EPIDEMIOLOGY AND MOLECULAR CHARACTERIZATION OF *AEROMONAS* SPECIES ISOLATED FROM FISHES, POULTRY AND DIARRHOEIC PATIENTS IN ZARIA, NIGERIA

BY

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DECLARATION

I hereby declare that the work presented in this dissertation is original. It was carried out by me under the supervision of Professors I. Ajogi and A.J. Nok in the Bacterial Zoonosis Laboratory, Department of Veterinary Public Health and Preventive Medicine and in the Mary Hallaway Teaching Laboratory, Department of Biochemistry, all of Ahmadu Bello University, Zaria, Nigeria.

References were made to the works of other investigators and were duly acknowledged. No part of this dissertation has been submitted for a degree or other qualifications at any other school or university.

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CERTIFICATION

This dissertation entitled **"EPIDEMIOLOGY AND MOLECULAR CHARACTERIZATION OF *AEROMONAS* SPECIES ISOLATED FROM FISHES, POULTRY AND DIARRHEAL PATIENTS IN ZARIA, NIGERIA**" by Samuel Mailafia meets the regulations, governing the award of the degree of Doctor of Philosophy (Ph.D) of Ahmadu Bello University, Zaria and is approved for its scientific contribution to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to: all scientists who contributed immensely to the field of bacteriology, public health and biotechnology but lost their lives at the course of their work.

It is also dedicated to:

1. God Almighty for the divine wisdom and protection during this work
2. My aged parents
3. My wife and my three children

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ABSTRACT

Bacteriological examination of feacal samples for isolation and identification of *Aeromonas* species was carried out in order to characterized them by molecular techniques. A total of 1,160 samples obtained from 400 fishes, 540 poultry and 220 diarrhoeic patients were collected in Zaria, Nigeria between October, 2005 and January 2006.

Fishes comprising *Tilapia zillii*, *Schilbe mystus*, *Synodontis filamentosus*, *Alestes nurse*, *Marcusenius senegalensis* and *Clarias gariepinus* were collected from 4 different locations (A.B.U. dam, Zaria dam, Samaru market and Sabon gari market). Poultry included layers (Brown harco) and Broilers (Anak giant) were collected from 5 areas (Zumunta farms, Lape farms, Zaria farms, Area 'C' and Area 'F'). Human samples were collected from 2 locations (ABU Teaching Hospital and ABU sick bay).

The samples were examined for *Aeromonas* species using the bacteriological culture method of isolation. Of the 1,160 samples examined from fishes, poultry and humans, 195 (16.8 %) yielded *Aeromonas* isolates. 84 (21 %) of the isolates were obtained from fish, out of which 24(23.76 %) were from *Tilapia zillii,* 11(16.41 %) from *Schilbe mystus* 18(22.78 %) from *Synodontis filamentosus,* 14(20.89 %) from *Clarias gariepinus*, 17(21.25 %) from *Marcusenius senegalensis* and none from *Alestes nurse.* The results did show that 82 (15.18 %) of the *Aeromonas* isolates obtained from poultry 67 (15.22 %) were from Layers and 15 (15 %) were from broilers. The least number 29 (13.8 %) of isolates were recovered from diarrhoeic patients 5(17.14 %) of which were from Adult males, 10(8.33 %) from adult females, 8(66.67 %) from young males and 6(33.33 %) from young females.

The isolates were characterized by both biochemical and molecular techniques their prevalence rates and distributions were determined. In fish, the isolates were *Aeromonas hydrophila* 47 (11.75 %), *A. caviae* 6 (1.50 %), *A. sobria* 12 (3.00 %) and *A. salmonicida* 19 (4.75 %). In poultry, the isolates were *A. hydrophila* 19 (3.52 %), *A. caviae* 23 (4.26 %), *A. sobria* 5 (0.93 %) and *A. salmonicida* 35 (6.48 %). In humans, the isolates were *A. hydrophila* 15 (6.81 %), *A. sobria* 6 (2.73 %) and *A. caviae* 8 (3.64 %) and *A. salmonicida* was not isolated from humans.

All the isolates were tested for hemolysis on 10 % sheep blood of which 72 (36.92 %) produced ß-hemolysins. The distribution of ß-hemolysins amongst different species of *Aeromonas* were *A.hydrophila* 31 (37.8 %), *A.caviae* 14 (37.83 %), *A. sobria* 9 (40.90 %) and *A. salmonicida* 18 (33.33 %).

The molecular characterization included extraction and quantification of soluble proteins from 26 selected isolates using protein analysis. In poultry, virulent proteins were *A.hydrophila* (3.58 g) *A.caviae* (4.00 g), *A.salmonicida*, (3.82 g) and *A.sobria* (0.00 g). In fish, the virulent proteins were *A.hydrophila* (3.11 g), *A sobria* (4.63 g), *A.caviae* (2.95 g) and *A.salmonicida* (2.74 g). Humans, the virulent proteins were *A.hydrophila* (4.07 g), *A. sobria* (3.58 g) and *A. caviae* (3.99 g). Electropherogrammes of soluble proteins using Sodium Dodecyl Polyacrilamide Gel Electrophoresis (SDS-PAGE) revealed that *Aeromonas* species produced 6 types of toxins. The relative molecular weight ranges between 35-73 kDa. Data obtained by analyzing *Aeromonas* protein profiles showed correlation coefficients of 0.70 and 0.64 which indicated that two strains could not be assigned to the same species.

The genomic deoxyribonucleic acid (DNA) of the 26 *Aeromonas* species was extracted using the Qiagen kit. The DNA extracts were of high purity, having optical density of 1.6-1.8. DNA quantification yielded total DNA in *A. hydrophila* (450 µg/ml) *A. caviae* 425 µg/ml, *A. sobria* (450 µg/ml) and *A. salmonicida* (375 µg/ml).

Electrophoresed DNA were further confirmed by digestion with rare-cutter restriction endonucleases (E*co* R1, B*am* HI and S*al* 1) which yielded restriction fragments (1.5 kb - 450 bp).

It is evident from this study that aeromoniasis is endemic affecting fishes, poultry and humans in Zaria, Nigeria. The epidemiological significance of these findings is discussed. Some observations on the zoonotic and public health implications of *Aeromonas* infections are presented. *Aeromonas* species were found to have developed some virulent factors which could be involved in pathogenicity. DNA analysis also demonstrates common evolutionary origin of these organisms. Further studies on molecular typing of *Aeromonas* is indicated.

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CHAPTER ONE

INTRODUCTION

1.2 STATEMENT OF THE PROBLEMS

There is an increasing demand on the fish and poultry industries in Nigeria for more fish and poultry production to enhance the availability of adequate supply of protein for the ever increasing population. Maximum production efficiency is therefore required of the fish and poultry population, if it is to meet the high protein requirement of the Nigerian population (Atsanda *et al*., 2000; Okpokwasili and Ogbulie, 2001).

Nigeria has estimated fish population of over 280 million and poultry population of over 240 million (FAO, 2005). About 80 % of total fish and poultry are harnessed by village farmers. Only about 20 % belong to the government owned fish and poultry centres and privately owned farms and fish ponds. Several constraints to poultry or fish production have been identified. These include: socio–cultural problems, poor nutrition, poor herd health management, diseases, poor housing, and other environmental factors directly or indirectly affecting production (Olanike, 1999; Okpokwasili and Ogbulie, 2001). The greatest obstacle to increased poultry and fish production is disease among which ectoparasites, helminthes, blood parasites, mycotic, bacterial and viral diseases are the most prominent.

Aeromoniasis is an emerging zoonotic disease of worldwide distribution. It is extremely common, but often made out to seem less important than it really is. In many countries, the native population in their way of life accepts diarrheal diseases and other gastroenteritis as inevitable occurrences nevertheless; they remain a significant cause of deaths especially in the young and elderly (Zhang *et al*., 2002).

Majority of food poisoning by *Aeromonas* species involves mishandling of fish and poultry products in either during processing, the home or street-vending places, with the foremost error being the use of improper handling procedure (Buchanan, 1986). *Aeromonas* infection of fish and poultry could originate from water that is contaminated with the infectious agents or feeding fish with poultry manure contaminated with *Aeromonas* species. Man also stands the risk of infection when handling or consuming improperly cooked fish or poultry meat (Zhang *et al*., 2002).

All members of the *Aeromonas hydrophila* complex are predominant in fish, meat, foods and poultry samples and contribute to the virulent taxons hybridization groups (HG 4 and HG 8). Although the significance of *Aeromonas* in foods remains undefined, the isolation of various *Aeromonas* strains from a variety of retail foods such as fish and poultry products can act as possible vehicles for the dissemination of food-borne *Aeromonas* gastroenteritis in humans (Bechet and Blondeau, 2003).

The infectious nature of *Aeromonas* species was first demonstrated in United States of America by Kluyver and Van Niel in 1936 as reported by Hernanz *et al*. (1998). Since then, interest in the organism has increased, because of its effect on human health ,poultry and aquatic animals (Quadri *et al*., 1976).

A. hydrophila have been recognized as causative agents of human meningitis, endocarditis, osteomyelitis, wound infections and gastro intestinal or diarrheal disease (Quadri *et al*., 1976; Karam *et al*., 1983). There can be septicemia and the organism can occur as opportunistic pathogen in immnnocompromised or HIV patients (Gracey *et al*., 1982).

Aeromonas species have been isolated from blood, cerebrospinal fluid, exudates from otitis media, urine, peritoneal fluid, necrotic muscle, infected heart valves and bones (Baron and Fine gold, 1990), and the disease is common in environments where fresh water fish are cultured widely, in many countries of the world.

Interest in the role of *Aeromonas* infection in humans, animals and food borne disease has increased in recent years (Krovacek *et al*., 1989). Although the "food poisoning" potential of the organism has not been completely established, the association with human gastroenteritis (Agger *et al*., 1985) strongly suggests that *A. hydrophila* produces a heat labile enterotoxin and a heat stable cytotoxic enterotoxin which is known to cause watery or bloody and mucoid diarrhea in infants as confirmed by Altwegg and Johl (1985). In many countries of the world like Japan United States of America (USA), little is known about the occurrence of other *Aeromonas* species except for *A. hydrophila* which is the most common and important pathogen in the gastrointestinal tract (GIT) of cultured fish (Santose *et al*., 1988; Cahill, 1990; Sugita *et al*., 1994).

1.2 IMPORTANCE OF AEROMONIASIS

Aeromonas organisms occur in a wide variety of retail fresh foods of animal origin such as sea foods, raw milk, meats and poultry (Waldroup, 1994). In humans, *A. hydrophila* occurs as an opportunistic pathogen in immunocompromised patients especially children or in adults with accompanied malignancies (Floyd and Francis-Reed, 2002). The organisms of the *A. hydrophila* group have been identified as zoonotic pathogens of public health significance (Hayes, 2000). It is characterized by intestinal symptoms including bacteremia, fever, chills, abdominal pain, nausea, vomiting and

diarrhea (Hayes, 1994); extra-intestinal symptoms which includes human meningitis, endocarditis, osteomyelitis and wound infections (Karam *et al.,* 1983). Other symptoms in humans includes cellulitis, septicemia, urinary tract infections, hepatobilliary infection, ear infections, and supurative arthritis in patients with leukaemia (Alvandi and Ananthan, 2005; Jordi *et al.,* 2005).

Aeromoniasis incures great socioeconomic loses to several communities affected by the disease. Africa is estimated to loose about two million fish and produce 70 times less fish and poultry proteins that might supplement beef as a result of infection by this organism (Olanike, 1999; Esuruoso, 1999). The degree of the loss due to the disease vary from one area to another depending on the intensity of the disease and the area and can vary within closely situated towns (Litchbag, 1987).

There could be decline in reproductive performance and carcasse quality during processing. In humans, the consequences might be serious leading to premature deaths, decreased productivity and hence economic losses (Lechevallier *et al*., 1982).

In Nigeria, if efforts by the federal government to boost the nation's poultry and fish industries must succeed and the need of health for all in the year 2015 must succeed, special attention must be given to developmental research especially in the area of veterinary public health and biotechnology. Information on molecular epidemiology of the disease is scarce inspite of the fact that it poses a major threat to man and animals.

1.3 JUSTIFICATION FOR THE WORK

Molecular epidemiology on *Aeromonas* in fish, poultry and humans in Nigeria is at its primordial stage and has not gained much ground. Cultural, biochemical and molecular evaluation will help to identify and elucidate the presence of these organisms and its epidemiology with the hope of revealing their virulence factors and characters (proteins or enzymes). This will pave a way for the development of drugs and vaccines that will minimize and control significant outbreaks of aeromoniasis in humans, poultry and fishes. The possibility of emerging resistant strains of these organisms is likely to lead to adverse outbreaks. There is thus need to alert the clinical personnel and the general public on possibilities of infection and ways to ensure that necessary control measures and interventions are adequately instituted in fish and poultry products in order to elucidate the epidemiology of these diseases in the country.

The link between human illness and this pathogen has not been thoroughly established as pathogenicity testing is needed. There is need for control and subsequent eradication of this disease. It is important to obtain basic understanding on the epidemiology of the disease before any meaningful strategy for control or eradication programme can be established. The phenotypic and genotypic characteristics between clinical and environmental isolates of *Aeromonas* are crucial to obtaining accurate evolution of the epidemiology of aeromoniasis among humans, fishes and poultry in Nigeria.

In Nigeria, little has been done on *Aeromonas* species to understand the molecular basis of enzymatic catalysis and the mechanism controlling the function of these enzymes. Several *Aeromonas* genes have been characterized in detail at the nucleotide

level but have not been specified to code for a known enzyme. Efficient genotypic typing technique is a prerequisite for effective surveillance of infections caused by members of the genus *Aeromonas.*

It is hoped that the results obtained from these investigations will lead to a better understanding of the epidemiology, public health significance, proteomic and genomic analysis of aeromoniasis in Nigeria and provide desired restriction fragments and the necessary information crucial for the formulation of strategies for treatment, vaccination, control and eradication of the disease in this country.

1.5 OBJECTIVES OF THE STUDY

The objectives of these investigations are:

1 Isolation and identification of *Aeromonas* species from the Gastrointestinal tract (GIT) content of fish, poultry feaces and human diarrhoeic stools.

2 Characterization and biotyping of the various species of motile and non-motile *Aeromonas* isolates.

3 To determine the prevalence and distribution of *Aeromonas* species amongst fishes, poultry and humans.

4 Conduct electrophoresis using Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE) to identify the various toxins and establishment of pathogenicity in *Aeromonas* species and to conduct colorimetric quantification (Biuret method) of the proteins (enzymes) associated with virulence in *Aeromonas* species.

5 Extraction, purification and quantification of *Aeromonas* genomic deoxyribonucleic acid (DNA); and production of restriction fragments based on conserved regions within the DNA binding sites, using rare-cutter restriction endonucleases such as B*am* H1, E*co* R1 and S*al* 1.

CHAPTER TWO

LITERATURE REVIEW

2.2 HISTORICAL BACKGROUND OF AEROMONIAISIS

The history, nomenclature and classification of the genus *Aeromonas* has been in the state of flux since these organisms were first described in the early 1890's and it is often difficult to know which *Aeromonas* species (in today's usage) a particular article in the literature deals with. History of *Aeromonas* is divided into three (3) different time period (Floyd, 2002).

In the period of 1890 to 1936 many different names were proposed for the *Aeromonas* strains isolated from a wide variety of sources and diseases. Within this period, environmental and animal isolates of *Aeromonas* were detected (Ainswort *et al.*,2006).

