore and from fish caught at a depth of 500 meters (W.H.O. 1991).

Association have also been found between the vibrios and Faunas. Investigation on the association between vibrios and marine plants, algae and planktons were carried out by various investigators at the "International Centre for Diarrhoeal Disease Research, Bangladesh" (ICDDR,B). It was observed that there is an association between the vibrios and two species of marine phytoplanktons:- Skeletonema costatum and chaetococero species (Tobiash **et al**, 1970; Nair **et al**, 1975; Islam **et al**, 1989; Lee **et al**, 1982;). Field studies in Bangladesh have also shown that vibrios are associated with hydrophitic fresh water plants. They were also found to survive while attached to a filamentous green algae - Rhyzoctonum fontanum and in the mucilaginous sheat of a blue-green algae - Anabaena virabilis (Islam and Aziz 1978 and 1981; Islam **et al**, 1989; Islam **et al**, 1990).

Vibrios are no longer restricted in transmission to areas marked by the presence of polluted water such as deltas, lagoons or brackish waters, but have also been identified in desert environments and in climatic conditions that were

formerly thought to be unfavourable to the survival of vibrios (Kamal, 1971; W.H.O, 1991). Transmission is often associated with movements and gathering of populations, resulting in short, sharp epidemics in places sometimes distant from endemic areas. Cholera spread along routes of communication - roads, ocean and rivers, Balast and holding tanks of ships travelling from contaminated harbours may help in the introduction of cholera to nonendemic areas. Souvenir of seafoods brought from endemic regions, occasions of ceremonies such as funerals, pilgrimages, feasts, or during mass movement of population such as nomads and refugees have been implicated in dissemination of cholera vibrios (W.H.O, 1991, Jean **et al**, 1993). Faecal-oral transmission of vibrios follow via dirty hands, dishes, locally contaminated foods and overcrowdedness (Farmer **et al**, 1985 ;Islam **et al**, 1990; W.H.O., 1991;).

Food-borne transmission of cholera vibrios have been observed in Africa. Such cereals as Millet (in Mali), rice eaten several hours after cooking and groundnut sauce (in Guinea), left over crab-meat (in Guinea Bissau) and chickpeas eaten without re-heating several hours after cooking (in Malawi), have been implicated in the transmission of cholera vibrios in Africa (W.H.O, 1991).

LABORATORY DIAGNOSIS OF VIBRIOS

Sometimes vibrio cultures will not grow well on highly selective media used to isolate other enteric pathogens. This is because, halophilic species of vibrios need added sodium

chloride for optimum growth and activity (Farmer **et al**, 1985)

Stools from patients with cholera will have large numbers of viable vibrio organisms, while there are few numbers of organisms in the stool of carriers (Vernon and Thomas, 1978; Cheesbrough, 1985; Islam **et al**, 1990). This makes the use of enrichment media necessary. Enrichment is best carried out by inoculating alkaline peptone water (1.5% peptone, 0.5% sodium chloride; pH 9.0) heavily with stool and incubating at 37°C for 6 - 12 hours. Bile-salt peptone broth may also be used. Where the sample will be preserved for longer times or is transported to long distances, requiring days, Cary - Blair transport media is recommended (Farmer, **et al**, 1985, Cheesbrough, 1985). Sub-culture is done on thiosulphate citrate bile-salt sucrose agar (TCBS). The T.C.B.S. agar brings immediate attention to a possible vibrio isolate whose colonies may not be obvious on other plating media (Sack **et al**, 1980; Farmer **et al**, 1985).

The approach adopted by a particular laboratory will probably depend on the frequency with which vibrio cultures are encountered. It also depends on the type of specimen collected for investigation. This explained why TCBS medium was not widely used in most states of United States of America as reported by Philip and colleagues (1989), where, according to their report, the incidence of vibrio infections is not well known in most states.

SAMPLE COLLECTION

Specimens which can yield vibrio organisms include, faeces, duodenal wash, vomitus, rectal swab, water, food remains and intestinal contents of aquatic organisms (Sack **et al**, 1980, Philip **et al**, 1989, Islam **et al**, 1992; Jean **et al**, 1992;)

Stool specimens should be collected early, preferably within the first 24 hours of illness and before administering antibiotics to the patient. Strips of blotting paper may be soaked in liquid stool and inserted into air tight plastic bags. Vibrios remain viable in this condition for 5 weeks (Sack **et al**, 1980;Farmer **et al**, 1985;). Samples of water are collected in presterilized 500ml narrow mouthed bottles. Other environmental samples such as water sediments planktons, and other water plants and animals are collected in sterilized wide mouthed glass containers (Kasthuri **et al**, 1989; Islam **et al**, 1992).

TRANSPORTATION OF SAMPLES

All field samples are transported to the laboratory in isullated ice-box with ice-packs and should be processed within 6-8 hours of collection.

The viability of vibrio species is well maintained at the alkaline pH of "rice water" stool, but unpredictable in formed stools. Faecal materials are therefore transported in transport media. This include, alkaline peptone water, Cary-

Blair transport medium which maintains its viability for upto 4 weeks or more. Others include Tellurite-taurocholate - peptone broth. Equal parts of 0.3m phosphate buffer and glycerol (pH 7.0), may be used. In the absence of transport medium, blotting paper strip is used to transport the specimen before placing in enrichment media. (Farmer **et al**, 1985; Islam, 1990).

SAMPLE PROCESSING

Several researchers and laboratories has employed different techniques to isolate vibrios from stool and other environmental specimens.

For the isolation of toxigenic vibrios from fresh water environment of Hiroshima, Japan, Kasthuri and his colleagues (1989), employed the spread plate method on R2A agar. They used the simple membrane filter, short-pad, pre-enrinchment technique to estimate the total viable count of vibrios in the water from the Ohta river. The membrane filter was used to filter about one litre of the water and 10mls of sediment. The filter was then placed on thiosulphate citrate bile-salt sucrose (TCBS) agar plate. Another set of the water and sediments were filtered and the membrane placed into two sets of alkaline peptone water and incubated at 37°C and 42°C respectively. The sets of peptone water were subcultured onto TCBS agar plates. Purified strains were grown in T_1N_1 agar (1% tryptone; 1% Nacl, 0.5% agar) for biochemical identifications.

Serotogical typing of isolates of vibrio parahaemolyticus was done with commercial O and K antisera by slide agglutination method. The toxigenicity of the isolated strains was screened on blood agar plates for haemolysis. The blood agar plates contain tripticase soy agar, supplemented with 5% defibrinated sheep blood (Kasthuri **et al**, 1989).

In another development, samples of water, water-plants, phytoplankton and sediments were collected from ponds in Bangladesh by Islam and his co-workers. They sampled at 15 days interval for one year. Water samples were collected in presterilized 500ml narrow mouthed plastic bottles. Water plants were collected in 120ml sterilized bottles. About 10grams of the water plants were homogenised in 100ml sterile normal saline in a blender. Also, 10ml of phytoplankton samples were homogenized in TEclon-tipped tissue grinder, using sted-fast-stirer. About 1ml of plant homogenate, 10ml phytoplankton homogenate, 50ml of each water sample and 1.0gram of sediments were enriched separately in bile-salt-peptone broth and incubated over night at 37°C. The samples were then plated onto TCBS agar and Taurocholate tellurite gelatine agar (TTGA) and incubated at 37°C for 18-24 hours (Islam **et al**, 1992).