In the period of 1936 to 1979, many different names were proposed for the *Aeromonas* strains isolated from a wide variety of sources and diseases. The first human isolate were first detected in 1936 by Kluyver and Van Niel; and it was first documented in the literature in 1937 (Miles and Halman, 1937). Many years thereafter, there were few reports. Lautrop (1961) reported *Aeromonas* as a predominant organism in the feces of patients with acute intestinal problem and assigned a possible etiological role. Lautrop (1961) reported a case of diarrhea that yielded *Aeromonas* and this was probably the first report to give any evidence on the etiological role of *Aeromonas.* In the early 1970's the genus *Aeromonas* was established, and many of the described species were studied and compared using techniques that measures phenotypic similarities and differences but not evolutionary distance (Ainswort *et al.,* 2006).

In the period 1979 to date phenotypic technique that measure evolutionary distance have been used to compare the species and to determine their genotypic relationship to other bacteria. This techniques have yielded some surprising result that form the basis of a more classification of the genus *Aeromonas* (Duplessis *et al*., 2006). Today many authors report on proteomics and genomics and molecular cell biology on *Aeromonas* and assign its etiological role (Floyd, 2002).

2.2 ORIGIN OF INFECTION, GENUS AND CLASIFICATION OF *AEROMONAS* SPECIES

Aeromonas species are ubiquitous, gram-negative, non-spore forming, motile aerobic or facultatively anaerobic rod bacteria or coccobacillary curved rods belonging to the genus *Aeromonas* and family *Vibrionaceae* (currently belongs to the family *Aeromonadaceae*) which includes the genus *vibrio* (Von-Graevenitz, 1968; Cipriano and Bullock, 2001).

The genus *Aeromonas* have high diversity and there are about fourteen deoxyribonucleic acid (DNA) phenospecies hybridization groups recognized (Figueras *et al.,* 2000). The current genomic groups of *Aeromonas* given by Figueras *et al.,* (2000) includes; *Aeromonas* (*A*) *hydrophila, A. bestiarum, A. salmonicida, A. caviae, A. media, A. eucrenophila, A. sobria, A. jandaei, A. veronii, A. schubertii, A. trota, A. allasaccharophila , A. encheleia* and *A. popoffi.*

Among these geno species, *A. hydrophila, A. caviae, A. veronii* biotype *sobria* and *A. salmonicida* are of clinical significance (Janda *et al.,* 1991; Janda and Abbott, 1998).

Organisms of this genus are straight rods, with rounded to coccoid ends, with peritrichous or single polar flagellum (Popoff and Veron, 1976; Popoff *et al.,* 1981). They are also facultatively anaerobic, glucose fermenting, gram-negative bacteria that are common to aquatic and terrestrial environments (Hazen *et al.,* 1978; Rau *et al.,* 2005).

They have been found in brackish, fresh, estuarine, marine, chlorinated and unchlorinated water supplies worldwide, with highest numbers obtained in warmer seasons of the year (Hazen *et al.,* 1978; Kapor *et al.,* 1981; Van der Kooj, 1988). Reports in California have shown that the number of these organisms increases with increase in water temperatures and decreases with decreased water temperature (Williams and LaRock, 1985). The cosmopolitan nature of *Aeromonas* species in aquatic environments provides ample opportunity for transmission to cold-blooded animals, particularly fishes and amphibians, which come into contact with and ingest these organisms (Hayes, 2000). Such may lead to infection, which depending on the species, pathogenicity and the virulence of the strains encountered may have life threatening consequences (Hayes, 2000). Carter *et al.,* (1995) envisaged that though *Aeromonas* species are pathogens of aquatic inhabitants but they can also infect other warm-blooded animals, including terrestrial and arborheal animals such as cattle, swine, dogs, horses, donkeys, several avian species, wild zoo and laboratory animals.

As a working compromise, it has been a common practice for members of the genus *Aeromonas* to be devided into non-motile psychrophiles which includes *A. salmonicida* and which is the cause of furunculosis and is characterized by “catariform” abscesses in salmonids. The motile mesophilic group (*A. hydrophila* group) which includes *A. hydrophila, A. sobria* and *A.caviae* (Gavriel *et al.,* 1998; Rau *et al.,* 2005).

Other diseases associated with *A. salmonicida* includes trout ulcer disease, goldfish ulcer disease, and carp erythro-dermatitis (Cipriano and Bullock, 2001).

A. hydrophila group of organisms causes red-leg disease in frogs but due to the association of fish and frogs with water, they can also infect fish regardless of its species (Austin and Allen–Austin, 1985; Williams and LarRock, 1985). The organisms especially *A. hydrophila* has been associated with several diseased conditions in fish including tail rot, fin rot, skin ulcer and haemorrahagic septicemia which is characterized by the presence of surface lesions which may lead to sloughing of the scales, haemorraging in the gills and anus (Hayes, 2005). There may also be ulcers, abcesses, exopthalmia, abdominal swelling, myonecrosis, cellulitis, ecthyma gangrenosum, necrosis of the scales and protrusion of the eye balls (Hayes, 2005).

Aeromonas infection in poultry is characterized by egg infection, cystic ovaries, salphingitis osteomyelitis, bladder infections and kidney problems (Calnek *et al.*, 1997).

2.4 GROWTH AND BIOCHEMICAL CHARACTERISTICS OF *AEROMONAS* SPECIES

Aeromonas species were recognized in 1891 as colonizers and pathogens of cold-blooded animals especially fish (Ewing *et al.,* 1961). There morphological characteristics were first detected by Kluyver and Van Niel in 1936. They were not regarded as human pathogens until in 1968 when Von-Graevenitz (1968) isolated it from diarrheal stools of humans.

The cells of *Aeromonas* species are straight, rod-shaped with round ends to coccoid or short filaments 0.3 – 1.0 μm by 1.03 μm. Flagella have wavelength of 1.7 μm. The organisms occur singly, in pairs or in short chains. They are non-sporing, gram

negative, and generally motile by a single polar flagellum. Cells of *A. salmonicida* are non-motile and atrichous (Krieg and Holt, 1984).

Acid is produced by all strains of *Aeromonas* species from glucose and maltose, but not from xylose, dulcitol, inositol, aldonitol, malonate and mucate (Popoff, 1969; Popoff *et al.*, 1981). All strains of *Aeromonas* possess enzymes such as decarboxylases, amylases, hemolysins, oxidase, catalase, gelatinase, deoxyribonuclease, ribonuclease and tween-80 esterase (Krieg and Holt, 1984).

Metabolism of glucose in *Aeromonas* species is both respiratory and fermentative. Carbohydrates are broken down into acid or acid and gas. The organisms ferments mannitol and inositol and are ornithine decarboxylase – negative (Funada and Matsuda, 1997).

Members are oxidase and catalase positive (Krieg and Holt, 1984). Optimum temperature for growth is 28 – 35^0 C. Some species are resistant to 2,4-diamino-6,7 di-isopropylpteridine (0/129). In motile *Aeromonas* species, the molecular weight and mole % of G + C of DNA is 57 – 63 bp. They are also chemo organotrophic, using variety of sugars and organic acid as carbon sources (Collier and Parker, 1987).

Nitrates are reduced to nitrites, indole is formed, gelatin is liquefied. Aesculin is hydrolysed, hydrogen sulphide is produced in cystein broth and most strains are Methyl Red (MR) positive and Voges-Proskauer (VP) negative (Baron and Fingold, 1990).

Aeromonas species produce siderophores indicating their ability to survive and grow in the iron deficient conditions (Alvandi and Ananthan, 2005). The organism grows well in ordinary media at 35^0 C and usually also at 37^0 C but the optimum temperature for many strains is 35^0 C. At this temperature acid and gas are produced in glucose, maltose,

sucrose and sorbitol (Merchant and Parker, 1977). Lactose is not quickly fermented but some strains will produce acid in few days. They produce 1 – to 3 mm colonies on a variety of media within 24 hours. Fermentation of lactose is variable but most strains of motile aeromonads are non-lactose fermenters, and do not form endospores or microcysts (Krieg and Holt, 1984). Fermentation of mannitol in Cefsulodin Irgasan Novobiocin (CIN) agar is characterized by red-centred colonies surrounded by a translucent halo.

On complex media, cells of *A. salmonicida* appears as coccobacilli, length is less than twice the width, with short chains and clumps (Smith, 1963; McCarthy, 1975). Most strains of *A. salmonicida* develop brown water-soluble pigment on media containing 0.1 % tyrosine or phenylalanine agar, hemolysis occurs rapidly and colonies becomes greenish after 7 days. The carbohydrates usually fermented by *A. salmonicida* include arabinose, trehalose, galactose, mannose, and dextrin (Krieg and Holt, 1984).

Altwegg (1994) reported that *Aeromonas* species are known to routinely grow on non-selective and selective media used for cultivation of gram-negative organisms (MacConkey, *Salmonella-shigella*, Hektoen enteric agar). The pH required for growth varies from 4.0 to 9.0, at NaCl concentrations between 0.00 % and 0.04 %. The transport medium used is the Carry-Blair or modified Stwart's transport medium (Altwegg and Bottone, 1994). Isolation frequencies of *A. hydrophila* can be increased by using enrichment media such as Alkaline Peptone Water (APW), Gram Negative Broth (G-N) Phosphate Buffered Saline (PBS). Glucose–Salt–Teepol Broth (GSTB), Thiosulphate Citrate–Bile–Salts Sucrose (TCBS) and Tethrathionate Broth (TB) (Bauman *et al.,* 1984, Kwaga *et al.*, 1988; Altwegg, 1994).

Aeromonas species produces greyish white, stippled, translucent, moist colonies, when grown on agar plate and a heavy turbidity with thick pedicle developed in broth (Merchant and Parker, 1977). Colonies on plain agar plate after 24 hours incubation are 1 – 3 mm in diameter, circular, smooth, convex, whitish and translucent, becoming light beige in colour on further incubation. It also grows on enriched media such as trypticase soy agar, blood agar and also on MacConkey's agar (Sugita *et al*., 1994). Colonies on blood agar may be hemolytic or non-hemolytic in most strains of *A. caviae* which are known to produce beta-hemolysis (Altwegg, 1994). On blood agar, there is a wide zone of β-hemolysis after one day and the growth becomes dark green after 2-3 days and this species also grows on selective media such as starch –ampicillin agar (Palumbo *et al.*, 1989).

2.4 BACTERIAL MICROFLORA OF FISH, POULTRY AND MAN

2.4.1 Fish

The microbial flora inhabiting the alimentary canal of fish are part of a complex ecosystem. The stability of the ecosystem is due to factors that are in part, host related and in part microbial. The result of interactions between host and microbe is an ecosystem comprised of many thousands of niches, each inhabited by the species or strain of microbe most aptly suited to that location. The niche dweller has successfully competed for that particular site (Hirsh *et al.*, 1990; Bruckner *et al*., 1998).

Many factors may influence the bacterial flora of fish. Some of these include common sources of stress such as poor water quality, overcrowding, or rough handling (Francis – Floyd, 2002). Also, the environment of the fish, its feeding habit, temperature

of the water and season of the year may influence bacterial flora of fish (Kaper *et al.*, 1981; Ellis, 1988). There is direct relationship between the number of bacteria found in the surrounding water or the environment and the bacteria on the surface of the skin of fish (Schewan and Hobbs, 1967). Fish also harbor bacteria in the gills, intestinal tract and the eggs, but among these, the intestinal tract tends to harbor the highest number (Aoki, 1988; Olanike, 1999). Fresh water fish such as *Tilapia* typically carry population of 10^2 – 10^3 bacteria per square centimeters of skin surface (Liston and Matches, 1976). The number in the intestinal tract depends on the amount of food present, and feeding habit and bacterial load ranging from very few to greater than 10^3 bacteria per gram of gut content (Liston and Matches, 1976).

The bacterial flora of healthy fish have been reported to be a reflection of their respective aqueous environments (Guelin, 1962; Geldrich and Clarke, 1966; Neito *et al.*, 1984; Silva and Widanapathirana, 1984; Buras *et al.*, 1987; Austin and Austin, 2002). In taxonomic study of aerobic heterotrophic bacteria isolated from temperate fresh water fish, Allen *et al.*, (1983); Dean *et al.*, (1998) reported the presence of *Acinetobacter, Aeromonas, Alcaligenes, Coryneforms, Enterobacter, Escherichia, Hafnia, Pseudomonas, Serratia, Vibrio, Yersinia, Agrobacterium, Arthrobacter, Bacillus, Bordetella, Cytophaga, Erwinia, Flavobacterium, Flexibacter, Klebsiella, Micrococcus, Moraxella* and *Staphylococcus.*

2.4.2 Poultry

Aeromonas hydrophila alone or in combination with other microorganisms may cause, localized and systemic infections of GIT in all avian species including poultry (Shane and Gifford, 1985; Siegmann *et al.*, 1989 and Calnek *et al.*, 1997).

The microbial flora or microflora of healthy or apparently healthy avian species includes the following: *Actinobacillus lignierensi, Corynebacterium pyogenes, Bacillus anthracis, Bactriodes fragilis, Bucella avium, Cytrobacter freundii, Cowdria ruminantum, Coxiella burnetii, Flaviobacterium meningosepticum, Francisella tularensis, Helicobacter pullorum, Klepsiella pneumoniae, Listeria monocytogenes, Moraxiella osloensis, Neissaria* species, *Nocardia* species, *Planococcus halophiles, Proteus vulgaris, Pseudomonas aeroginosa, Rothia* species, *Shigella boydii, Streptobacillus moniliformis, Campylobacter* species, *and Aeromonas hydrophila* (Shane and Gifford, 1985; Calnek *et al.*, 1997).

2.4.3 Man

The microorganisms associated with food contamination and infection of the human alimentary canal are diverse. Many organisms if present in sufficient numbers in fish, poultry or food may cause intestinal upset in some of those who consume it (Bryan, 1978).

The most common bacterial groups associated with food poisoning in terms of intoxications which is caused by ingestion of food containing viable bacteria which then grow and establish themselves in the host, resulting in illness (Cruickshank *et al.*, 1975; Scientific status summary, 1988).

Organisms associated with food poisoning and intoxication in man includes: *Salmonella typhimurium, Salmonella enteritidis, Campylobacter jejuni, Shigella dysentriae, Shigella flexineri, Shigella boydii, Shigella sonnei, Listeria monocytogenes, Listeria seeligeri, Listeria ivanovii, Clostridium perfrigens* types B, C, D and E. Others

include *Yersinia, enterocolitica, Aeromonas hydrophila, Plesiomonas shigeliodes* and *Escherichia coli.*

Most of these organisms are associated with consumption of fresh foods including fish, meat, poultry, raw milk and salad vegetables, as well as water (Adams and Moss, 1985).

2.5 SOURCES OF TRANSMISSION

Aeromonas species are ubiquitous and heterogeneous group of organisms occurring in both fresh and chlorinated water and cause disease in fish under stress condition or in concert with infection by other pathogens (Stevenson, 1988). *A. hydrophila* are known pathogens of frogs (Austin and Allen-Austin, 1985) but due to association of frogs and fish with water, the organisms can also infect fish regardless of their species (Palumbo *et al.,* 1992). Frogs, channel catfish, Eels, Tilapia and Ayu are all infected by members of *A. hydrophila* group (Lechevallier *et al.*, 1982).

Understanding the disease caused by these organisms in various fishes, amphibians, poultry, reptiles and man is very important because of its pathogenicity (Qadri *et al.,* 1976; Karam *et al.,* 1983 and Palumbo *et al.,* 1992). Affected humans exhibit meningitis, endocarditis, osteomyelitis, wound infections and gastroenteritis (diarrheal) symptoms and is common with children or aged individuals, and the organism can occur as opportunistic pathogen in immunocompromised (HIV) patients (Karam *et al.,* 1983).