In the bid to identify the source of cholera outbreak among attendees of a private picnic in Maryland, United States of America, Jean and his colleagues (1993), collected stool samples, food remnants and sera of the infected patients.

Sewage samples at the Maryland Municipal Sewage treatment plant was collected at weekly intervals using the "Moore Swab" technique. The Moore Swabs were placed at four sewage outlets. The stool and food samples were cultured on enrichment medium (alkaline peptone water) for 18-24 hours and then subcultured onto TCBS agar plates. The Swabs were also cultured on alkaline peptone water and then subcultured on TCBS agar plates. The plates were incubated at 37°C for 18-24 hours. serum samples were tested for vibriocidal antibodies using the standard Ogawa and inaba test strains. Antibodies to vibrio cholerae toxin were measured using the Enzyme linked Immunosorbent assay (ELISA) method (Jean **et al**, 1993).

In their study of the epidemiology and spectrum of vibrio infection in a chesapeake Bay Community in the U.S.A., Charles and his colleagues (1989) used MacConkey agar, Bismuth Sulphide, xylose-lysine deoxycholate (XLD) agar, Kektoen and TCBS agar media for the isolation of vibrio organisms from various samples. Blood and wound specimens were cultured on 5% sheep blood agar plates. The isolated organisms were tested for oxidase reactivity and other biochemical tests (Charles **et al**, 1989).

Philip and his co-workers (1989) in a separate epidemiological study of vibrio gastroenteritis among attendees of a scientific congress in New Orlean, Louisiana, U.S.A., collected stool swabs from the attendees and transported the samples to their laboratoy in Cary-Blair

transport medium. The swabs were cultured on TCBS agar, salmonella Shigella agar (SSA) and campylobacter medium (Butzler medium). The seafoods consumed by the attendees - (Shellfish, and Oysters), cooked and raw were transported to their laboratory in ice maintained at 4°C. At the laboratory, the samples were placed in alkaline peptone water and incubated at 35°C for 18-24 hours. The peptone water cultures were then subcultured on TCBS agar. Water used by the attendees and homogenised food samples were treated using the most probable number technique (MPN), to quantitate the amount of vibrio organisms in the samples (Philip **et al**, 1989).

Robert and Nathaniel (1984) described an indirect fluorescent antibody staining method used to detect viable vibrio cholerae serogroup 01 in environmental waters, developed by Dr. R.R. Colwell. This method was said to be more sensitive than most conventional culture techniques (Robert and Nathaniel, 1984). This technique was improved upon in 1992, when colwell and his colleagues developed a rapid, simple sensitive monoclonal antibody based co-agglutination test for direct detection of vibrio cholerae 01 (Colwell **et al**, 1992). Using this technique known as cholera screen, Carillo and co-workers (1994) detected the presence of vibrio cholerae 01 in the stool of a Peruvian cholera patient. The technique utilizes a monoclonal antibody to the A factor of vibrio cholerae 01 lipopolysaccharide. The antibody is adsorbed to heat-killed staphylococcus aureus cells. This is

described as a slide agglutination test where a drop of the antibody is added to that of the sample and observed for agglutination (Carillo **et al**, 1994).

Pablo **et al**, (1994), developed a more sensitive direct method to detect vibrio cholerae in stool samples using polymerase chain reaction (PCR) procedure that do not require a DNA purification step. PCR allows detection in a few hour of organisms present in low numbers by amplifying specific DNA segments. They squeezed out rectal swab of cholera patients into a micro centrifuge tube. This is boiled for 5 minutes and centrifuged at 16,000 x g for 5 minutes to remove the debris. Two consecutive PCR assay were carried out with 2 ul portion of 1:100 dilution of supernatant using nested primers to achieve the desired sensitivity.

Islam and co-workers (1990) in another work to determine the association of vibrios and algae, homogenised the algae A. viriabilis in a glass homogeniser with 0.9ml phosphate buffered saline. They prepared a tenfold dilution of the homogenate. About 0.1ml of the homogenate dilutions was spread on trypticase soy agar plates. Water samples were also cultured on trypticase soy agar plates. The plates were incubated at 37°C for 18-24 hours. The bacterial isolates were estimated after incubation by colony count. Unhomogenized algae samples were examined using the phase contrast microscope after mixing with a drop of physiological

aline on a glassslide and covered with glass coverslips. lso some algae samples were spread on polytetrafluoroethylene PTFE) coated glass slide-wells. The slides were air-dried nd flame-fixed. 20ul of 2% bovine serum albumin (BSA) was dded to the wells and the mixture was incubated at 37°C for 0 minutes in a moist chamber. After incubation, the slides ere rinsed in phosphate buffered saline (PBS), gently blotted nd dried. *Vibrio cholerae* polyvalent antisera-20ul was added o the wells. The slide were again incubated at 37°C for 30 inutes. The slide were then gently washed in phosphate uffered saline (PBS), blotted and dried. Fluorescent sothiocyanate (FITC), conjugated with anti-rabbit globulin-oat serum in PBS and 20Ul of 0.1% Evan's blue was added to he wells. The wells were again incubated for 30 minutes at 7°C. After the incubation the slides were again washed, lried and covered with coverslips using buffered glycerol PH8.9). The slides were examined under the epifluorescent iicroscope (Islam **et al**, 1990).

CONTROL MEASURES:

The continued widespread of epidemic cholera caused by *ibrio* organisms have been attributed to several reasons, among which are:- (a) The absence of pre-established plan for controlling the epidemics (b) inadequate health infrastructures or lack of supplies and equipments, (c) Few health workers, trained in the control of diarrhoeal diseases,

(d) inadequate treatment (e) Lack of preparedness, (f) late reporting or treatment by patients, (g) misdirection of treatment, like vaccination and (h) lack of treated or inadequate supply of portable water (Nig. Bull., 1991, W.H.O, 1991; Rodman, 1992; Huq **et al**, 1996). Several control measures have been postulated by various researchers. Since inadequate sanitation and lack of safe water is known as a major cause of cholera infections, greater emphasis must be laid on safe water and sanitary conditions, if a sustainable solution to the threat by cholera is to be found (Hughes **et al**, 1982; Huq **et al**, 1996). Most contamination of foods and water arises from direct and indirect transfer of vibrio organisms from faeces of infected persons to food and water. The vibrios are known to persist in several aquatic environments such as seaweeds (Islam **et al**, 1989, 1990), and aquatic foods such as shellfish, Oysters and CrustaCians (Hood **et al**, 1981, Philip **et al**, 1989). They may be persistently contaminated or intermittently contaminated even in the absence of recent exposure to human faecal materials (Robert and Nathaniel, 1984). They have been shown to survive in frozen and canned foods for long periods (Jean **et al**, 1993). Such foods pose risks to the consumers if they are eaten raw or allowed to contaminate other foods before being cooked.

Several measures have been adopted at present in the control of the cholera diseases. Most of the attention has focused on cholera vaccine production with biochemical or

genetic modifications (or both) of the cholera toxin or deletion to the toxin entirely (Sciortino, 1989).

Ann-Merri **et al**, (1984) studied the mucosal and systemic response to an oral cholera vaccine consisting of the B-subunit of vibrio enterotoxin (CT) plus killed vibrios, in Bangladesh. The vaccine was found to have a 90% protection. Several other attempts on the construction of attenuated live oral vaccine by Fasano and Colleagues in 1991 and Bernadette and her colleagues in 1992 proved unexpectedly difficult (Fasano **et al**, 1991; Bernadette **et al**, 1992). This involved the use of recombinant DNA (rDNA) technology which has led to the construction of several attenuated vibrio cholerae strains administered as oral vaccine in recent times. However, most of these strains have been found to be capable of causing mild to moderate diarrhoea, when tested in volunteers (Fasano **et al**, 1991). The discovery of the "Zonula Occludens toxin" (ZOT) gene encoding for toxin production in attenuated vibrio cholerae is a potential highly significant step in the development of a safe live oral vaccine against cholera vibrios (Bernadette **et al**, 1992).

Huq **et al.,** (1996) developed a simple and inexpensive method for removing phytoplanktons associated with vibrio cholerae in raw water supplies. This involves the filtration of water before use with either nylon nets or different types of "Sari" (Lace) materials. This method indicated a 99% removal of vibrios cholerae attached to phytoplanktons. Other

simple methods of preventing cholera includes the boiling of water prior to drinking, to kill the vibrios attached to cyclops and other aquatic plants (Yohalem and Fry, 1991).

Moreover, health education of the public is most essential for cholera control. Mass media, community leaders, school authorities, health and health related workers and socio-cultural groups are highly necessary in the control of cholera vibrios especially in Nigeria (W.H.O., 1991).

CHAPTER THREE

MATERIALS AND METHODS.

3.0 STUDY AREAS.

The study areas covered Giwa, Zaria and sabon gari local government areas of Kaduna state, Nigeria.

FIELD OBSERVATION

3.1 GIWA LOCAL GOVERNMENT

Giwa local government area is relatively a small rural suburb. The local government has a population of about 170,253 inhabitants (population census, 1991). The local government is situated on longitude 8° and 9° and latitude 10° and 11° and is bound on the east by Sabon Gari L.G.A on the West by Katsina State on the North by Makarfi L.G.A and on the South by Zaria Local government area of Kaduna State. There are few human activities taking place in the area. The inhabitants are predominantly farmers. They live in small settlements except for few areas such as shika and the Giwa central Market area where there are relatively large settlements. There are few number of man-made ponds and streams and limited human activities taking place around the surroundings of the streams and ponds. There are also few number of Ducks and other domestic animals left to run around the areas. The environment around the houses are relatively clean and sewage and refuse are properly disposed of or channelled to ponds away from the houses.

The main source of water for domestic use in the local government are wells, which are located in private homes. There are few public wells and most of the public wells are out of use.

3.2 ZARIA LOCAL GOVERNMENT AREA.

Zaria local government has a population of about 277,187 inhabitants (population census, 1991). The local government is relatively larger than Giwa local Government in terms of population. It is situated on longitude 8° and latitude 9". It is a moderate urban set up. It is bounded on the east by Soba L.G.A and west by Giwa L.G.A and on the North by Sabon Gari L.G.A and on the South by Igabi L.G.A.

The houses are crowded together, and there are much activities such as trading and craft works carried out by the inhabitants of the area, who are predominantly of the Hausa/Fulani origin. There are more domestic animals such as Ducks, goats and sheep, than in Giwa local government area. There are man-made ponds situated at almost 100meters intervals within the residential areas. Rivers and several natural ponds found outside the urban settlements are used extensively by cattle as well as humans. In the urban area, there are several artificial ponds where domestic waters from kitchens and other wastes from homes are directed. These ponds provide play-grounds for Ducks. Toilet facilities are poor in this local government, as some inhabitants are openly

seen passing faeces around refuse dumps which is seen littering most open places, farmlands, near streams and other bodies of water.

There were reports of cholera gastroenteritis epidemic outbreak in the local government within the period preceding the month of this survey in the local government area.

3.3 SABON GARI LOCAL GOVERNMENT AREA.

Sabon gari local government area is the largest of the three local government areas under study, in terms of population and structural development. The local government is predominantly inhabited by "non-indigens", who are not of the Hausa/Fulani origin. The local government is a very large urban setup with a population of 246,544 people (Population census, 1991). The local government is situated along longitude 8° and 9° degree and latitude 10 and 11 degree in the world map and is bounded on the East by Soba L.G.A and on West by Giwa L.G.A on the North by Makarfi L.G.A and on the south by Zaria L.G.A area.

There are greater number of activities usually associated with most modern cities going on in the local government. The sanitary condition of the environment is extremely poor, though the inhabitants are more of civil servants, artisan-workers and traders. There are lots of domestic animals let loose to roam the streets, such animals include pigs, Ducks, Dogs, Goats and other domestic animals which help in creating

favourable conditions for disease causing organisms. There are poor drainages in most areas of the local government. The waters provide recess avenues for pigs and ducks. There are many ponds and streams which are used by cattle rearers and individuals for their daily domestic activities.

Generally, the vicinities of the ponds and streams in the local government area is in very poor sanitary conditions. Farmlands surounding the bodies of water are used as toilets, as there are few public and even private toilet systems in some areas. There are also many public wells which are not covered and they have no unified container for collecting water from them.

3.4 SAMPLE COLLECTION

Water samples and some samples of water-letus, were collected from wells, ponds, streams and rivers in Giwa, Zaria and Sabon-gari local government areas of Kaduna State, Nigeria; Stool samples were also collected from ducks and healthy individuals with or without history of gastroenteritis, from the three local government areas.

3.4.1 WELLS

50 mililitres of water samples were collected from the various wells, using sterilized 50 millilitre narrow mouthed glass bottles. The water samples were drawn from the wells using the buckets and methods used by the inhabitants of the

various areas when drawing water from the well.

3.4.2 PONDS

50 millilitres of water samples and water sediments were collected using 50 millilitre narrow mouthed sterilized glass bottles. The water was collected by immersing the sterilized 50millilitre bottle deep into the pond, after agitating the water using sterile glass-rod. The ponds in the various local government areas are used for various domestic purposes including bathing, farm irrigation, cooking and for cattles.

3.4.3 STREAMS AND RIVERS

50 millilitres of water samples and water sediments were collected from streams and rivers by facing the narrow mouthed sterilized 50 millilitre glass bottles against the current of the flow of the body of water. The water was agitated to obtain the sediment which was collected with the water.

3.4.4 AQUATIC PLANT (WATER-LETUS)

Five Water-letus were collected from different ponds, streams and rivers using sterile polyethylene bags. All the plants were collected from the Sabon-gari local government area. The few number of water-letus sampled was due to the sampling period(November-April), which does not coincide with the period of the bloom of this plant in the area.