All species of fishes including salmon, trout, charr and grayling are susceptible to *Aeromonas* infection (Cipriano and Bullock, 2001). In some cold water fishes such as

rainbow trout, *Aeromonas smithia* and *masoucida* produce atypical forms which is manifested by dermal ulcerations and external pathology with or without subsequent septicemia (Holt *et al.,* 1984; Thompkins *et al.*, 1986).

In salmonids, such as *Salmo salar*, the organism produces stress related diseases, particularly when the temperature of water rises (Groberg *et al.,* 1978; Mills, 1993). In *Tilapia* acute bacterial haemorrahgic septicemia (motile *Aeromonas* septicemia) occurs as a systemic disease causing swelling of the body cavity and haemorrhages in body organs including fins and skin (Stevenson, 1988). The abdomen may be distended and eye balls protruded (Floyd, 2002). In other cases there may be mortalities without external indication. Damage may also occur as a result of injuries caused by external parasites including leech species. There can be fluid accumulation in the abdomen, liver, spleen and intestine. Leeches may carry various *Aeromonas* species from water. These bacteria are symbionts, aiding digestion of blood for the leech and providing nutrient for proliferation of the bacteria. There may be localized infection at sites where parasites such as leech attached. The ability of the leech (*Hirudo medicinalis*) to parasitize the fish, increases infection with the organism (Graf, 2002). In frogs, *A. hydrophila* causes red leg disease. In snakes the organisms may cause septicemia and stomatitis. In other fish species the organism may infect them and may be a secondary invader of virus infected fish. The organism also occur widely in fresh foods of animal origin such as fish, sea foods, raw milk, meats, poultry and fish eggs (Williams and larRock, 1985). Specific diseases caused by *Aeromons salmonicida* includes, furunculosis in salmonids, ulcer disease in gold fish and erythrodermatitis in carp (Holt *et al.*, 1984).

In humans, *Aeromonas hydrophila* group of organism have been described as emergent food borne pathogen of increasing public health importance (Dalsgaard *et al.*, 1998). The organism have a variety of virulent factors which have the ability to adhere the intestinal mucosa and invade through the human epithelia (HEP-2cell) (Paulo,1998). They also possess pili, fimbriae, protein and lipopolysaccharides which provides bacteria adhesion in the buccal epithelia and erythrocytes (Bruckner *et al.,* 1998).

The organisms are also recognized as important pathogens of humans in which they cause various extra-intestinal (meningitis and endocarditis) and intestinal (diarrheal) symptoms (Daily *et al*., 1985; Callister and Agger, 1987). It has been demonstrated that human infection is caused by biotype I of the species (Hazen *et al.,* 1978). In humans *A. hydrophila* group are recognized as opportunistic pathogens in imunocompromised patients and persons with underlying malignancies (Harris *et al*., 1985; Janda and Brenden, 1987; Kralzae and Golembock; 1987; Menge *et al.,* 1987; Sherlock *et al*., 1987). The organisms are frequently isolated from individuals from underdeveloped countries, particularly children (Altwegg and Johl, 1985).

A. hydrophila produces a heat labile enterotoxin and a heat stable cytotoxic enterotoxin (Baron and Fineglod, 1990) and show resistance to vibriostatic compounds (Sugita *et al.,* 1994). The pathogenicity of *A. hydrophila* has caused concern to public health authorities and probably account for significantly more cases of gastroenteritis than is apparent at present. Indeed, a large proportion of cases of gastroenteritis are still been classified with unknown or unidentified aetiology (Gavriel *et al.,* 1998). It also causes diarrhea in susceptible host and in animals (Taylor *et al.,* 1986). It is found in frogs where it produces a condition known as "red leg" disease due to hemorrhagic

erosion found in the skin and between toes and limbs attachments (Merchant and Parker, 1977). The condition is also found in salamanders and fish which may be kept in tanks in laboratories without the circulation of cold running water (Oniye *et al.,* 1998). It has also been isolated from stored fish eggs where it causes a condition characterized by the term "black rot" (Merchant and Parker, 1977). All species of fishes, including salmon, trout, charr and grayling are susceptible to *Aeromonas* infections (Cipriano and Bullock, 2001).

A. hydrophila have been occasionally recovered from ducks and chickens with salphingitis (Bisgaard, 1991); septicemia or air sacculitis (Watts *et al.,* 1993). *Aeromonas formicans* has also been isolated frequently from arthritic lesions in muscovi ducks at processing plants (Bisgaard, 1995).

Some fish species may harbor latent infection of *Aeromonas* species and can transmit the pathogen horizontally by either physical contact or shading of the bacteria into the water column which represents a significant threat for horizontal transfer of the pathogen to naïve fish within fresh water (Ezura *et al.,* 1984).

Transmission may lead to epizootics and is particularly common at marine culture. *Aeromonas* species carrier smolts may be transported into the ponds (Hammel, 1995). Under such conditions, transports often facilitate contagion and the physiological stress encountered with osmo-regulation may enhance disease (Ezura *et al.,* 1984). Transmission within the natural water ways may be further enhanced by the escape of infected individuals from culture facilities or sea farms and the subsequent movement of this fish within the feral environment (Johnson and Jenson 1994). Large aggregation of fish in deep ponds or beneath waterfalls in response to favourable water temperature may facilitate transmission (Johnson and Jensen 1994).

Although some workers may not transmit the organism via gastrointestinal route Klontz *et al*. (1973); McCarthy, (1975), observed that clinical furunculosis in other fishes resulted from the ingestion of infected Atlantic salmon (*Oncorhynchus kisutch*). Miyazaki and Kubota (1975), have also provided histopathological evidence for both perbronchial and percutaneous routes of infection.

The present information suggests that the reservoirs or vectors of infection and transmission of *A. salmonicida* is probably similar to that which has been described for other species of *Aeromonas* (Wiklund and Dalsgaard, 1988). Dermal scarification studies by Tokahashi *et al*., (1975) envisaged that gold ulcer disease was most severe among fishes whose dermis has been severally eroded by either ectoparasites or handling. *Aeromonas salmonicida* has been isolated from sea louse (*Lepeophtheirius salmonis*) and in marine environment from *Argulus corregoni*, in fresh water environment from *Tetrahymena pyriformis* (King and Shotts, 1988; Nese and Enger; 1993). These ectoparsites are capable of inducing severe bronchial or dermal damage. Thus, it may act as vectors in the spread of aeromoniasis.

Horizontal transmission of aeromoniasis has been demonstrated where mollusks have been co-cultured with susceptible hosts (Bjoershol *et al*., 1999; Starliper, 2001). The use of wrasse in maintaining husbandry techniques in a fish pond may act as a novel source of horizontal transmission of *Aeromonas* species. Infected wrasse may act as a further reservoir of contagion for salmon with which they are co-cultured (Collins *et al*., 1991; Laidler *et al*., 1999).

Cohabitation or fish to fish contact is an important source of *Aeromonas* transmission (Enger *et al*., 1992). Aerosol or droplets is also an important means of

transmission in man and poultry (Starliper, 2001). Aerosol transmission of *Aeromonas* species is problematic in culture system which utilize sprays bars within race ways or acquaria in close proximity to one another (Enger *et al.*, 1992). Also fish fed with poultry manure can also get infection.

Presence of *Aeromonas* species in ovaries and testes of infected fish may vertically transmit *Aeromonas* species to fingerlings (Bullock and Stucky, 1987).

2.6 OCCURRENCE AND EPIDEMIOLOGY OF AEROMONIASIS

The number, diversity and distribution of fish species that are susceptible to *Aeromonas* species enhances this bacterium's distribution worldwide (Cipriano and Bullock, 2001). However, both typical and atypical subspecies may not be common to all geographic areas (Hammel, 1995).

The epidemiology of aeromoniasis has been ascertained since the past thirty years and particularly during the past two decades. The infection is reported to be widespread among cattle, swine, dogs, fish, amphibians, horses, several avian species, wild, zoo and laboratory animals (Carter *et al.*, 1995). Its detection in USA in 1936 was followed by reports from Australia in 1948 and Sweden, 1961. The study on the occurrence of *A. hydrophila* in fishes has been reported from most countries of the world including India, Saudi Arabia, Australia and New Zealand (Bhat *et al.*, 1974; Hammel, 1995; Ibrahim *et al.*, 1996).

Incidence of the disease in a specific geographic area may be as a result of introduction of a typical variant of virulent strains of *Aeromonas* (Humphrey and Ashburner, 1993). Similarly, *A. salmonicida* was reported from salmonids in Croatia,

Canada and New Zealand (Humphrey and Ashburner, 1993; Hammel, 1995). The organism spread to other parts of Brazil and Australia (Hammel, 1995). The incidence of the disease is highest between October and February period in fish, but clinical observations, together with the results of agglutination tests and the isolation of *Aeromonas* from fish indicates that the infection is far more extensive than suspected and, furthermore, is a very important cause of furunculosis in fish (Stevenson, 1988).

A. hydrophila is a cosmopolitan organism of global distribution occurring in both fresh, sewage and chlorinated water (Krieg and Holt, 1984). The organism is known to occur in Europe, America (Carnahan *et al*., 1989) and most developing countries especially Africa (Ellis, 1988). A study in Cote d'Ivoire where peri-urban and rural fish farming is gaining ground shows that the organism exists in such areas (Spore, 2000). High levels of *Aeromonas* are also present in fish ponds cultured in Sweden and in fish ponds in Hungary and India (Olah, 1986). *Aeromonas* species has also been reported in fish cultured ponds in Kenya and Tanzania (Olah, 1986).

In the Philippines and Thailand, outbreak appear most severe as a result of stress, when water temperature as well as nutritional status of fish decline, *A. hydrophila* becomes an opportunistic infection of public health consideration. The spectrum of pathogenicity in this case is mild asymptomatic. When temperature and nutritional status increases, the spectrum of pathogenicity rises to severe disease. Hence, low level of infection leads to asymptomatic infection while high level infection may lead to severe infection (Thune *et al*., 1982). In Costa Rica and in Democratic Republic of the Congo, the organisms has been isolated from fresh vegetables at various times of the year

(Vandepitte *et al*., 1974; Oniye *et al*., 1998). Ibrahim *et al*., (1996) also isolated *Aeromonas* species from human diarrheal stools in Saudi Arabia.

In the United States of America, Russia and Middle-East countries such as Iraq and Kuwait, *Aeromonas* disease causes significant loss in their poultry industry. The infection is also wide spread and of major public health significance in the poultry industry (Calnek *et al.,* 1997). All avian species including wild and domestic birds are susceptible to infection (Shane and Gifford, 1985; Glunder and Siegmann, 1989).

In the United Kingdom (Bisgaard, 1991); ducks and other water birds are known to be reservoirs of this organisms and their association with domestic or commercial poultry could lead to infection. Also the association of this birds with contaminated water through the act of swimming in ponds or dams or drinking the water were paramount sources of infection especially in the United Kingdom (Bisgaard; 1995). In all species of avians, morbidity ranges between 20-100 %.

Little is known about the economic impact of this disease in the developing countries, but the cost are likely to be significant. Results of study conducted in Africa (Cote d'Ivoire) and Asia (Bangladesh, China, India, Thailand) and Central America (Mexico) suggests that *Aeromonas* is a significant cause of diarrhea in children (WHO, 1994). Epidemiological studies suggests that aeromoniasis occurs as a result of repeated exposure and *A. hydrophila* being an opportunistic infection of stressed fish or a secondary invader of parasite injuries. Evidence suggested that protection is immunologically mediated and indicates that vaccine development is a possibility (Stevenson, 1988).

2.7 VIRULENCE FACTORS

Virulence factors of *Aeromonas* organisms are associated with structural components of the bacteria cell and with exotoxins that are secreted during bacteria metabolism (Olivier, 1992; Dean *et al*., 1998). The nature of virulence in *Aeromonas* species is complex and apparently varied between strains. Some of the virulent factors produced by *Aeromonas* species include the following:

2.7.1 A layer Protein

Uddey (1978) showed that virulent *A. salmonicida* possessed an additional layer (A-layer) associated with the external surface of the membrane of the cell wall. Evenberg *et al.* (1988) determined that the A-layer consisted of major Additional Cell Envelopes (ACE) protein that was immunologically similar among virulent isolates. The protein is water insoluble, hydrophobic and similar to the K 88 adhesive fimbriae of enteropathogenic *E.coli*. The protein was described to have a moleculer weight of 49 kilodaltons (KDa), which was expressed in a regularly separating tetragonal array beyond the bacteria cell wall (Kay and Trust, 1991), and was present within typical and atypical strain of the pathogen (Hamilton *et al*., 1981). Kay and Trust (1991) shows that the tetragonal array is tethered to the cell surface via the O-antigen side chain of the bacteria lipopolysaccharide (LPS). A-layer protein array apparently blocks bacteriophage receptors on the outer membrane of *A. salmonicida* to present chemically refractile and somewhat impermanent barrier that buffers the underlining membrane and its associated proteins from chemical modification involved with host defense including elements of

both the immune response and cytotoxic effects of the complements pathway. (Trust *et al.*, 1981; Kay and Trust, 1991).

2.7.2 Glycerophospholipid Cholestrol Acyltranferase (GCAT)

GCAT is a protein produced by *Aeromonas* species and is hemolytic for fish erythrocytes; cytolytic, leucocytolytic, cytotoxic and lethal for atlantic salmon when injected at a concentration of 0.045 µg protein per gram of body weight (Paterson and Fryer, 1974).

2.7.3 Bacteria Proteases

The *Aeromonas* species produce severe leucopenia when observed histo-pathologically in lesion development (Klontz *et al.*, 1973). Proteases were also extracted from the extra cellular materials (Shieh and Maclean, 1975) that induced the development of furuncle like lesions in fish (Sakai, 1986), and yet others expressed cytotoxic activity to rainbow trout (*Oncorynchus mykiss*) and gonad cell in tissue culture were hemolytic for trout erythrocytes and lethal in brook trout (*Salvelinus frontinalis*) regardless of the virulence of the strain from which they were extracted (Sakai, 1986).

2.7.4 Seridophore Production and Iron Regulation

Most typical and atypical isolates of *A. salmonicida* can grow under condition of iron restriction as evidenced *in vitro* by multiplication in the presence of iron chelators. Seridophore production was detected only among typical strains and it behaved as a 2,3 – diphenol catechol (Hirsh *et al.*, 1990).

2.7.5 Hemolysin

Hemolysin is one of the virulent factors produced by motile aeromonads and has been cloned from different strains of *Aeromonas* (Hirono *et al*., 1972; Wong *et al*., 1998). *A. hydrophila* secretes at least two types of hemolysin. One of them is aerolysin (Aero A) which can oligomerise on erythrocyte cell membrane, form channels and lead to cell lysis of the RBC (Wicklund *et al*., 1998).

The second is a non channel forming hemolysin (Hly A) and is proposed to be a *Vibrio cholerae* – Hly A like hemolysin (Wong *et al*., 1998). These two types of hemolytic toxins are low in hemology and believed to be distinct. They contribute to virulence in *A. hydrophila* and a widespread within virulent strains of motile aeromonads (Hirono *et al.*, 1972; Wong *et al*., 1998).

2.7.6 Histone – Like – Protein (F 52)

These are small basic heat – stable molecules and binds to signal and double stranded DNA (Drlica and Rouviere-Yaniv, 1987). They are known to alter DNA recognition by changing DNA dynamic flexibility and accessibility (Flashner and Gralla, 1988). The histone – like – protein has psychological functions and have been demonstrated to have a potential role in the pathogenesis of *Streptococcus* - induced tissue inflammation (Buckner *et al.,* 1978).

2.8 *AEROMONAS* INFECTION IN NIGERIA

In Nigeria there is increasing awareness of the organism with its economic and public health significance. Some studies in Nigeria have incriminated *Aeromonas* as a

fish pathogen. Okafor and Nzeoko (1985) isolated the organism from fish (*Clarias gariepinus*) from Ogun river and have reported *A. salmonicida* to be pathogenic for fish.

Okpokwasili and Alipiki (1990) isolated *A. hydrophila* with other associated flora from fresh water fish (*Oreochromis niloticus*) in Port Harcourt. Okpokwasili and Okpokwasili (1993) incriminated aeromoniasis in cultured fish to be associated with water as well as animal and poultry waste. This validates the findings of Healing and Greenland (1991) who did similar work on aeromoniasis in England and found that the spread of this organism was via faecal contamination. Okpokwasili and Ogbulie (1993) reported the presence of different *Aeromonas* specie in different fishes in Rivers State. Okpokwasili and Ogbulie (1999) evaluated the histological and hematological responses of fish to different *Aeromonas* species. Okpokwasili and Ogbulie (2001) established that *Aeromonas* species were associated "Brown Patch" syndrome in *Tilapia* and ascertained the fact that the syndrome was higher in the dry season than in raining season.

Ogbondeminu and Okaeme (1986) found that *Aeromonas* infection is associated with extensive accumulation of organic manure in Kainji Lake, New Bussa, Niger State. *Aeromonas hydrophila* is known to compete with nitrosomonas to reduce nitrate in organic matter aerobically by assimilatory reduction via nitrate to ammonium (Pelczer and Chan, 1979; Horsely *et al*., 1982). Other species like *A. salmonicida* were attributed to cause ulclerative syndrome in *Tilapia* (Okpokwasili and Eleke, 1996).

In Shagamu, Ogun State, Ayeni (1998) reported the isolation of *A. hydrophila* in some cultured fish. Ocholi (1991) characterized *Aeromonas* species isolated from various species of wild birds in Vom and its surrounding environments.

In Zaria Kaduna State, *Aeromonas* species were isolated from the GIT, gills and skins of various fishes, from both fresh, sewage, chlorinated and non chlorinated water (Fasanya *et al.,* 1981; Kwaga *et al.,*1988; Aminu *et al.,* 1998; Yakubu *et al.,* 2005). The extent and level of infection and ubiquitous nature of *Aeromonas* species was assessed by Godwin (1999) who isolated the organism from smoked dried fish, fish trucks and fish cold rooms in Zaria market. This establishes the fact that humans handling fish and fish products without proper disinfection may contact infection.

Abbey and Etang (1988) conducted studies on incidents and biotyping of *Aeromonas* species on snails and water samples in Port Harcourt, rivers state found out that these samples were likely reservoirs and therefore constitute high sources of human infection. Low incidence rates were reported from poultry, cattle and humans, thus suggesting that these may be transient hosts (Yakubu *et al*., 2005). Reports on human cases of aeromoniasis in Nigeria is gaining ground. *A. hydrophila* has been isolated from children with diarrheal in Zaria, PortHarcourt and Lagos (Abbey and Etang, 1988; Olayinka, 2004).

2.9 PREDISPOSING FACTORS TO INFECTION

A. hydrophila may not cause disease in healthy fish, but can act as opportunistic pathogen in those fish that have been exposed to infection as a result of stress (Esch and Hazen, 1980). Stressful environment may result from chemical pollution as well as some environmental factor (Heat, ectoparasitic) (Pettibone *et al.,* 1996). Pollution level may influence the genetic make up of resident bacteria and higher incidence of antibiotic resistance have been reported from polluted aquatic environment than from less polluted

sites (McNicol *et al.,* 1980). Pollution level may also influence plasmid content and greater bacteria from sites polluted by toxic chemicals have been reported to have a higher incidence for plasmids mediated resistance to antimicrobial agents than those obtained from cleaner sites (Baya *et al*., 1986). Several years of accumulation of organic and inorganic pollutants concentrated in the sediments are potentially harmful to resident fish population. Fish infected by resistant strains of *A. hydrophila* may serve as a reservoir and aid in their dissemination (Baron and Finegold, 1990).

A variety of pathogenic *Aeromonas* have been found in water (Sharon *et al*., 1998). Humans and other animals may contact infection via drinking and inhalation or by exposure to skin or mucus membrane (Sharon *et al.,* 1998). In adults, predisposing risk factors include antibiotic treatment and any underlining enteric condition such as gastric and colonic surgery (Auckland, 2003).

Outbreak of disease are usually associated with change in environmental conditions, including overcrowding, high temperature, a sudden change of temperature, rough handling, transfer of fish, low dissolved oxygen, poor nutritional status and parasitic infection contributes to physiological change and hightened susceptibility to infection (Hayes, 2000).

Water birds, ducks and geese that come in contact with water and are exposed through swimming and drinking will serve as a reservoir hosts which may transmit the disease to other poultry species and susceptible host. Feeding poultry with fish as component of their diet may be a source of transmission to healthy birds (Calnek *et al.*, 1997). Understanding the farm ecology may help us to gain insight into problem of the disease, that is, from feeding troughs, drinkers and litters.

2.10 AEROMONIASIS IN HUMANS

The bacteria belonging to *Aeromonas* species presents three types of infection as described by Auckland (2003). Systemic infection occurs usually in people who are immunocompromised; wound infection and diarrhea (Auckland, 2003). Many food borne outbreaks have occurred overseas involving fish, oysters, prawns, soup and egg salad. It is believed that an enterotoxin is produced which is responsible for symptom or clinical presentation (watery diarrhea) of *Aeromonas* associated gastroenteritis (Sharon *et al*., 1998).

Gastroenteritis associated with *Aeromonas* is the second or third most common gastroenteritis in children below 5 years, but can occur in any age group (Auckland, 2003). The incubation period is usually about 1 to 2 days after exposure. It eventually presents a self limiting illness of 1 to 7 days although diarrhea may persist in up to one third of the patient for a period of two weeks to several months, (Cumberbatch *et al*., 1979). The most common presentation of symptoms is a mild fever associated with typical watery diarrhea (Kingombe, 2002). The infective dose required to cause gastroentenitis are levels of 10^7 to 10^9 CFU (Sharon *et al.*, 1998). In children, under two years of age, vomiting may also occur. There may be a presentation of blood mucus and white blood cell in focal samples (Sharon *et al.,* 1998).

2.11 PATHOGENISIS IN MAN

When the organism is incidentally ingested during swimming or water contact, it produces toxins (Baron and Finegold, 1990). These toxins could be ß-hemolysin or aerolysin (cytotoxic), extracellular heat labile cytotonic aenterotoxins, non-haemolytic

toxins, heamaglutinins, proteases and elastases (Plum, 1984; Burke *et al.,* 1987; Chakraborty *et al.,* 1987; Ljung, 1982 and Trust *et al.,* 1987).

The motility of the GIT and the ability of the organism to adhere to the intestinal epithelium are important factors in the pathogenicity (Baron and Finegold, 1990). The action of the toxins is to activate adenylate cyclase of the intestinal mucosal cells, which inturns raises the intracellular level of cyclic adenosine monophosphate (cAMP), ultimately causing active secretion of electrolytes into the intestinal lumen by altering ion transport within the cell membrane of the gastrointestinal epithelial cells. The outpouring of ions is followed by release of water (Baron and Finegold, 1990). If diarrhea persists, death may occur between twelve hours to four days (Baron and Finegold, 1990).

The organism is believed to enter the blood stream via the gastrointestinal tract. It therefore invades the lymphatic system (Bibberstien and Zee, 1990). The invasive potential is mediated by proteases especially in patients with underlying hepatic immunocompromising disease (Baron and Finegold, 1990).

The disease may manifest itself either as direct damage to host structures and functions by enterotoxins or damage due to host reactions such as those triggered by hosts cells, which may stimulate particularly in macrophages, the secretion of mediator substances such as interleukin – 1 which elicits manifestations such as enterotoxaemia, meningitis and endocarditis (Trust *et al.,* 1987).

Gastroenteritis associated with *Aeromonas* is most often reported in children but can occur in any age group. *Aeromonas* species are associated with the diarrhea disease which produces symptoms ranging from a mild diarrhea to a febrile dysentery- like illness. The most common is a mild self limiting watery diarrhea of short duration, the

frequency of other clinical features including bloody or mucoid stools, abdominal cramps, fever and vomiting varies considerably (Khalifa *et al.,* 2001).

Incubation period is about 1-2 days after exposure (Auckland, 2003). It usually presents a self limiting illness of 1-7 days and symptoms may persist in up to one third of patient for periods from 2 weeks to several months. The most common symptom is a mild fever associated with typical watery diarrhea (Auckland, 2003). In children under 2 years of age, vomiting is predominant. Less common is a presentation of blood, mucus and white blood cells may be found in fecal samples. Auckland (2003) suggested that *Aeromonas* is the second or third most common cause of gastroenteritis in children during which fed infants appear to have an increased risk of infection with *Aeromonas caviae* in the first three years. Predisposing risk factor include antibiotic treatment and an underlying enteric condition such as gastrointestinal surgery (Auckland, 2003). The infective dose required to cause gastroenteritis according to Auckland (2003) are levels of 10^7 to 10^9 CFU.

2.12 VIRULENCE MARKERS OF *AEROMONAS* SPECIES

The genus *Aeromonas* includes 14 species (Figueras *et al.,* 2000; Pidiyar *et al*., 2002), *A. veronii* var *sobria*, *A. caviae* and *A. hydrophila* being the most prevalent in clinical samples, although other species such as *A. Jandaei* are also isolated (Carnahan *et al*., 1991; Joseph *et al*., 1991; Janda and Abbott, 1998; Gonzalez *et al*., 2001; Carson *et al*., 2001; Valera and Esteve, 2002; and Sarma, 2002).

Genetic studies on *A. jandaei* showed that the species caused septicemia and intestinal diseases in humans (Janda and Abbott, 1998). The organism is also known to

show resistance to antimicrobial agents other than clinical species such as *A. schubertii*, *A. trota* and *A. veronii* biotype *veronii* (Rose *et al*., 1989; Janda *et al*., 1991; Ferguson *et al*., 1997).

Several virulence factors have been studied in *Aeromonas* including Aerolysin, hemolysin, enterotoxins, proteases, lipase and deoxyribonucleases (Chopra *et al*., 2000; Janda, 2001; Chacon *et al*., 2003). Studies by Albert *et al*., (2000) has revealed a significant statistical association between two genes, the *ast* gene that codifies for a heat stable enterotoxin (Chopra *et al*., 2000), in strains isolated from patients with chronic gastroenteritis (Chopra *et al*., 1993) and the A*lt* gene that codifies for a heat labile enterotoxin (Chopra *et al*., 2000; Sha *et al*., 2002), in strains isolated from patients with diarrhea. It was postulated that both genes act synergistically to induce severe diarrhea. Moreover, double deletion mutants for *ast* and *alt* genes lead to important fluid secretion reduction in a mouse model (Sha *et al*., 2002).

The incidence of virulence genes in *A. jandaei* have been only rarely investigated (Kingombe *et al*., 1999; Albert *et al*., 2000; Kirov *et al*., 2002; Chacon *et al*., 2003). On investigation of the virulence genes by Soler *et al*., (2005) reveals the presence of aerolysin, hemolysin *act* and *laf* A genes, using amplification of the Enterobacterial Repetivtive Intergenic Cencus (ERIC), a good typing technique for *Aeromonas* (Soler *et al*., 2003).

In order to avoid considering duplicate isolates when studying virulence gene distribution, the isolates should be typed by the ERIC-PCR method, which was proved to be highly discriminatory in other *Aeromonas* species (Soler *et al*., 2003).

Detection of virulence gene could be through primer design and PCR amplification (Soler *et al*., 2005). In this reaction, sequences deposited in the Gen Bank and in the European Molecular Biology Laboratory (EMBL) under the gene denomination A*st* (Sha *et al*., 2002). Lag (A1 and A2) Kirov *et al*., 2002); Gavin *et al*., 2002) and aid (Chopra *et al*., 1993) were used for primer design using primer design 3 software (Scientific Educational Software Durham, USA). The selected primer were then computer aligned with the sequences in the Gen Bank and EMBL databases by "BLAST" analysis to ensure their specificity for the mentioned *Aeromonas* genes (Chopra *et al.*, 2000).

The list of primer used included *Laf* A-F and *Laf* A-R which bind to the genes *Laf* A, *Laf* A_1 and *Laf* A_2 and amplified similar fragments for the consensus region of those genes (Dorsch *et al*., 1994; Soler *et al*., 2002; Chacon *et al*., 2003). PRC amplifications for the remaining genes were performed in a final volume of 50 µl containing 3 mg of DNA, 0.3 mm deoxynucleotide mix, IX PRC buffer (Gibco-BRL, Barcelona, Spain), $Mgcl_{2,}$ 0.3 µm of primer and 2.5 units of taq polymerase (Gibco-BRL) PCRS were carried out in a Perkin-Elmer Gene Amp 2400 PCR system.

Dot blot experiments were performed for the detection of *ast, Laf A* and aerolysin or hemolysin genes. *Aeromonas hydrophila* also secretes extra cellular proteins which are Aerolysin, Glycerolphospholipid Cholesterol Acyltransferase (GCAT), serine protease and hemolysin (Albert *et al*., 2000).

2.13 PHYLOGENETIC RELATIONSHIP OF *AEROMONAS* SPECIES

Soler *et al*. (2005) identified unrooted phylogenetic trees for *gyr* B *rpo* D gene sequences. They were constructed from derived genetic matrices. All obtained phylogenies showed considerable divergence (branch lengths) between all *Aeromonas* species under study. Strain grouping is consistent in all three trees, and is also in agreement with a preliminary phylogenetic study based on *gyr* B (Yanez *et al*., 2003).

Some differences in topology at the deepest branching points of single *gyr* B and *rpo* D trees are due to the differences in the interspecies nucleotide substitution rates found between *gyr* B and *rpo* D sequences (Soler *et al*., 2005). Since *rpo* D yielded a better resolution than *gyr* B, it helped in spilling *A. salmonicida* from *A bestriarum* and the opposite was found for other closely related species (*A. encheleia, A. veronii* and *A. culicicola*) the phylogeny of the genes *Aeromonas* was expected to be improved by constructing the *gyr* B-*rpo* D tree, as it comprises the combined capacities of both molecular blocks. It has been known to improve reliability of phylogenies (Stackebrandt *et al*., 2002).

rpo D analysis to the systematics of the genus *Aeromonas* has increased the advantages previously found on the basis of *gyr* B sequences (Yanez *et al*., 2003), compared to referenced 16 S rDNA phylogeny (Martinez-Murcia *et al*., 1992; Martinez-Murcia *et al.*, 1999). Pairs of species like *A. trota, A caviae, A. hydrophila, A. media, A culicicola* and *A. jandaei* show 1,3 and 1 nucleotide difference respectively in their 16 S rDNAs, are clearly separated by *gyr* B sequences (7.3 %, 6.5 % and 6.3 % respectively and even more *rpo* D, (Soler, 2005).

2.14 BACTERIAL GENOMICS OF *AEROMONAS* SPECIES

Genomics is the main focus of modern biotechnology. This comparative study and analysis of multiple genomes provides substantially more information on the physiology and evolution of different organisms and expands our ability to better assign putative functions to predicted coding sequences (Danchin and Yuen, 1993; Wang and Leung, 2000).