FAECAL SAMPLES

3.4.5 Human: Faecal Samples were collected from diarrhoeal and non-diarrhoal patients attending the General hospitals and clinics in the three local government areas in Kaduna State, including Giwa General Hospital, National Agricultural Production Research Institute (NAPRI), Shika, staff clinic; Ahmadu Bello University, Zaria, Samaru campus, Sick Bay and the Kofan Gayan, General hospital Zaria. The stool samples were collected in clean washed, dried screw-capped wide-mouthed glass bottles and plastic containers.

3.4.6 Ducks: Dried and fresh faecal samples from domestic ducks were collected into sterile polyethylene bags from the various local government areas.

The various samples were transported to the laboratory in insulated plastic containers.

3.5.0 SAMPLE PROCESSING

The methods of Islam **et al**, (1989 and 1992), and Kasthuri **et al**, (1989) were used 10 millilitres of each specimen (water and water sediments from ponds, wells, streams and rivers), were asceptically transferred into 20 millilitre sterile alkaline peptone water and Cary-Blair transport medium respectively. The tubes were incubated at 37°C for 6-8 hours for the alkaline peptone waters and 7-8 days for the Carry-Blair transport medium respectively.

About 5 grams of the water-letus were homegenised using a sterile mortar and 10 millilitres of the homogenate were transferred into 20 millilitres of sterile alkaline peptone water and Cary-Blair transport medium respectively. The alkaline peptone water cultures were incubated for 6-8 hours and the Cary Blair transport medium for 7-8days at 37°C. 5 grammes of human faecal samples were inoculated into 20 millilitres alkaline peptone water and Cary-Blair transport medium and incubated at 37°C for between 7-8 hours and 18-24 hours respectively. Similarly, between 2 grammes and 5 grammes of the duck stool samples were inoculated into sterilized alkaline peptone water and Cary-Blair transport medium and incubated for 18-24 hours at 37°C. After incubation, a loopful of the various specimens were collected from the alkaline peptone water and Cary-Blair transport medium and streaked on thiosulphate citrate bile salt sucrose agar (TCBS) and taurocholate tellurite gelatine agar (TTGA)plates using sterile wireloop. The plates were incubated at 37°C for 18-24 hours. (For preparation of TTGA see Appendix B).

The methods for the identification of vibrio cholerae -01 and non-01 vibrio species is summarised in appendix 1 (flow chart for identification of vibrios).

3.6.0 ISOLATION

Colonies with characteristic yellow appearance, 2-4mm in diametres and with smooth surface and also those with characteristic green colonies on the TCBS agar plates including colonies surrounded by clear zones on TTGA agar plates were suspected to be vibrios. The colonies were picked with sterile wireloop and purified on nutrient agar plates. The purified colonies were inoculated on T_1N_1 agar slants (1% tryptone; 1% sodium chloride and 0.5% agar), and stored in refridgerator for biochemical tests and identification. The organisms were also plated on nutrient agar plates for oxidase test (see appendix ?) for preparation of T_1N_1 agar see appendix E.

3.7.0 OXIDASE TESTS

The suspected colonies were tested for oxidase reaction. About 0.1 gramme of oxidase reagent (NNN'N-tetramethyl-P-phenylene-diamine dihydrochloride) (BDH chemicals-England), was dissolved in 5millilitres of distilled water. Wathman No.1 filter papers were soaked in the oxidase reagent solution and allowed to drain. The edge of a glass-slide was dipped into absolute ethanol and flamed over a burnsen flame to sterilize it. Suspected vibrio colonies on the nutrient agar plates were picked using the edge of the sterilized glass-slides and smeared on the filter papers soaked in the oxidase reagent solution and observed for colour change. The change of colour

from light-ash or light-Brown to deep violet colour at the area smeared with the organism within 2-3 seconds depicted a positive oxidase reaction.

3.8.0 SUGAR FERMENTATION AND AMINO ACID UTILIZATION TESTS.

The oxidase positive colonies were picked from the nutrient agar plates using sterilized straight-wire. The organisms were streaked on the slant of Kliglers' Iron agar (KIA) and the butt was stabbed, using the sterile straight wire. The sterile straight-wire was also used to pick the organisms and stabbed into motility-indole-urea medium (MIU) and smeared into each of the different sugar broths, namely, Arabinose, Mannose, sucrose, glucose and Inositol. The organisms were also smeared into 2 millilitre of different concentrations of sodium chloride solutions, that is -0%; 1% 3%; 8%; and 10% respectively.

Amino acid utilization tests were performed using the Falkows and Moellers methods (For preparations- see appendix C and D), (Lennette **et al**, 1978; Cowan, 1981; Albert **et al**, 1991). Using sterile wireloop, tubes containing lysine, ornithine, and arginine broth were inoculated with the test organisms respectively. The tubes were incubated at 35°C for 24 hours. After incubation, a drop of 40% sodium hydroxide was added to each of the tubes. Also 1 millilitre of Ninhydrin solution (1% ninhydrin in chloroform), was added to each tube and observed for colour change. A colour change

from blue to purple, appearing in the chloroform phase within 5 minutes, represented a positive test (Lennette et al, 1978; Cowan, 1981).

3.9.0 MODIFIED FALKOWS AND MOELLERS METHODS.

In order to confirm or compare the result of the Falkows 1958 and Moellers 1955 methods, the modified Falkows methods (Lennette et al, 1978), for lysine and ornithine decarboxylase test was employed. Sterile straight wire was used to pick the organisms and inoculated into each 2ml amounts of sterilized lysine, ornithine and arginine broth. Sterile liquid paraffin was added to each tube to make a height of about 5mm. The tubes were then incubated at 37°C for 4 days and checked for colour change from yellow to purple for positive ornithine, lysine or arginine test (Cowan, 1981/ Lennethe et al, 1991).

3.10.1 SEROLOGICAL TESTS.

The slide agglutination tests, using commercially prepared antisera against vibrio cholerae -01 (polyvalent and monovalent -DIFCO U.S.A) was used. The polyvalent antisera was use^f to identify cholera vibrio species. The specific monovalent antisera against inaba and ogawa serogroups was used to identify the vibrio cholerae serogroup 01.

Using sterile wireloop, the organisms were picked from the nutrient agar plates. A loopful of the oragnisms was emulsified in a drop of sterile normal saline placed on a

clean greasefree glass slide. A drop of the polyvalent antisera was then dropped on the emulsion and then mixed. The glass slide was rocked for 2-3 minutes and was observed for agglutination. Colonies which agglutinated in the polyvalent antisera were further tested for agglutination using the monovalent antisera against inaba and ogawa serogroups, using the same procedure as for polyvalent antisera above.

3.10.2 CHICKEN RED CELL AGGLUTINATION TEST.

Slide agglutination test using chicken red blood cells was performed. This procedure was used to differentiate the vibrio cholerae serogroup 01, (Ogawa and Inaba) into classical and EL Tor biotypes (Lennette **et al**, 1978; Farmer **et al**, 1985).