Proteomics on the other hand is the application of high technology towards the characterization of the proteins in a given organism (Tan *et al*., 1998). Proteomics can have global, integrated view of disease processes; cellular processes and networks at the protein level (Zhang *et al*., 2002). Analysis of virulence genes or proteins using these two approaches will be invaluable for the development of urgently needed new vaccines and drugs to fight against infectious diseases (Tan, 1998).

Current research by Danchin and Yuen (1999) on the genomics and proteomics of bacterial pathogens such as *Aeromonas hydrophila, Edwarsiella tarda* and *Vibrio* species enable identification of their virulence phenotypes and characterization of their genes and proteins to enhance analyzing their genomes. The genes have been identified by subtraction hybridization and transposons tagging (Ljungh *et al.,* 1987; Janda, 2001). The construction of proteome maps followed by mass spectrometric determination of the protein sequence and structure can present all virulence proteins in an integrated manner (Joseph and Carnahan, 2000; Wang and Leung, 2000).

Importance of genomics and proteomics lies in their usefulness in gene mining and drug discovery, hence, spinning up the development of DNA and peptide chips for rapid and sensitive diagnostic kits, vaccine development, food safety and quality control,

and screening of new drugs against fish or poultry and human diseases (Tan *et al*., 1998). Genomics is also useful in the understanding of evolution and microbial phylogenetics (Nelson *et al*., 2001). Genomics helps the growing biotechnology industry globally and is also in line with its national strategy to promote value added industries (Fricker and Thompsett, 1989; Wang and Leung, 2000).

2.15 MOLECULAR AND GENETIC ANALYSIS OF *AEROMONAS* SPECIES

Researchers studying *Aeromonas* species have identified a large array of virulent associated factors, including proteasis, hemolysins, lipases, adhesions, agglutinins, pili, enterotoxins, various enzymes and outer membrane arrays (Joseph and Carnahan, 2002; Soler *et al.,* 2002).

Filamentous structures on aeromonads include several different types of pili, incuding those that are short and rigid and those that are long and wavy (Joseph and Carnahan, 2000). On agar medium, the organism has lateral flagella in addition to the polar flagellum (Joseph and Carnahan, 2000). Researchers are beginning to identify the genetic determinants of these structures. For example the type IV pilus gene cluster (Tap A, – B – C – D) for *Aeromonas* encodes a 17 – kda pilus (Carnahan, 2000).

Other genes involved in type II secretion pathway are grouped into two operons, exe AB and exe C – N, which form an outer membrane secretion port (Millership, 1996). Molecular apparatus, phosphate bonds hydrolysis and proton motif force are required for molecules to be secreted through this port. The gene products exe A and exe B form an inner membrane complex that apparently plays a role in energy – dependent gating of the port (Joseph and Carnahan, 2000). Some *Aeromonas* species possess type iv pili, with

molecular mass of 19 – 23 kDa, occur singly or in bundles and are closer to type IVA than type IVB with close homology to Mannose Sensitive Hemagglutination Antigen (MSHA) (Joseph and Carnahan, 2000; Labeelund and Sorum, 2000).

Laf genes are present in 35 % *Aeromonas* isolates and lateral flagella is expressed when grown on solid medium 8 hours at 37^0 C (Joseph and Carnahan, 2000).

Genetic analysis provides a more stable basis for microbiological investigation than phenotypic method (Soule *et al.,* 2005). Several molecular techniques including finger-printing by randomly amplified DNA polymorphism (RAPD – PCR), Amplified Fragment Length Polymorphism (AFLP), Pulsed Field Gel Electrophoresis (PFGE), Restriction Enzyme Fragmentation Patterns (REFP), plasmid profiling and ribotyping have been used to study different strains of *Aeromonas* species (Miyata *et al.*, 1995; Inglis *et al.*, 1996; Lund *et al.,* 2003 and Soule *et al.*, 2005).

The results generally support phenotypical evidence that typical strains are generally homogeneous and may be clonal, and that atypical isolates are heterogeneous, there is little congruence between the techniques in establishing relationships between subspecies, strains and isolates (Austin *et al.*, 1998).

The use of genome arrays containing whole genomes or large sets of genes, either as a result of high – coverage genome sequencing or selected after suppressive subtractive hybridization have been used to study genome variability among strains of bacterial pathogens including *Aeromonas* species (Ong *et al.,* 2004). To examine the diversity of virulence factors among clinical isolates of *A.salmonicida* and other aeromonads, a DNA microarray of 2024 selected genes from *A. salmonicida* strain A 449

will be constructed, A449 is a wild typical virulent isolate which has been sequenced and is currently undergoing annotation (Taboada *et al.*, 1998).

Table 2.1 Current Genospecies and Phenospecies Within the Genus *Aeromonas*

DNA Hybridization	Type of strain or HG Strain	Genomospecies	Phenospecies	Remarks
1	ATCC7966^T	*A. hydrophila*	*A. hydrophila*	Isolated from clinical specimens
2	ATCC 14715^T	*A. bestrarum*	*A. hydrophila* like	Isolated from clinical specimens
3	ATCC 3359^T	*A. salmonicida*	*A. salmonicida* Subsp. achromogenes	Nil
3	ATCC727013^T	*A. salmonicida*	*A. salmonicida* subsp. *masaucida*[a]	Nil
4	ATCC15468^T	*A. caviae*	*A. caviae*	Isolated from clinical specimens
6	ATCC 23309^T	*A. eucrenophila*[c]	*A. eucrenophila*[c]	Nil
8Y	ATCC 9071	*A. veronii*	*A. veronii* biovar *sobria*	Isolated from clinical specimens

Source: Borrel *et al.* (1998); Joseph and Carnahan (2000).

2.16 DIAGNOSIS OF *AEROMONAS* INFECTIONS IN FISH, HUMANS AND POULTRY

Aeromoniasis is a zoonotic disease of public health and economic importance. The cosmopolitan nature of the agent warrants diagnosis of its infection. The diagnosis of this infection can be therefore based on history, clinical signs, serology, bacterial isolation, biochemical characterization and rapid molecular characterization.

2.16.1 History

The cosmopolitan nature of the organism in different aquatic environments is suggestive of infection (Palumbo *et al.*, 1989). History of exposure to previous contaminated water through drinking, swimming, fishing and other recreational activities may be a basis for diagnosis (Santose *et al.*, 1988). In poultry, history of incorporation of fish meal into poultry diet or feeding birds with contaminated water may be a basis for diagnosis. In fish, history of prevalence of aeromoniasis amongst frogs, shrimps and other aquatic animals may be suggestive of evidence of transmission of aeromoniasis to susceptible fishes, poultry or humans (Palumbo *et al.*, 1989).

2.16.2 Clinical Signs and Pathological Lesions

(i) Fish

Presumptive diagnosis of a typical variant of aeromoniasis from clinically diseased or moribund fish is based on clinical indications of disease (Esch and Hazen, 1980; Cipriano and Bullock, 2001). Infection is evident in fish ponds which are characterized by improper handling, transportation problems, overcrowding, poor growth

and debilitation (Esuruoso, 1999). The clinical signs are similar to those given by Groberg *et al.* (1978) and Stevenson (1988).

In fish, the pathognomonic signs of motile aeromonad septicemia are seen as ulcerative skin lesions, which may be on surface of organs or deep within the tissues (Aoki and Hirono, 1991). There is necrosis of fins or tail (fin rot), ulcers on flanks or dorsal area leaving a bright heamorrhagic surface. As ulcer necrotizes, it goes brown and may be secondarily invaded by a fungus, *Saprolegnia diclina* (Thorpe and Roberts, 1972). Hemorrhagic septicemia is often characterized by the presence of small surface lesions, often leading to sloughing off of the scales, hemorrhaging in the gills and anus (Hayes, 2000). In European carp culture, there may be signs of ulcers, abscesses and exophthalmia (bulging eyes), and abdominal swelling (dropsy) (Miyazaki and Kaige, 1985). There is inapetance, swimming abnormalities, pale gills with bloating (Rollins, 1997).

Gross pathological lesions in fish are seen as presence of ascitic fluid in the peritoneal cavity, anaemia, and swelling of the kidney and liver (Miyazaki and Kaige, 1985). As in *A. hydrophila* infections, there may be redness of the fin which is characterized by the presence of surface haemorrhages and scale erosion (Miyazaki and Kaige, 1985). There may be hyperemia with visceral haemorrhage over the mesenteries and within visceral and parietal peritoneum. There is liquefactive necrosis of the kidneys (Aoki and Hirono, 1991). There may be haemorrhagic mucus–desquamative catarrah. Toxic metabolites are absorbed from the intestine leading to capillary haemorrhage in the dermis, fins, trunk and submucosa of stomach. There is degeneration of hepatic cells and renal tubules and glomeruli resulting in exudates and fibrin (Miyazaki and Johl, 1985).

Histologically, in fish, there may be generalized hyperemic hemorrhagic capillary beds, with a high level of macrophages and other leucocytes migrating from them (Bach *et al.,* 1978). There may be large amount of melanin and lipofucsin from ruptured haemophoietic melanomarcrophage centres (Roberts, 1989).

(ii) Poultry

Clinical signs of aeromoniasis in poultry include; salphingitis, infertility, septicemia, air-sacculits, respiratory distress, influenza like symptoms of coughs, sneezing, restlessness and arthritic lesions. Morbidity ranges between 20 – 100 % (Bisgaard, 1995).

Gross pathological lesions is seen as swelling of the base of the phallus, ulceration and eventual scaring of the mucosa often making reproduction impossible (Calnek *et al*., 1997; Hinton *et al*., 2003). There is necrosis of the beak (Cheng *et al*., 1976). Intercellular infection is characterized by desquamation of endothelial cells (Julian and Galt, 1980). There may be obliteration of the air capillaries dominated by intercellular organisms. Interlobular septa are distended and contains inflammatory cells which are clearly detected by Periodic Acid Schiff (PAS) and Heamatoxylin and Eosin (H & E) stains (Calnek *et al*., 1997). Granulomatous lesions are seen in the liver and osteomyelitis in the hock joint are common in turkeys and are often detected during processing. This may lead to excessive condemnation of such part during meat inspection (Julian and Galt, 1980).

(iii) Man

In humans, bacteremia is the most common pathogenic manifestation of aeromoniasis (Hayes, 2000). Mild symptoms include fever and chills, but patients who become septic with *Aeromonas* infection often exhibit abdominal pain, nausea, vomiting and diarrhea (Hayes, 2000).

The gastroenteritis in man, characterized by diarrhea, especially in children is noticed by acute, profuse watery diarrhea, fever and vomiting to a dysentry-like illness with cramps and abdominal pain, or a mild, chronic diarrhea of more than 10 days duration and septicemia (Altwegg, 1994).

Extra-intestinal infections due to *Aeromonas* species is characterized by wound infections, typically occurring after trauma and water contact. Septicemia is usually associated with immunocompromised patients (Altwegg, 1994). Wound infections can have fatal or serious debilitating outcomes, such as amputations. *Aeromonas* wounds fall into three categories in order of the increasing severity of damage caused; cellulitis, myonecrosis and ecthyma gangrenosum (Hayes, 2000).

Cellulitis is an acute inflammation of subcutaneous tissue characterized by redness and induration that may arise from injury or secondary sepsis (Musher, 1980). Myonecrosis and ecthyma gangrenosum are less commonly seen types of *Aeromonas* infections and are characterized by bullous lesions, liquefaction of muscles with blackening of the tissue which may be gangrenous with gas formation (Hayes, 2000). Ecthyma grangrenosum is a cutaneous necrotic or gangrenous pustule that occur secondary to sepsis. Lesions have an erythematous border surrounding a vesicle which

can progress to necrosis of the soft tissue within 24 hours. The infection is usually fatal (Musher, 1980).

2.16.3 Bacterial Isolation

Cultures can be made from GIT, affected organs or lesions. The characteristic growth of the *A. hydrophila* or other *Aeromonas* species on MacConkey blood, nutrient Xylose Lysine Desoxycholate (XLD) and Xylose Citrate Desoxycholate (XCD) agar are evaluated similar to those given by Cowan and Steel, (1974); Krieg and Holt (1984); Collier and Parker (1987) and Kwaga *et al*. (1988). These characteristics are diagnostic of infection.

2.16.4 Biochemical Method

Biochemical tests include oxidase tests, catalase tests, coccobacillary growth in pairs in solid or in liquid media, fermentation of sugars, aesculin hydrolysis, gelatin liquefaction, indole production, TSI, citrate utilization, hydrogen sulphide production and motility tests (Cowan and Steel, 1974, Kreig and Holt, 1984) are sufficient for the complete identification of the organism.

2.16.5 Serology

Serological tests such as Slide Agglutination Tests (SAT) and Tube Agglutination Tests (TAT) can be carried out to determine antibody titres in man, poultry and fish (Ellis, 1988). H and O antigens are used for immunization using whole live or killed

bacterins. Antisera are produced from precipitins of sheep labile extra cellular proteases (Thune *et al*., 1982).

Apart from cultural and serological methods, a number of other related techniques have been reported for detection of *Aeromonas* species from different sources (Kingombe *et al*., 2002). These methods include fluorescent antibody microcolony technique, shortened liquid enrichment, enrichment serology, conductance, radiometry, immunosensors, bacteriophages, gene probes, and separation and concentration techniques (Kingombe *et al*., 2002).

2.16.6 Rapid Molecular Diagnosis

One of the recent rapid techniques used in the detection of *Aeromonas hydrophila* includes the Enzyme Linked Immunosorbent Assay (ELISA) (Chopra and Houston, 2000). Another rapid, sensitive and specific technique for detecting *Aeromonas* and other fish pathogens is the Polymerase Chain Reaction (PCR) and multiplex PCR (Kingombe, 2002). In this reaction, probes are designed as DNA primer pairs for use. The primers targets the 16 S ribosomal RNA genes, a conserved region of the bacterial genome that contains information unique to each specie of bacterium (Kingombe, 2002). In addition, a system based on an internal gene probe was developed to verify the accuracy of the DNA sequences amplified by PCR in respect to the bacterial fish pathogens (Chopra and Houston, 2000). The advantage of the PCR is that it is used as a tool for the early detection of bacterial pathogens in farmed and wild fish, poultry and man (Kingombe *et al.*, 2002).

2.16.7 Differential Diagnosis

In fish, the disease can be confused with bacterial septicemia and salmonellosis (Ellis, 1988). In man, the disease may be confused with amoebiosis, colibacillosis, meningitis, rotavirus infection in infants and children, campylobacteriosis, giardiasis, *Clostridium difficile* infection, cytomegalovirus infection, shigellosis and salmonellosis (Collier and Parker, 1987; Ellis, 1988; Stevenson, 1988, and Ibrahim *et al*., 1996).

2.17 TREATMENT, PREVENTION AND CONTROL IN FISH

2.17.1 Treatment and Prevention

Aeromoniasis infects a wide variety of hosts in a diversity of habitats. Epidemiological control is directed towards its management as a geographically ubiquitous, but obligate pathogen (Cipriano, 2001). The pandemic distribution and prevalence of this bacterium, should never diminish the gravity of its pathogenic ramification in the minds of fish culturists and resource managers (Jarb *et al*., 1993). Risks associated with outbreaks such as migration of infected to uninfected fish into water supplies, sharing of personnel, equipments and population density of fishes (Jarb *et al*., 1993).