A loopful of the vibrio cholerae serogroup 01 positive, ogawa and inaba strains were emulsified in a drop of sterile normal saline on a greasefree glass slide respectively. A drop of purified chiken red blood cells was added to each of the suspension and mixed. The mixture was rocked for two minutes and observed for agglutination. The presence of agglutination shows the presence of EL Tor biotype while the absence of agglutination shows the presence of the classical biotype of vibrio cholerae serogroup 01.

CHAPTER FOUR

RESULT

The result is as shown in table 1,and 2, and appendix I, II, III. Thirty seven (37) cholera vibrio organisms were identified using biochemical and serological identification methods. Sixteen (16 = 43.2%) of the cholera vibrios were identified as vibrio cholerae serogroup 01. Thirteen (13 = 81.3%) of the vibrio cholerae serogroup 01 were identified as vibrio cholerae serogroup 01, ogawa biotype while one (1 = 6.3%) was of the inaba biotype. Two (2 = 12.5%) of the vibrios exhibiting the biochemical characteristics of the vibrio cholerae serogroup 01 but could not agglutinate in either ogawa or inaba antisera, and was presumptively diagnosed as non-agglutinating vibrio cholerae serogroup 0139. Out of the 21 vibrio cholerae non-01 serogroup identified, 11 (52.3%) were vibrio parahaemolyticus while 10 (47.6%) were of the other speices of vibrios.

From the different local Government areas - table 2 and appendix I, II and III, 3 isolates of cholera vibrios were recorded from Giwa L.G.A., of which 1 isolate was identified as vibrio cholerae serogroup 01 while the other 2 were vibrio cholerae non 01 serogroup. 17 cholera vibrios were isolated from Zaria L.G.A. 6 were identified as vibrio cholerae serogroup 01 while 11 were vibrio cholerae non-01. 17 cholera vibrios were isolated from Sabon-Gari L.G.A. 9 were vibrio cholerae serogroup 01 while 7 were vibrio cholerae non-01.

The non-01 vibrios included vibrio parahaemolyticus and other vibrio species.

From the different ecological nitches 13 cholera vibrios were isloated from pond waters, 1 vibrio cholerae non-01 from Giwa, 4 from Zaria and 3 from Sabon Gari Local Government areas. 1 vibrio cholerae serogroup 01 from Zaria, 4 from Sabon Gari local government areas and none from Giwa local government area. 6 cholera vibrios were isolated from well waters. Non from Giwa L.G.A., 3 from Zaria and 3 from Sabon gari local Government. From stream waters, 10 vibrio organisms were isolated. 1 from Giwa L.G.A. 5 from Zaria and 4 from Sabon gari L.G.As. 4 organisms were isolated from human stools, 1 from Giwa, 3 from Zaria and none from Sabon gari L.G.As. Likewise 4 vibrios were isolated from Duck stools, non from Giwa, 1 from Zaria and 3 from Sabon Gari L.G.As. No cholera vibrio was isolated from the water letus collected from Sabon Gari L.G.A.

TABLE 1: Total no. of vibrio cholerae 01 and Non-01 isolates fr different ecosystems.

	POND WATER	WELL WATER	STREAM WATER	HUMAN STOOL	DUCK STOOL	ROOTS WATER-LETUS	TOTAL
SAMPLE SIZE	150	150	150	150	150	5	755
NO OF VIBRIO CHOLERAE -01	5	3	-	4	4	-	16
NO OF VIBRIO CHOLERAE NON-01	8	3	10	-	-	-	21
TOTALS	13	6	10	4	4		37=9.8%/ 755

All vibrio cholerae 01 Ogawa or Inaba Serotype belong to EL T biotype

TABLE 2: Total No of vibrio cholerae-01 and Non-01 isolates from different ecosystems by local government areas.

	SAMPLE SOURCE	POND WATER	WELL WATER	STREAM WATER	HUMAN STOOL	DUCK STOOL	WATER-LETUS	ISOLATED TOTAL	TOTAL VIBRIOS/ L.G.A
GIWA L.G.A.	NO OF VIBRIO CHOLERAE -01 ISOLATES	-	-	-	1	-	-	1	
	NO OF VIBRIO CHOLERAE NON-01 ISOLATES	1	-	1	-	-	-	2	3
ZARIA LGA	NO OF VIBRIO CHOLERAE -01 ISOLATES	1	1	1	3	1	-	6	
	NO OF VIBRIO NON-01 ISOLATES	4	2	5	-	-	-	11	17
SABON GARI LGA	NO OF VIBRIO CHOLERAE -01 ISOLATES	4	2	-	-	3	-	9	
	NO OF VIBRIO NON-01 ISOLATES	3	1	4	-	-	-	8	17

CHAPTER FIVE

DISCUSSION

The presence of cholera vibrios in all the environmental ecosystems studied may be seen as an indication of low level of environmental and personal hygiene among the inhabitants of the three local government areas studied. The presence of vibrio cholerae serogroup 01 in ponds, wells and streams in these areas is an indication of a possible epidemic of cholera in these areas, in no distant future. The fact that these ecological nitches serve as reservoirs of vibrio organisms, possibly provides the basis for the endemicity of these organisms in these parts of Kaduna state (Nig. Bull of Epid. 1991). The occurrence of the vibrio organisms in fresh water environments have also been reported by other workers (Smith, 1968; Vernon and Thomas, 1978; Robert and Nathaniel, 1984; Sarkar **et al**, 1985; Islam **et al**, 1989 and 1990), and have been found to be reservoirs of the cholera vibrios.

Vibrio cholerae Serogroup 01, Ogawa, EL Tor biotype ranked high, among the vibrio organisms isolated from ponds and well water samples studied (table 1). The relative high occurrence of this biotype which has been implicated as the agent of the 7th pandemic cholera, which is presently ravaging the world, is of great concern. The vibrio cholerae serogroup 01, is known, more than other vibrio species for causing epidemic cholera. The El Tor biotype has been reported to cause more infections and survives longer than the classical

biotype in water and sewage enviroments (Vernon and Thomas, 1978; Pelzer **et al**, 1982; Farmer **et al**, 1985; Popovic **et al** 1995). The absence of the vibrio cholerae serogroup 01 from the stream water sampled is not unexpected. This is because of the subsequent dilution of the bacterial load along the line of flow of the streams.

The presence of vibrio parahaemolyticus in these fresh water environments (Ponds, wells and streams) is as important as that of the vibrio cholerae serogroup 01. This is because, vibrio parahaemolyticus is a well documented causative agent of acute gastroenteritis (Philip **et al**, 1989). It has been involved in several endemic and sporadic cholera epidemics. This includes the sporadic epidemic outbreak of cholera in Louisiana, Maryland, in the United States of America, in 1986 (Charles **et al**, 1989), and in the Chesapeake Bay Community, also in the United States of America, in 1986 (Robert **et al**, 1992).