The best prevention model for motile aeromonads septicemia is good husbandry (Kapor *et al*., 1981). In a situation where these conditions are not well improved, antibiotics particularly oxytetracycline or the nitrofurans can be used for both treatment and prophylaxis (Thune *et al*., 1982). However, extensive use of antibiotics in fish ponds have serious drawback of increasing plasmid-encoded antibiotic resistance in *A. hydrophila* (Stevenson, 1988; Carnahan *et al*., 1989). Therefore, the use of vaccines

against *A. hydrophila* in agriculture and aquaculture becomes an attractive option when there is limited flexibility to improve environmental and management conditions (Stevenson, 1988). Wu *et al*., (1981) proposed the use of bacteriophage lysis as a control measure, but the problems associated with resistance and host range of phages makes this impractical. Lamers and Van Muiswinkel (1986) identified the use of bacterins in prevention of *A. hydrophila* infection.

2.17.2 Immunization and Vaccination

Immunogens are emulsified in oil adjuvant and delivered by intraperitoneal injection. Thus, providing long–lasting protection and their use is promoted in commercial aquaculture (Ellis, 1977; Lillehuag *et al*., 1992). Both aqueous and oil adjuvanted vaccines are commercially produced and may induce immunity at water temperature as low as 2^0 C (Ellis, 1977). Serologic relationship between strains of *A. salmonicida* suggests that immunization of fishes against atypical forms of this pathogen is also a realistic possibility (Evenberg *et al*., 1988). Immunogens used in fish aquaculture includes; cell envelope preparations, lipopolysacharrides (LPS), purified A – layer protein, formalized whole cell bacterins and detoxified extracellular protein (ECP) (Gudmondsdottir and Gudmondsdottir, 1997).

A vaccine against motile Aeromonad diseases will be cost-effective in fish culture where there is a recurrent disease problem (Stevenson, 1988). The use of vaccine is also an in-expensive replacement for antibiotics in intensive culture. The vaccines which are available commercially includes *A. salmonicida* bacterin, code 2035:00 (09MAR90) and bacterin, code 2051:00 (11APR91). Reports have also shown that the use of aro A.

mutant of *A. hydrophila* is effective in the control of aeromoniasis in fish (Plumb, 1984; Hernanz *et al.*, 1998). All these vaccines are polyvalent in nature and contain antigens representing all strains that fish might encounter (Plumb, 1984). Vaccines are also available against furunculosis caused by *A. salmonicida* and bacterial septicemia caused by *A. hydrophila* (Dooley *et al*., 1986).

The first vaccination trials of fish against *A. hydrophila* were carried out by Lamers and Van Muiswinkel (1986). In which two methods were described, namely the dip immersion technique and the intraperitoneal injection technique. The bacterins were prepared from lipopolysaccharides of sheep erythrocytes coated with bacterial antisera. Formalin treatment is known to alter *A. hydrophila* antigenic structure and consequent processing of the antigens by macrophages, while heating and breakage could release more antigenic material (Lamers and Van Muiswinkel, 1986). The duration of immunity is 8 months and booster dose can be given at 1 – 3 months (Lamers, *et al*., 1986). Dose rate of 0.01 – 0.2 units of the vaccine is known to confer immunity to the agent in fish with antibody titres of 1:64 are considered immuned (Stevenson, 1988).

Vaccination against *A. hydrophila* increases non-specific, natural immune mechanisms (Stevenson, 1988). Antigen localization is of two phases; non-specific which induces cell-mediated immune mechanism and specific which localizes to the melanomacrophage centres of tissues at the onset of antibody production (Lamers and De Haas, 1985). Melanomacrophage centres appear to be the site of long term antigen localization (Stevenson, 1988). *A. hydrophila* antigens remained in lymphoid tissue for 12 months after fish were injected with 10^9 cells (Lamers and De Haas, 1985).

2.17.3 Genetic Control

Genetic control of aeromoniasis through selective breeding programs has been developed in salmonids (Cipriano, 1997). Genetic control enhances experimental and field resistance to furunculosis. Resistant progeny produced are known to grow faster and lay more eggs than susceptible fish (Kincaid *et al*., 1997).

Heritability of mortality exists amongst Atlantic salmon from sire to dam (Gjedrem *et al*., 1991). This implies that resistance to furunculosis can be effectively improved by selective breeding (Gjedrem and Gjoen, 1995). Resistance in fish to furunculosis is associated with exon 2 sequences encoding the major part of the peptide – binding region among the fish types (Langefors *et al.,* 2001). Variable degrees of resistance has also been noticed amongst salmonids and non-salmonids in response to *A. salmonicida* infection. Resistance is known to reduce with age and is noticed by a shift from sub-acute to chronic forms (Egusa, 1971; Langefors *et al*., 2001).

2.17.4 Therapy

Chemotherapeutic agents are used for the treatment of aeromoniasis in fish farms. Isolates of *A. hydrophila* are known to be sensitive to chloramphenicol, florfenicol, tetracycline, sulphonamide, nitrofuran derivatives and pyrodine-carboxylic acids (Aoki and Egusa, 1971; Parker, 1987). In hatchery operations, terramycin has been effective when incorporated into pellated fish feed and fed at 3.5 grams of active drug per 100 pounds of fish per day (Wicklund *et al*., 1998).

2.18 TREATMENT, PREVENTION AND CONTROL OF *AEROMONAS* INFECTIONS IN MAN

In man, aeromonad associated diarrhea is self limiting and normally does not require treatment. In acute cases of diarrhea, fluid and electrolyte therapy is required for the patient (Collier and Parker, 1987). For protracted bloody or chronic diarrhea and for most severe infections, several antimicrobial agents are effective. The organisms can be treated with quinolones, gentamicin, trimethoprim – sulfamethoxazole third generation cephalosporins, aminoglycosides, chloramphenicol and polymyxin (Prescott and Baggot, 1990). The organism is also known to be resistant to ampicilin, penicillin, tetracycline, first and second generation cephalosporins (Bhat *et al.,* 1974; Baron and Finegold, 1990). Treatment is done systematically and strictly on the recommendation of the physician.

Proper sanitary measures and use of gloves during fish handling and proper washing and disinfection of hands after handling is important (Collier and Parker, 1987). Public health education and awareness is very important in management of infectious zoonoses (Umoh, 1998; Spore, 2000).

The WHO committee on the control of bacterial zoonoses in 1988, proposed a number of measures that can be adopted in control of aeromoniasis. These include decontamination of infected vectors, prevention of spread of *Aeromonas* during fish transportation and handling via use of gloves and masks for protective purposes (WHO, 2000).

2.19 MANAGEMENT OF *AEROMONAS* INFECTIONS IN FISH, POULTRY AND MAN

In confined heavily stocked commercial fish systems, aeromoniasis can proceed rapidly, therefore, prevention is essential to any management scheme (Reed, 2002). The steps involve; avoidance of adverse environmental conditions such as high water temperatures, low water flows, low oxygen levels or crowding (Warren, 1991). Also, avoidance of stressors which may favour outbreak such as poor nutrition, poor water quality, improper handling during vaccination exercises, overcrowding and presence of other diseased causing agents that will increase the fish's chances of contacting infections. Use of copper compounds is effective for infectious disease control though such compounds can be harsh on fish (Reed, 2002). Quarantine of new fish and good sanitation practices should be done at all times, that will minimize the spread of *Aeromonas* from infected and non-infected fish should outbreak occur (Reed and Francis – Floyd, 2002). Tanks and culture facilities should be kept clean and free from unnecessary waste materials.

As provided by WHO (1988) control of *Aeromonas* infections remains a public health problem. The widespread distribution of the organism in the environment complicates the problem of control. This problem can be solved by eliminating *Aeromonas* from fish and water environment and improving fish handling practices. Surveillance of processed fish to identify infected suspects and public health education and awareness of the disease is important in the control of this disease.

2.20 ECONOMIC AND PUBLIC HEALTH SIGNIFICANCE OF MESOPHILIC *AEROMONAS*

The mesophilic *Aeromonas* (motile *Aeromonas*) are important components of the bacterial flora in environmental drinking water and aquatic animals and fish (Sartory, 2002). When temperature and nutrient conditions are favourable, they can rapidly proliferate in water systems (Sartory, 2002). These bacteria can also express a range of virulence factors and some researchers have assessed drinking water supplies as a source of *Aeromonas* gastroenteritis and is responsible for the deterioration in the bacteriological quality of water in fish farms (Daily *et al.*, 1985; Davies and Wrays, 1995; Sartory, 2002). Recipient rivers that are supplied by such water will therefore be contaminated by *Aeromonas*. This may lead to increased abundance and activity of the bacteria in the affected rivers (Carr and Goulder, 1993).

Drinking water supplies have previously been shown to be a source of mesophilic *Aeromonas* species (Burke *et al.*, 1987). The presence of these organisms coupled with ineffective disinfection at water treatment plants, post-treatment infiltration or as a result of after growth within the distribution system will be a source of danger of infection to man and other aquatic animals (Lechevallier *et al.*, 1982; Burke *et al.*, 1987; Knochel and Jeppenson, 1990).

Current evidence by WHO in 1988 suggested that the predominant typical *Aeromonas* species found in drinking water are of the same DNA homology groups as those isolated from cases of gastroenteritis. Numbers in drinking water are generally low compared to those found in food and fish. Proliferation of these bacteria in water systems may be responsible for the deterioration in the bacteriological quality of the water and has

been associated with the development of biofilms on pipe walls (Carr and Goulder, 1993; Davies and Goulder, 1993; Davies, 1994).

A. hydrophila was isolated from grocery store produce, ponds, foods and soil (Callister and Agger, 1987). This may cause economic losses in fish pond in the form of stunting, poor reproductive performance, debilitation and subsequent death (Esuroso, 1999). The economic implication is decrease in fish protein that will supplement beef (Olanike, 1999). The zoonotic significance of *A. hydrophila* has shown that the infection may result in bone, nervous, heart and gastroenteritic disorders in man (Qadri *et al*., 1976). This may adversely affect human population (Karam *et al*., 1983).

Public health advances aimed at controlling this infection on several fronts have had tremendous impact in diminishing fish and water bone disease transmission in industrialized countries (Burke *et al*., 1987). These advances as provided by the WHO (1988) include improvement of water quality, use of antimicrobial agents in feed, general environmental sanitation, and general fish and poultry hygiene.

2.21 CONTROL MEASURES OF *AEROMONAS* INFECTIONS IN NIGERIA

Due to the cosmopolitan nature of the organism and its pathogenicity, its control remains a major problem in tropical Africa including Nigeria (Olanike, 1999). At present, little systematic work has been done on pathogenicity, virulent factors of the organism and immunogenicity of the bacteria (Paulo, 1998). The use of antimicrobial agents for fish or poultry and symptomatic treatment of infected humans have not achieved much success. Detailed laboratory investigation of the organism is affected by drawback such as high cost of drugs and laboratory reagents, increasing resistance of the pathogen to

existing drugs, absence of new drugs and technologies to identify and treat aeromoniasis, toxicity of some drugs, and lack of human awareness of its zoonotic importance (Paulo, 1998).

To date, the need to control aeromoniasis in Nigeria has received little attention. It is not even listed among the reportable diseases. In developed nations, vaccination of fish against aeromoniasis prior to stocking have been shown to give good protection. A commercially prepared vaccine has been used successfully in fish ponds as soon as a diagnosis of *Aeromonas* infection is made.

Control measures in man will primarily depend on the control of the disease in fish, poultry and other animals. Safe handling of contaminated fish (Oniye *et al*., 1998), avoidance of feeding poultry with contaminated water or incorporating contaminated fish in poultry feed, and treatment of infected fish and man with erythromycin, kanamycin, neomycin and oxytetracycline have also been recommended (Okpokwasili and Ogbulie, 2001). There should be proper regulations guiding conduct of workers, maintenance and operations of cold rooms and refrigerated fish trucks as they play prominent roles in successful preservation of fish and possible transmission of microbes (Oniye *et al*., 1998).

In Nigeria, there is lack of flow of information from manufacturer of antimicrobial agents and appropriate professional advice to the fish farmers and final consumers. This information could have contributed immensely to the increased resistance associated with older drugs use in Nigerian culture systems. Fish and poultry farmers should seek professional advice before using certain antibiotics in their farms.

This will enhance safety in terms of dose and dosage rates, route of administration and periods of therapy (Ogbulie and Okpokwasili, 1998).

2.22 GENERAL PROCEDURE FOR CULTURING, ISOLATION AND DENTIFICATION OF *AEROMONAS* SPECIES

Detailed strain identification is essential for complete biochemical and epidemiological investigations, prevention, and control of outbreaks (Stevenson, 1988).

However, isolation must preceed identification (Le Minor, 1984; Krieg and Holt, 1984). Numerous techniques and methods have been described for the isolation of *Aeromonas* from different types of specimens (Kwaga *et al*., 1988; Kingombe *et al*., 2002). Indeed, it is not possible to recommend a particular technique because of lack of comparative evidence of the efficacy of the isolation methods in current use (Kwaga *et al.,* 1988). It is well known that the procedures that can be used for isolation of bacteria in a sample are seldom suitable for isolation of other organisms in the same situations (Fricker, 1987; Wray *et al.,* 1996). Therefore, for efficient isolation of *Aeromonas* in a sample, a scheme including some or all of the following stages are required: resuscitation and pre-enrichment, enrichment culture in a liquid medium, isolation as individual colonies in a selective medium or differential solid media and confirmation of the identity of the organisms using phenotypic or molecular tests (Fricker, 1987; and Blackburn, 1993).

Under normal circumstances, organisms which are present as a major component of the flora of a sample are usually much easily isolated than those present in minority when grown under particular conditions (Fricker, 1987). For example, isolation of *Aeromonas* species from GIT of fishes, frogs, and contaminated water is of little

difficulty whereas there is often more difficulty isolation of same species in vegetables, humans and poultry, infected wounds and from feeds and processed foods (Chan, 1979; Fricker, 1987).

I BACTERIAL CULTURE AND ISOLATION

a. Pre-Enrichment Media

Pre-enrichment media are used for culturing samples where *Aeromonas* are sub-lethally damaged as may occur by inculcating fish in meals during poultry feed manufacture, in dry samples and environmental samples which the number of organism is low or where disinfectant, have been applied (Wray *et al.,* 1996). Pre enrichment culture therefore allows the number of organism to increase hence, reducing the detrimental effect of initial killing by selective media (Altwegg, 1994).

Also, it enhances the repair of any bacterial lesion and promotes their regain in resistance to selective agents prior to enrichement (Fricker, 1987). According to D'Aoust (1981); Harvey and Price, (1979); and Wray *et al.,* (1996), the widely used pre-enrichment media are buffered peptone water, although lactose broth, Ringer's solution and nutrient broth can be used as well. The recommended incubation period in pre-enrichment media is 16 – 24 hours (Fricker, 1987; and Blackburn, 1993; Altwegg, 1994).

b. Enrichment Media

Enrichment media are used for culture of samples which are likely to be contaminated e.g. faeces, cloacal swabs, environmental samples or subculture from pre-enrichment media (Wray *et al.,* 1996). The aim of using the enrichment media is to

increase the ratio of *Aeromonas* to competitor organisms such as *Salmonella, Shigella, E. coli*, and *Pseudomonas* (Blackburn, 1993). According to Fricker (1987) enrichment protocols may either be selective or elective. Elective procedures involves the use of media which will allow the growth of a single or small number types of bacteria based on the unique combination of nutritional and physiological attributes of the organism required. On the other hand, selective enrichment procedures involve inhibitory substances or procedures to impede the growth of most organisms but permit, though not necessarily encourage the growth of the desired organisms. The second protocol is the one which is widely used for isolating bacteria in clinical, veterinary and food microbiology.