The occurence of other species of vibrios other than vibrio cholerae serogroup 01 and vibrio parahaemolyticus in these fresh water environments cannot be neglected. The level of these organisms in these bodies of water are unacceptable. This is because, several of these vibrio species have been found involved in several diarrhoeal diseases (Carrol **et al**, 1984; Farmer **et al**, 1985), though the severity of the diarrhoea associated with these vibrios are known to be of lesser magnitude. They have also been involved in several

non-diarrhoeal infections such as infection of wounds, sepsis, pneumonia, meningitis and other invasive diseases (Carol **et al**, 1984; Farmer **et al**, 1985; Judith **et al**, 1994; Popovic **et al**, 1995; Dilip **et al**, 1996).

The importance of these ecological nitches such as ponds, wells and streams to the lives of the inhabitants of these local government areas cannot be overemphasized. These fresh water environments form the main backbone of the survival of the inhabitants of the areas understudy. Water from the wells ponds, and streams are used for several domestic purposes in these areas, such as washing, bathing and cooking. They are also used for feeding of animals and for irrigation of farmlands. Water from these sources is extensively used for irrigation during the dry season farming. They are used for irrigating vegetable farmlands and also for washing the vegetables after harvesting. Vegetables grown through irrigation include garden-eggs, carrots, cucumber, tomatoes, pepper and other fruits. These human activities around the bodies of water, ensure contact with possible pathogens contained in them. It can therefore be deduced that foci of vibrio cholerae infection will be found in such communities whose socio-economic activities revolves around such bodies of water.

The isolation of vibrio cholerae serogroup 01, ogawa, EL Tor biotype from both human and duck stools shows the importance of man and animals as carriers of these organisms.

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This observation was also made by Farmer **et al**, (1985). There is the possibility that the human and animal carriers are the main agents for disseminating these organisms in the environment especially in the fresh water ecosystems. The poor sanitary systems, public hygiene and social behaviour of the people in the three local government areas understudy predisposes the bodies of water to contamination with the vibrio organisms. Also, the presence of animals like Ducks, Pigs and cattles, which are in constant contact with human faeces, in these local government areas, may be a prime factor in the dissemination of these organisms, considering their presence in these water environments used for domestic purposes.

The two vibrio isolates from stool samples of Ducks, having the characteristics of the vibrio cholerae serogroup 01, but were unable to agglutinate in either inaba or Ogawa antiserum, were presumptively diagnosed as the strains of vibrio cholerae serogroup 0139, synonym Bengal. The presumptive diagnosis is based on the fact that the vibrio cholerae serogroup 0139 strains are reported to be closely related to the vibrio cholerae serogroup 01, both in morphology, culture and even in fimbrial antigen, cholera toxin (CT), gene for zonnula occludens toxin (zot) and accessory cholera enterotoxin (ace), capsular expression and structural genes (Johnson **et al**, 1992; Albert, 1994; Popovic **et al**, 1995). The possibility of the vibrio cholerae serogroup 0139 representing —

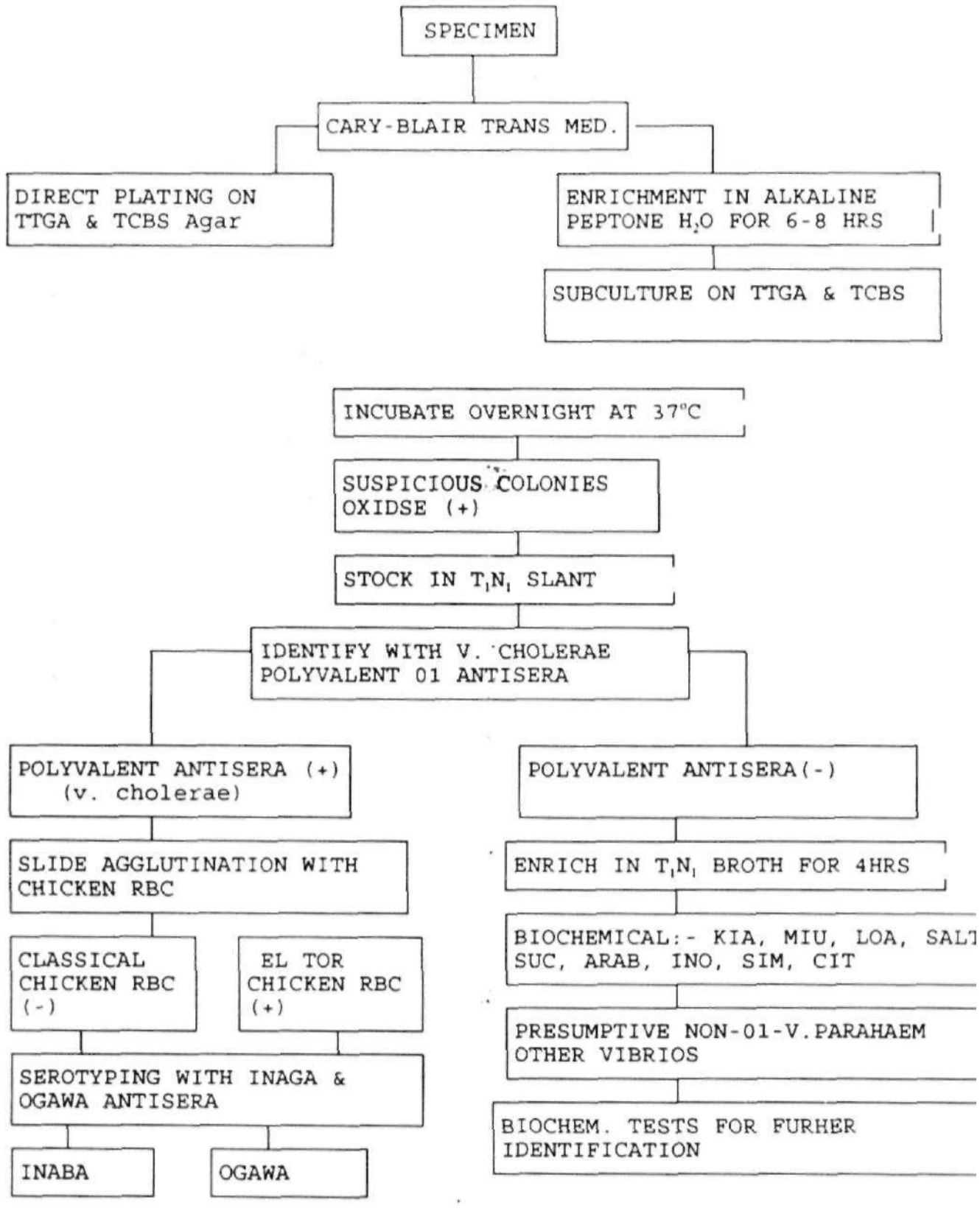
a mutant of the vibrio cholerae 01,, EL Tor biotype has been suggested (Rivas **et al**, 1993). Although, vibrio cholerae serogroup 0139 has not been reported in Nigeria, materials for its full identification are hard to obtain.

The presence of vibrios in human, animal and environmental sources calls for a well articulated control measures especially, health education programme. The people need to be educated on the importance of contamination of their environment either by man or by animals. The sanitary and personal behaviour of the local inhabitants of these areas should be improved through health education. Governments at the different levels (Local, State and Federal governments), should be able to provide as a matter of urgency, adequate safe drinking water such as pipe-borne water and or borholes. Where these facilities cannot be provided, adequate health education on the provision of safe water, such as by using clean cloth materials for filteration (Huq **et al**, 1996), and or boiling before use, will go a long way in limiting the contact between the people and these disease organisms. The inhabitants of these areas, where these social facilities are not provided, should be encouraged to provide for themselves such facilities as deep-wells and water tanks. The importance of protection of these facilities (through provision of covers for the wells and tanks), against external contaminations must be emphasised. Moreover, the control of domestic animals such as pigs, cattles and birds, by keeping them in pens and cages

and the practice of safe disposal of human faeces, through the provision of water closet-systems and ventilated-pit-latrine (V.I.P Latrine), will adequately control the presence of the cholera vibrios in these environments. In this way, resources usually used for treatment of these diseases, (for purchase of vaccines, drugs, oral rehydration salts, equipments and personnel costs), will be saved for more pressing needs of the people.

MATERIALS AND METHODS
APPENDIX A

FLOWSHEET FOR IDENTIFICATION OF V. CHOLERAE -01, NON-01 AND V PARAHAEMOLYTICUS



1. T_1N_1 BROTH STOCK

Trypticase	-	10g
Sodium chloride	-	10g
Distilled water	-	1000ml

The reagents were weighed as shown above. The weighed reagents were mixed in the distilled water in a 2 liter conical flask and the pH adjusted to 7.4. 10millilitres of 0.2% bromocresol purple was then added to the mixture and the mixture autoclaved at 121°C for 15 minutes and was stocked for further use.

2. SUGAR BROTHS

100millilitre of the stock T_1N_1 broth was mixed with measured amounts of the various sugars as shown below and dispensed in 2 millilitre amount into screw capped bijou bottles and autoclaved at 121°C for 15 min.

(a) T_1N_1 broth - 100ml
Mannose - 0.5g.

(b) T_1N_1 broth - 100ml
Sucrose - 0.5g.

(c) T_1N_1 broth - 100ml
Glucose - 0.5g.

(d) T_1N_1 broth - 100ml

Inositol - 0.5g.

(e) T_1N_1 broth - 100ml

Mannitol - 0.3g

The sugar and amino acid mixtures were dispensed in 3ml amount respectively into test tubes and covered with cotton wool. The tubes containing the mixtures were then autoclaved at 15lbs pressure for 15 minutes and stored in the refrigerator for use in biochemical tests.

APPENDIX C

PREPARATION OF SOME UNCONVENTIONAL MEDIA AND REAGENTS.

MONSURS MEDIA pH 8.5 - 90(ie. TAUROCHOLATE TELLURITE GELATINE AGAR-TTGA).

Reagents includes:-

Sodium chloride	- 10g
Trypticase	- 10g
Sodium taurocholate	- 5g
Gelatine powder	- 30g
Agar	- 16g
Distilled water	- 1000ml.

The reagents were mixed in 2 litres conical flask and boiled to dissolve completely the reagents. The pH was adjusted to between 8.5 - 9.0. The mixture was autoclaved at 15lbs for 15minutes. It was then cooled to 50°C. 5ml of 0.1% potassium tellurite solution was then added. The medium was poured into sterile petridishes in 20ml amounts and allowed to solidify and stored in refrigerator (Monsur, (1963).

T.N. AGAR

Materials:-

Sodium chloride	- 10g
Trypticase	- 10g
Agar	- 15g

pistilled water - 100ml

The reagents were dissolved completely in the distilled water and the pH adjusted to 7.4. The mixture was then boiled and allowed to cool. It was then dispensed in 2 - 3ml amount in screw capped bijou bottles and tubes and autoclaved at I5lbs pressure for 15minutes. The tubes were left upright and some in slanting positions to solidify. They were stored in refrigerator pending use.

APPENDIX D

PREPARATION OF LYSINE ARGININE AND ORNITHINE BROTH MEDIA.

1. moellers, (1955) METHOD

Peptone - **5g**

Beef extract - **5g**

Pyrodoxal - 5mg

Glucose - 0.5g

Distilled water - **1 litre**

Bromocresol blue (0.2%) solution - 5ml

Cresol red (0.2%) solution - 2.5ml

The solids were dissolved in the distilled water l heating. The pH then adjusted to 6.0. The indicators we mixed (i.e. Bromocresol blue and cresol red solutions). T solution was distributed into 4 equal volumes and steriliz at 115°C for 20 minutes.

For use the following additions were made respectivel

(1) L - arginine hydrochloride - **1%**

(2) L - Lysine hydrochloride - **1%**

(3) L - Ornithine hydrochloride **1%**

The pH was readjusted to 6.0. The media were distribut in 2ml amount into bijou bottles containing sterilized liqu paraffin to a height of about 5mm. The mixture were r sterilized at 115°C for 10mins and stored for use.

II FALCOWS (1958) METHOD

Peptone - 5g

Yeast extract - 3g

Glucose - 1g

Distille water - 1 litre

Bromocresol purple (0.2%) solution - 100ml

The solids were dissolved in the distilled water and the pH adjusted to 6.7. The indicator solution was then added. The mixture was sterilized at 115°C for 20 minutes.

The basal medium was divided into 4 equal parts and the following additions were made.

(1) L - arginine hydrochloride - 0.5%

(2) L - Lysine hydrochloride - 0.5%

(3) L - Ornithine hydrochloride - 0.5%

The pH was readjusted to 6.7 and the mixture dispensed in 2ml amount respectively in bijou bottles and resterilized at 115°C for 10 minutes and then stored for use.

III MODIFIED FALKOWS METHOD

L - aminoacid (Lysine, Arginine and Ornithine monohydrochloric acid) - 0.5g

Monopotassium phosphate - 1.0g

Bromocresol green - 0.002g

The reagents were measured respectively as specified above and mixed with 100ml distilled water in a 250ml conical flask. The pH was adjusted to 4.4. The mixture is stored in a screw-capped bottle over a slight excess of chloroform and dispensed as needed in 1ml amount into 13mm screw capped bijou bottles.

APPENDIX I
BIOCHEMICAL IDENTIFICATION OF ISOLATES FROM GIWA L.G.A.

	SODIUM CHLORIDE (%)					AMINO ACIDS			SUGARS						K. I. A.			M. I. U.					
I.D. CODE	0	1	3	8	10	ORNI	ARGI	LYS	ARAB	MANN	SUC	INO	GLU	MANI	SLANT	BUTT	H_2S	UREA	IND.	MOTI	GELAT	OXID	IDENTIFIED ORGANISMS
C_{P25_g}	-	+	+	+	+	+	-	+	-	+	-	-	+	+	K	A	-	-	+	+		+	V.Parahaemo
C_{S1^2g}	-	+	+	+	+	+	-	+	+	+	+	-	+	-	K	A	-	-	+	+		+	V.Parahaemo.
G_{H14}	+	+	+	+	+	+	-	+	-	+	+	-	+	-	K	A	-	-	+	+	+	+	V. cholerae

NB: ORNI = Ornithine; ARGI = Arginine; ARABI = Arabinose; SUC = Sucrose; INO = Inositol; GLU = Glucose; MANI = Manitol; MANN = Mannose; IND = Indole; MOTI = Motility; GELA = Gelatinose OXID = Oxidase.