At the time being, tetrathionate broth, Alkaline Phosphate Water (APW), Thiosulphate Citrate Bile Salts (TCBS), Phosphate Buffered Saline (PBS) and Gram – Negative (G-N) broth are the enrichment media which are in common use (Wray *et al.*, 1996). Apart from these, other enrichment media which have been used over the years include; the Mueller–Kauffman tetrathionate broth, MacConkey broth, Strantium chloride, gram negative broth, bismuth sulphide broth, Rappaports magnesium chloride, Malachite green broth (Fricker, 1987; Harvey and Price 1979, Nastasi *et al.*, 1991; and Altwegg, 1994).

Depending on the type of sample being studied and species of *Aeromonas* likely to be present, one must decide upon which medium or media to use since it is not easily possible to recommend one as the best for all purposes (Fricker, 1987; Harvey and Price 1979; and Wray *et al.*, 1996). Comparing Rappaport's broth and selenite broth, the

former was reported to be superior for isolation of *Aeromonas* from pre-enriched samples (Fricker, 1987).

In order to inhibit enteric bacteria other than *Aeromonas*, most of the enrichment broths are incubated at 35 – 37^0 C (Wray *et al*., 1996) and the recommended incubation period is 18 – 48 hours (Blackburn, 1993).

c. Plating Media

The use of suitable plating media is crucial for efficient isolation of all *Aeromonas* species which are able to grow on solid media (Fricker, 1987). An effective plating media therefore, should support the growth of a wide range of strains of the particular specie required and wherever possible inhibit growth of other bacteria not desired (Fricker, 1987). In addition, it should allow easy recognition of the organisms required from other bacteria which are able to form colonies from the same medium (Fricker, 1987). Medium which are common in use today include MacConkey agar, blood agar, and nutrient agar (Cruickshank *et al.*, 1975). In order to increase the chance of isolation and to be more accurate, it is recommended that at least two plating media should be used simultaneously for isolation of *Aeromonas* (Kwaga *et al.*, 1988).

II PHENOTYPIC AND MOLECULAR TYPING METHODS IN *AEROMONAS* SPECIES

A typing method refers to any method which can be used to differentiate the bacteria below the species level (Oslen *et al*, 1993; and Towner and Cockayne, 1993). Such typing methods are becoming increasingly important in characterization of bacteria involved in diseased outbreaks, defining sources of infection, determining mode of transmission, and route of spread of infection in susceptible population (Towner and

Cockayne, 1993). Infectious microorganisms are clonal that is, they have a common origin hence they are genetically identical or nearly so. Orskov and Orskov (1983), used the 'clone' to denote bacterial cultures isolated independently from different sources, in different locations, and perhaps at different times but revealing so many identical phenotypic and genetic characteristics and therefore the most likely explanation for this identity was a common origin. The subdivision of bacterial species into distinct clonal lines becomes possible when typing methods are applied (Oslen *et al.*, 1993).

An ideal typing method therefore should have a good discriminatory power, show good reproducibility and typeability (Maslow *et al.*, 1993; and Oslen *et al.*, 1993). It is worth noting that, the combination of suitable typing methods is recommended in epidemiological studies of an outbreak of aeromoniasis (Christensen *et al*., 1995).

In general, bacterial typing methods are categorized into two major groups, the phenotypic typing methods which comprises the conventional and modern phenotypic typing methods and the genotypic methods (Maslow *et al*., 1993). Both phenotypic and genotypic methods have been used for typing *Aeromonas hydrophila* (Abbott *et al.*, 1992).

a. Conventional Phenotypic Typing Methods

This category comprises techniques that detect characteristics expressed by microorganisms such as biotyping, serotyping, phagetyping and antibiogram (Cipriano and Bullock, 2001).

Biotyping is a typing method that separates strains of a species based on differences in selected biochemical tests (Cipriano and Bullock, 2001). This method has been used for years in typing *Aeromonas veronii* into serovars and biovars. For example,

Aeromonas veronii biovar *hydrophila* and *sobria* which belonged to serovar *veronii* can be typed by this method (Krieg and Holt, 1984). However, biotyping is time consuming and the tests are organism specific, hence phenotypic results may vary depending on environmental and media related conditions which may cause divergence in different laboratories (Cruickshank *et al.*, 1975).

Due to this reason, this method is useful in limited short term local investigations of an outbreak (Altwegg, 1994). Therefore, for long term investigations covering different epidemiological outbreaks, the method should be used in conjunction with other typing methods (Kwaga *et al.*, 1988).

On the basis of extensive diversity of O, H and VI antigens, strains of *Aeromonas* species are classified into different serotypes (Thune *et al*., 1982). *Aeromonas salmonicida* subspecies *achromogens* serovar *masaucida* which belonged to serogroup D according to Thune *et al.* (1982) is one of the over one hundred recognized serovars (Le Minor, 1984; Threlfall and Frost, 1990; and Shivaprasad, 1997).

Serotyping is highly discriminative, relatively stable and reliable typing method (Oslen *et al*., 1993). The major drawback of this typing procedure is associated with problems of antisera production and standardization methodology (Towner and Cockyane, 1993). Also, this typing procedure requires specialized reagents which may be available to only specialized reference laboratory (Towner and Cockayne, 1993). However, in spite of the limitations, one cannot deal with DNA based typing methods without serotyping, thus such methods are secondary and supplementary to it.

Phage typing is a sensitive method for identification and characterisation of bacterial strains based on susceptibility of defined collections of bacteriophage (Maslow

et al., 1993). This method is relatively cheap and it has been used widely for public health and epidemiological purposes through out the world (Oslen *et al.,* 1993). Austin *et al* (1998) typed *Aeromonas hydrophila* by means of bacteriophage lamda using two *A. hydrophila* and four, 2 phase types and 5 phase types of *A. caviae* were demonstrated (Austin *et al*., 1998). 2-phage typing has no application to a new organism or to a sudden emerging problem. In addition, this typing method becomes inadequate when one particular type becomes dominant (Austin *et al.,* 1998).

b. Conventional Molecular Typing Methods

Molecular typing methods are categorized into four major typing groups based on type of macromolecules carrying enough information, which allow the study of the microbial diversity. Such methods are lipopolysaccharrides, fatty acid, protein, and nucleic acid based methods (Zyskind and Bernstein, 1992).

According to Olivier (1992), lipopolysaccharrides, fatty acid, and protein-based methods are classified as modern phenotypic typing method, which depend on expression of phenotypic properties. Through, like the traditional typing methods, they are influenced by the environmental factors like the growth conditions and the genotype of the bacteria. These methods have been reported to have minimal use for characterization of *Aeromonas* strains due to their reported poor discriminatory power (Olivier, 1992).

Rapid methods for extraction and analysis of plasmid DNA have become available and hence plasmid typing has been used to supplement the traditional methods for typing the bacterial strains (Egusa, 1971; Threlfall and Frost, 1990). This method is simple yet powerful for identifying strains of *Aeromonas* (Swami-Nathan and Matol, 1993). It has been useful for sub typing several *Aeromonas* strains (Christensen *et al.*,

1995). However, plasmid profiling is advocated for use only in strains, which harbor several plasmid of different molecular weights. Based on this fact therefore the method has limited use in different strains lacking these features (Thompkins *et al.,* 1986; Oslen *et al.,* 1993; and Towner and Cockyane, 1993; Dorsch *et al,* 1994; Christensen *et al.,* 1995).

Sometimes however result from plasmid profiling can be interpreted wrongly due to three major reasons. Either when a single plasmid contains different molecular form or when the preparations contain large plasmid, and lastly when two dissimilar plasmid have identical molecular weight (Towner and Cockayne, 1993). To overcome such problems, restriction enfonucleases are used in cleaving plasmid DNA to generate plasmid fingerprints (Towner and Cockayne, 1993). These enzymes recognize specific DNA sequences thereby cutting the plasmid DNA into a number of fragments depending on the number of enzyme recognition (Oslen *et al.,* 1993; Brock *et al.,* 1994). Generally, plasmid fingerprinting provides a means of plasmid relatedness, which can be useful tool in tracing the evolutionary origin of plasmids and of their host bacterial strains (Threlfall and Frost, 1990). The use of plasmid profiling and restriction analysis for typing *Aeromonas* was highlighted by (Wiklund *et al.,* 1998). It is therefore reported that many *Aeromonas* species may have identical restriction endonuclease digestion patterns which may be difficult to differentiate by this technique. Therefore, more defined and unchangeable molecular techniques are indispensable (Estaben *et al.,* 1993).

Typing methods that base on chromosomal DNA can be applied to overcome problems that arise in analysis of plasmid profiles and fingerprints (Towner and Cockyne, 1993). So far, all strains are typable when chromosomal DNA based typing methods are

applied as opposed to plasmid DNA based typing method (Christensen *et al.,* 1995). In this category of typing methods, ribotyping and Pulsed Field Gel Electrophoresis (PFGE) are the most widely used typing techniques. Their applications in epidemiological investigations of *Aeromonas hydrophila* was demonstrated by Cockayne, (1993).

Chromosomal fingerprinting is a modern genotypic typing method that based on chromosomal DNA (Inglis *et al.,* 1996, Soule *et al.,* 2005). Following *in-vitro* digestion of the isolated chromosomal DNA with restriction endonucleases, a multiple of chromosomal fragments are obtained which may be considerably difficult to interprete (Threlfall and Frost, 1990; Popovic *et al.,* 1993). The fragment patterns can evolve up to 200 fragments of different sizes (Threlfall and Frost, 1990). In order to make such results interpretable, different techniques are applied including hybridization techniques and the use of rare cutters restriction endonucleases. In the hybridization technique, following digestion of chromosomal DNA, generated fragments are separated by agrose electrophoresis, then transferred onto hybridization membrane (Towner and Cockayne, 1993). Membranes with DNA fragments are then hybridized with 165 and 235 ribosomal RNA sequences probe or by insertion sequence 15200 probe (Grimont and Grimont, 1986; and Stanley *et al.,* 1993).

When 16 S and 235 ribosomal RNA sequences are applied, only genes that code for rRNA are hybridized by a probe (Popovic *et al.,* 1993). RRNA genes are theoretically present in all bacteria and they have been highly conserved during evolution (Brock *et al.,* 1994). The banding pattern obtained after hybridization is small (7- 12 bands) to allow interpretation (Popovic *et al.,* 1993). This technique is called ribotyping since it is based on rRNA genes. Since 1986 when it was described for the first time by Grimont

and Grimont (1986), it has been extensively used in typing of *Aeromonas* species (Nielson *et al.,* 1994; Nelson *et al.,* 2001). Ribotyping was previously applied in typing *Aeromonas salmonicida* subspecies *masaucida* (Nielson *et al.,* 1994). In a typing studies using *E. coli* H*ind* III and *Sma*I restriction enzymes, two, 13 and one riboytpes were generated respectively (Nielson *et al.,*1994). From this study, it was concluded that ribotyping using H*ind* III was more discriminatory than other enzymes. In another study, ribotyping using E*co* R1, H*ind* III, and S*ma* I restriction enzymes was used to compare *A. hydrophila* from Denmark and Germany origins and all enzymes generated identical ribotypes irrespective of origins. It was concluded that aeromoniasis in Denmark originated from Germany (Nielson *et al.,* 1994). Further more, this technique was applied by Oslen *et al.* (1993) using small, and all isolates were typable all through and all had some ribotype which is found in many *Aeromonas* serotypes but not in other members of the family *Enterobacteriaceae* (Nash *et al.*,2006).

Another way of resolving problem caused by complex finger print patterns is the use of "rear culture" restriction endonuclease, which cuts the bacteria genome at few sites resulting to large restriction fragment (Oslen *et al.,* 1993). However, the resulting DNA fragments are large and cannot be separated readily by conventional agarose electrophoesis. Therefore, these segments are separated by special techniques called Pulse Field Gel Electrophoresis (PFGE) (Schwartz *et al.,* 1983). This technique is capable of separating high molecular weight DNA molecules ranging from 12 kb to 50 kb. It is a very powerful technique in epidemiological investigation (Schwartz *et al.,* 1983).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 STUDY AREA

The area of this study is Zaria town. Zaria area is located in Kaduna State in the north central region of Nigeria, on a plateau at a mean height of about 715 metres above sea level. The area is immediately surrounded by settlements like Giwa, Soba, Makarfi, Shika and Jaji and many others. Zaria emirate is bounded by Bauchi State in the East, Kano State in north, Katsina State in the north west and Plateau in the south east (FRN, 2000).

The town is situated between 20^0 33' south, 7^0 34' west, 20^0 37' north and 9^0 25' east (FRN, 2000). The urban area is divided into two Local Government Areas (LGAs) wich includes Zaria LGA to the south and Sabon gari LGA to the north (Fig 3.1).

The town experiences 3 climatic conditions namely, hot dry season within the months of February to April, cold harmmattan periods between the periods of December to February and raining season between May to October (FRN, 2000). Zaria has an estimated area of about 45 square km with estimated population of about 1.5 million people. About 60 % of the people are immigrants while 40 % are indigenous. Majority of the tribes are Hausa and Fulani while Kanuri, Yoruba, Igbo, Nupe and Tiv make up other significant ethnic tribes (FRN, 2000).

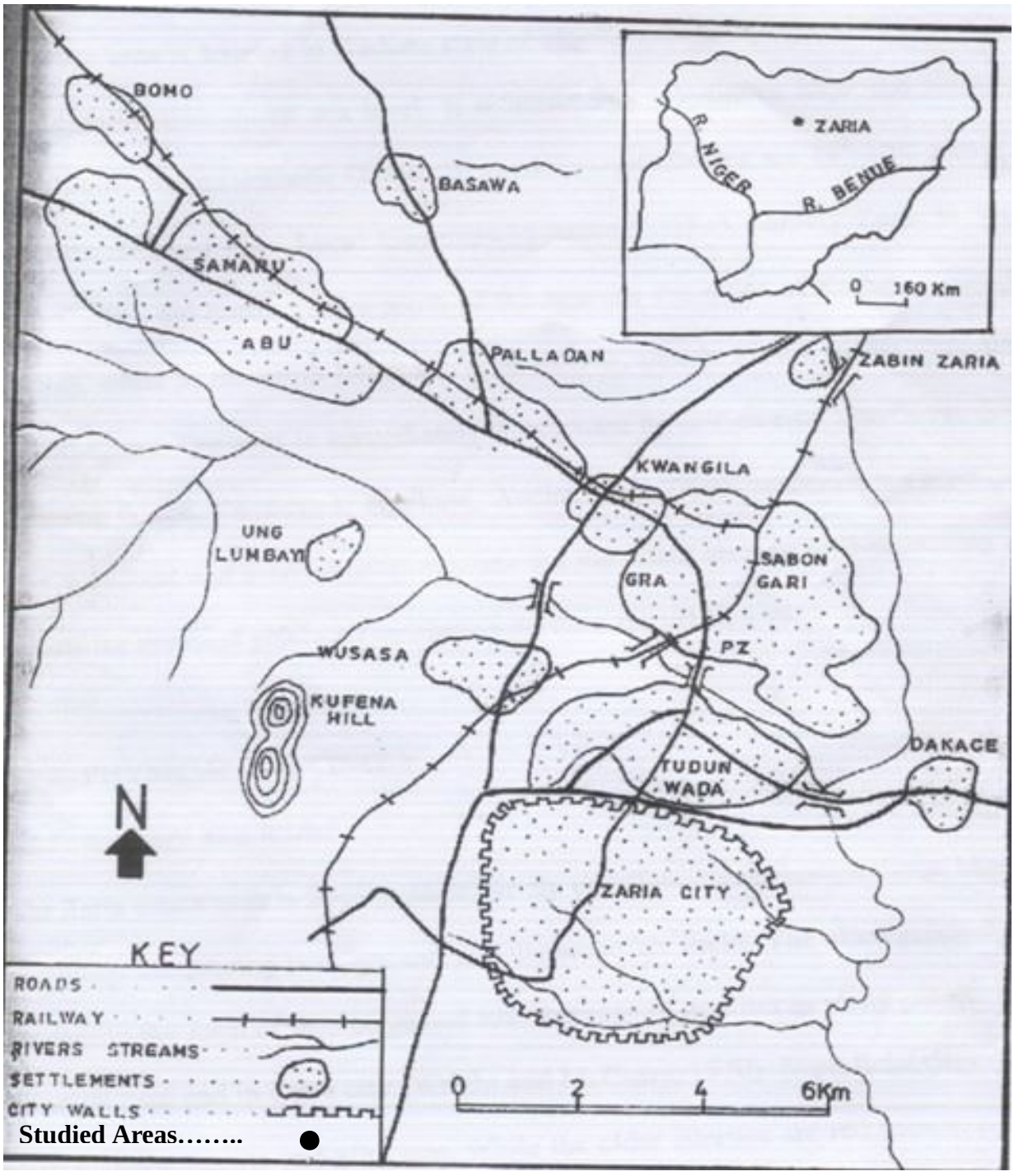

Fig. 3.1 Map showing some locations where samples were collected.
Source: Mohammed, (2006).