KEY: G = Giwa L.G.A.
P = Pond Water
Qs = Stream Water
CH = Human Stool.

APPENDIX II

BIOCHEMICAL IDENTIFICTION ISOLATES FROM ZARIA L.G.A.

	SODIUM CHLORIDE (%)					AMINO ACIDS			SUGARS						KIA			MIU					
I. D. CODE	0	1	3	8	10	ORNI	ARGI	LYS	ARAB	MANN	SUC.	INO	GLU	MANT	SLANT	BUTT	H_2S	UREA	INDO	MOTI	GEL	OXID	IDENTIFIED ORGANISMS
Z_{S1G}	-	+	+	+	+	+	-	+	-	+	-	-	+	-	K	A	-	-	+	+		+	V. parahaemoly
Z_{S1^3G}	-	+	+	+	+	+	-	+	+	+	-	-	+	-	K	A	-	-	+	+		+	V. parahaemoly
Z_{S2^1G}	-	+	+	+	+	+	-	+	-	+	-	-	+	-	K	A	-	-	+	+		+	V.parahemoly
Z_{S3C}	-	+	+	-	-	+	-	+	-	+	+	-	+	+	K	A	-	-	+	+		+	Vobrio Spp.
Z_{S3^2G}	-	+	+	-	-	-	-	+	-	+	-	-	+	-	K	A	-	-	+	+		+	Vibrio Spp.
Z_{D22^1}	+	+	+	+	+	+	-	+	d	+	+	-	+	-	K	A	-	-	+	+		+	V. Cholerae
Z_{H2}	+	+	+	+	+	+	-	+	-	+	+	-	+	+	K	A	-	-	+	+	+	+	V. Cholerae
Z_{H2^2}	+	+	+	+	+	+	-	+	-	+	+	-	+	-	K	A	-	-	+	+	+	+	V. Cholerae
Z_{H10^3}	+	+	+	+	+	+	-	+	-	+	+	-	+	-	K	A	-	-	+	+	+	+	V. Cholerae
Z_{P4^2G}	-	+	+	+	+	+	-	+	+	+	-	-	+	-	K	A	-	-	+	+		+	V.parahaemoly
Z_{P5^2G}	-	+	+	+	+	+	-	+	+	+	-	-	+	-	K	A	-	-	+	+		+	V. parahaemoly
Z_{P11}	-	+	+	+	-	-	+	+	-	+	+	-	+	-	K	A	-	-	+	+		+	Vibrio Spp.
Z_{P12^1G}	-	+	+	+	-	-	-	+	+	+	+	-	+	-	K	A	-	-	+	+		+	V. parahaem.
Z_{P24}	+	+	+	+	+	+	-	+	-	+	+	-	+	+	K	A	-	-	+	+	+	+	V. Chalerae
Z_{P24}	-	+	+	+	-	+	-	+	-	+	-	-	+	-	K	A	-	-	+	+		+	V.parahaem.
Z_{W8}	+	+	+	+	+	+	-	+	-	+	+	-	+	+	K	A	-	-	+	+	+	+	V.chalerae
Z_{W21^2}	-	+	+	-	-	-	-	+	-	+	+	-	+	-	K	A	-	-	+	+		+	Vibrio Spp.
Z_{W21^5}																							

KEY: Z = ZARIA L.G.A.
S = Stream water samples
D = Duck Stool
H = HUMAN STOOL SAMPLES
P = Pond wate samples
W = Well water samples.

APPENDIX III

BIOCHEMICAL IDENTIFICATION OF ISOLATES FROM SABON-GARI L.G.A.

	SODIUM CHLORIDE (%)					AMINO ACIDS			SUGARS				K I A					M I U					
I.D. CODE	0	1	3	8	10	ORNI	ARGI	LYS	ARAB	MANN	SUC	INO.	GLU	MANT	SLANT	BUTT	H_2S	UREA	INDO	MOTI	GEL	OXID	IDENTIFIED ORGANISMS
S_{P6}^{2}	+	+	+	+	+	+	-	+	-	+	+	-	+	+	K	A	-	-	+	+		+	V. Cholerae
S_{P11}^{2}	+	+	+	+	+	+	-	+	-	+	+	-	+	-	K	A	-	-	+	+		+	V. Cholerae
S_{P24}	+	+	+	+	-	+	-	+	-	+	+	-	+	-	K	A	-	-	+	+		+	V. Cholerae
S_{P33}^{2}	-	+	+	-	-	-	+	+	-	+	-	-	+	-	K	A	-	-	+	+		+	Viberio Spp
S_{P33}^{2}	-	+	+	-	-	-	+	+	-	+	+	-	+	+	K	A	-	-	+	+		+	V. Chloerae
S_{P34}^{1}	+	+	+	+	+	+	-	+	-	+	-	-	+	+	K	A	-	-	+	+		+	Vibrio Spp.
S_{P36}^{1}	+	+	+	-	-	-	+	-	-	+	+	-	+	-	K	A	-	-	+	+		+	V.Parahaem.
S_{P39}^{2}	-	+	+	+	+	+	-	+	-	+	-	-	+	-	K	A	-	-	+	+		+	Vibrio Spp.
S_{S1}^{3}	-	+	+	-	-	-	-	+	-	+	+	-	+	-	K	A	-	-	+	+		+	V.parahaem
S_{S1}^{1}	-	+	+	+	+	+	-	+	-	+	+	-	+	-	K	A	-	-	+	+		+	Vibrio Spp.
S_{S12}	-	+	+	-	-	+	+	+	-	+	+	-	+	-	K	A	-	-	+	+		+	Vibrio Spp.
S_{S14}^{2}	-	+	+	+	-	-	+	+	-	+	+	-	+	+	K	A	-	-	+	+		+	V. Chloerae
S_{W6}^{2}	+	+	+	+	+	+	-	+	-	+	+	-	+	+	K	A	-	-	+	+		+	Vibro Spp.
S_{W7}^{2}	-	+	+	-	-	-	-	+	-	+	+	-	+	+	K	A	-	-	+	+		+	V.Cholerae
S_{W9}^{1}	+	+	+	+	+	+	-	+	-	+	+	-	+	+	K	A	-	-	+	+		+	V. Cholerae
S_{D10}^{1}	+	+	+	+	+	+	-	+	-	+	+	-	+	+	K	A	-	-	+	+		+	V.Cholcae
S_{D20}^{1}	+	+	+	+	+	+	-	+	-	+	+	-	+	+	K	A	-	-	+	+		+	V.Cholerae.
S_{D28}^{3}	+	+	+	+	+	+	-	+	-	+	+	-	+	+	K	A	-	-	+	+		+	

KEY: S = Sabongari, P = Pond Water, S = Stream Water
W = Well Water, D = Duck Stool.

65c

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