3.2 EQUIPMENT, MATERIALS AND REAGENTS

3.2.1 Equipments, Materials and their Uses

- A phase contrast microscope: used for viewing *Aeromonas* species.
- Analytical balance: used for weighing reagents and chemicals .
- Metler balance: used for weighing culture media.
- Bench top centrifuge: used for centrifugation of broth culture.
- Quartz cuvette: 0.5 ml glass used for measuring absorbance.
- Spectrophotometer: machine used for taking absorbance readings.
- Petri dishes: used for plating agar.
- Test tubes: used for the various biochemical reactions.
- Eppendorf tubes: used for DNA storage or isolation.
- Micropipettes: used to pick microlitres of proteins or DNA or reagents from a given sample.
- Platinium loop and straight wire: used to pick *Aeromonas* from the culture media.
- Glass slides and Cover slips: used for catalase test and gram staining.
- Shandom tank: used for bacterial lysis.
- Water bath: used to heat and dissolve crystal, chemicals and reagents.
- Electrophoresis power pack and tank: used to run SDS-PAGE.
- UV – lamp: used to supply UV light
- UV– transilluminator: used to view DNA and restriction fragments after AGE.
- Agarose gel electrophoresis tray and power pack: used to run AGE.
- Microcentrifuge: used for centrifugation of broth culture.
- Culture plates and bottles: used for storage of *Aeromonas* culture broth.

- Incubator: used for growth of *Aeromonas* in agar plate of broth culture.
- Biurets: used for suspending of *Aeromonas* proteins.
- Hand gloves: used for protection against *Aeronomas* infection.
- Mask: used for protection against inhalation or contact with zoonotic *Aeromonas*.
- Laboratory coat: used for protecting body contact against *Aeromonas*.
- Conical flask: used for preparation of buffers.
- Gas oven: used for sterilizing platinium wire or loop.
- Refrigerator: used for storing isolates.
- Heating mantle: used for heating samples.
- PH meter: used to determine PH of buffers.
- Autoclave: used for sterilization of *Aeromonas* culture media.
- Microwave sterilizer: used for sterilization of eppendorf tubes

This were all laboratory materials of the bacterial zoonosis laboratory Department of Veterinary Public Health and Preventive Medicine and Mary Hallaway Teaching Laboratory the Department of Biochemistry Ahmadu Bello University, Zaria.

3.2.2 Chemicals and Reagents

Acetic acid (CH_3COOH), Electrophoresis standard, Acrylamide, Bis-acrylamide, Tris Buffer, TEMED, proteinase K, MacConkeys' agar, Nutrient agar, Distilled water, microscope, Esculin reagent, Grams reagent, Hydrogen peroxide, Citrate agar, BHI, SIM agar, Sugars, Gelatin agar, Urease agar, Ureas base, MR-VP medium, Sugars for fermentation, Sheep blood, blood agar, Sodium dodecyl sulphate, Agarose gel, bromophenol blue, glycerol, Coomasie brilliant blue, iodine.

British Drug house, England supplied the following; Acetic acid, Sulfuric acid, Diabasic sodium phosphate, Monobasic sodium phosphate, Sodium hydroxide, Periodic acid, Sodium arsenite and Sodium chloride.

Qiagen kit, salmon sperm DNA, Agarose, Ethidium bromide, Restriction enzymes such as B*am* HI, E*co* R1 and S*al* 1, Electrophoresis standards, Acrylamide, Bis-acryladmide and Ammonium persulfate. All other reagents were of analytical grade and were purchased from Sigma Chemical Company, St. Louis U.S.A.

3.3 SOURCES AND COLLECTION OF SAMPLES FROM FISHES POULTRY AND HUMANS

3.3.1 Collection And Processing Of Fish Samples

A total of four hundred (400) live fishes were purchased from fish vendors by convinience sampling which was based on the availability of different species of fish at the following four (4) locations in Zaria: ABU dam, Samaru market, Sabon gari market and Zaria dam. They were carried in plastic buckets containing water from which the fish were cought and transported alive to the bacterial zoonosis laboratory of the Faculty of Veterinary Medicine for examination. This method is most appropriate as it reduces the rate of cross infection (Cottral, 1978; Atsanda *et al*., 2000).

Fish samples were brought alive from the field in plastic buckets. They were immediately processed on arrival at the laboratory. The stomach contents were removed by searing an area of the stomach wall with heated knife, longitudinal and transverse incision were made to expose the GIT contents and transferring about 2 g of the sample using a platinum wire loop into the prepared tetrathionate broth.

The fishes were identified to the species level based on published methods (Reed *et al*., 1967; and Lewis, 1974) and with the assistance of Prof. S. Oniye and Dr. J. Auta of the Department of Biological Sciences, Ahmadu Bello University Zaria.

Processing of samples was done based on established methods (Cottral, 1978; Kwaga *et al*., 1988; Mailafia, 2003). Each fish was sacrificed by pitting before examination. The hood was cleaned with disinfectant (Purit ®) which contains cyclohexidine glucognate (BP) 0.03 % w/v and cetrimide 3.0 % w/v. The dead fish was placed in the hood dorsally and the abdomen raised upward. A clean cotton wool was soaked in alcohol and used to swab the abdomen to reduce bacterial load. A broad clean, knife was heated to red hot on a bunsen flame and used to sear the area of incision on the abdomen. The abdominal wall was incised with a sterile surgical blade. Incision was made from the vent and cut anteriorly, then laterally and a transverse incision was made to remove the flesh and expose the visceral organs, that is, the gut, liver, gall bladder, swim bladder, gonads, spleen and kidney within the body cavity. Fish GIT was incised thinly from the aesophagus to the anus at the longitudinal edge to expose the GIT contents.

3.3.2 Collection And Processing Of Poultry Samples

All the owners or management of the respective locations were visited and the purpose of the study explained to them. Poultry were sampled from nine (9) farms which included: Zumunta, Zaria, Lape, Shika, National Animal Production Research Institute (NAPRI), and Institute for Agricultural Research (IAR) farms, some backyard poultry in ABU staff quarters in Area ‘C’, Area ‘F’, and Area ‘A’ were visited. The criteria used to

select the locations in the poultry study area included: consent of the farmers, owners or proprietors, and willingness of the owners approached to cooperate or participate. This came from different strata of Zaria. Therefore, the major sampling method used was based on convenience, availability and willingness of various areas visited to participate in the study.

Only five (5) locations of poultry agreed to participate in the study and they included: Area 'C', Zumunta farms, Area 'F', Lape farms and Zaria farms.

Five hundred and forty (540) cloacal swabs were obtained from five locations of poultry using sterile cotton swab stick from the following locations; Zumunta farms, Lape farms, Zaria farms, Area 'C' and Area 'F' in Zaria metropolis. Breeds of poultry were identified with assistance of specialists in the National Animal Production Research Institute (NAPRI) Shika, Zaria.

Swab sticks were dipped into the cloaca by gentle rotatory movement and were sent from the field in sterile bijou bottles in ice packs and were cultured in tetrathionate broth immediately on arrival to the laboratory.

The samples were transported on ice from the field to the bacterial zoonoses laboratory of the department of veterinary public health and preventive medicine. Standard biochemical methods were employed for the culturing, isolation and identification of the *Aeromonas* spcies (Ewing, 1986).

3.3.3 Collection And Processing Of Human Samples

Ethical clearance and permission was sought from the matrons, Doctors or Heads of Department of Microbiology in the various hostpitals in which the samples were collected.

About eight (8) health institutions or establishments, clinics were approached with a letter of introduction seeking consent of appropriate authorities for inclusion in the study. These clinics or health institutions included: Salama, Savannah , Basawa, Jama'a, Ilimi, ABU Sickbay, Ahmadu Bello University Teaching Hospital (ABUTH) Shika, Ahmadu Bello University Teaching Hospital (ABUTH) Tudun wada.

The criteria used to select the locations in the study area included: consent of the authorities, availability of microbiological units in the hospitals and willingness of the owners approached to cooporate. This came from different strata of Zaria.

Only two of the areas sampled participated in the study and this includes ABU teaching hospital and ABU sickbay. Two hundred and twenty (220) human stool samples were collected from the two locations (ABU Teaching Hospital and ABU Sick bay) with previous arrangement made with the doctors or matrons in each hospital or health establishment. About 5 gm or 5 ml of each fecal sample was submitted for the study. The samples were collected when freshly voided fecal specimens were submitted to the various laboratories for bacteriological or parasitological examination. The samples were scooped with a spatula and decanted, respectively into a labelled screw cap polystrene biofreeze bottle and tightly screwed. Care was taken to avoid gross contamination with environmental material. A corresponding questionnaire was given to the appropriate caregiver for final submission. It was used to collect information on age, sex, type of food, source of water for domestic use, presence of vomiting, fever and episodes of diarrhea.

There was no control samples collected from apparently normal healthy humans. The problem of non availability of samples and unwillingness of apparently healthy

humans to participate or donate samples at the time the research was conducted has made the collection of such samples difficult.

3.4 PERIODS OF SAMPLE COLLECTION

The periods of sample collection lasted for about four months that is, October 1st 2005 to 31st January 2006. One thousand one hundred and sixty (1,160) samples were collected mostly in the mornings and evenings from the fishes, poultry and humans when available each day from Mondays through Sundays, at the respective dams, markets, farms and hospitals.

3.5 PREPARATION OF MEDIA AND CULTIVATION

The samples were transported to the bacterial zoonoses laboratory for examination. Stool samples were taken with the aid of sterilized cotton swabs and were dipped into the prepared tetrathionate broth. It was incubated at 37^0 C for 24 hours. If not used immediately, it was sent to the Artificial Insemination Unit Laboratory of the Nigerian Agricultural Research and Laison Service (NEARLS), and was stored in liquid nitrogen (-196^o C) until ready for culture and isolation.

The conventional culture and isolation method for detection of *Aeromonas* as described by Cowan and Steel (1974) and Cottral *et al.* (1978) were used. This involves enrichment, selective plating, detection of colonies, preliminary identification and complete biochemical identification.

Bacterial culture was based on established methods (Bhat *et al.*, 1974; Krieg and Holt, 1984; Alam *et al.*, 2006). The isolates were picked from the tetrathionate broth with

the aid of a sterile platinum loop and inoculated onto prepared MacConkey's agar. Other media that would have been used to isolate *Aeromonas* organisms included: Xylose Lysine Desoxycholate Agar (XLDA) and Xylose Desoxycholate Citrate Agar (XDCA). But because of non availability at the time this research was conducted, they were not used.

Sterile cotton swabs were dipped into the fecal samples by rotatory movement and suspended unto prepared tetrathionate enrichment broth which was incubated at 37^0 C covernight. They were later streaked on MacConkey's agar and incubated at 37^0 C overnight. From MacConkeys agar (MCA) plates circular colonies (about 2 – 3 mm in diameter) with characteristic pale, translucent, or in a few strains white or cream colour were picked using coiled platinum wire loop and subcultured on sterile nutrient agar slants. These were stored in the refrigerator for further biochemical tests.

3.6 LABORATORY IDENTIFICATION OF ISOLATES

Non-lactose fermenting colonies were picked and tested for production of oxidase. Oxidase positive organisms were purified by restreaking on nutrient agar slants and pure colonies inoculated onto the surface of prepared nutrient agar slants. They were incubated for 24 hrs at 37^0 C and stored in the refrigerator for further biochemical tests.

3.7 BIOCHEMICAL CHARACTERIZATION OF *AEROMONAS* ISOLATES

Biochemical identification was based on standard techniques (Cowan and Steel, 1974; McWhorter – Murlin and Hickman – Brenner, 1994). All the media and reagents for biochemical tests were prepared according to the instructions of their manufacturers.

Characteristic non-lactose fermenting colonies on MacConkey's agar were gram-stained and observed using x 100 objective lens of the light microscope (oil immersion). Gram staining was employed for diagnostic identification of various organisms (Cuickshank *et al.,* 1975). Gram positive organisms and fibrin are stained violet in colour. Gram – negative organisms, nuclei and protoplasm of pus cells and tissue cells are stained pink (Cruickshank *et al.,* 1975). Gram – negative coccobacillary rods that take on the colour of safranin (red) indicated *Aeromonas*. The isolates on MCA were subjected to the following preliminary tests.

3.7.1 Catalase Test

Isolates were emulsified in a drop of 30 % hydrogen perioxide (H_2O_2) solution on a clean glass slide. The evolution of abundant gas bubbles indicated the catalase positive. Absence of bubbles indicated catalase negative (Cruickshank *et al.,* 1975).

3.7.2 Oxidase Test

Non lactose fermenting colonies were subjected to oxidase test. This test was done based on standard method given by Cruickshank *et al.,* (1975). The colonies were picked from the agar using a sterilized glass rod onto a clean filter paper. Two to three drops of 1% solution of tetramethyl – p – phenylene – diamine dihydrochloride were placed on the filter paper so as to cover the surface of the organism. Oxidase positive organisms rapidly developed a purple colour.

3.7.3 Triple Sugar Iron (TSI) Test

On TSI, *Aeromonas* produced a pink (alkaline) slant over a yellowish (acid) butt with little blackening along the stabling line, indicative of H_2S production. There could be gas production, evidenced by air space below the butt or air bubbles along the stab line (Cruickshank *et al.,* 1975).

3.7.4 Urease Test

When *Aeromonas* isolates were inoculated on urea slant, the yellow colour of the urea will remain unchanged, while urease positive reaction is evidenced by a change from yellow colour of the urea to deep red (Cruickshank *et al.,* 1975).

3.7.5 Hydrogen sulphide, Indole and Motility (SIM) Test

The SIM (hydrogen sulphide, indole and motility) medium was used to test for H_2S production, indole and motility. Each SIM tube was inoculated by stabing to a depth of about 5 mm with a platinum straight wire followed by incubation overnight of 37^0 C. H_2S production was noticed by blackening in the tube along the stab line. Motile organisms migrated through the medium which became turbid while growth of non-motile organism is confined to the stab line.

For the indole test, a few drops of Kovac's reagent was added into the SIM tube which was then shaken. A red colour in the reagent layer indicated indole production (Cruickshank *et al.,* 1975).

3.7.6 Citrate Utilization

Slants of Simmon's citrate agar (oxoid) were inoculated by making a single streak over the surface of the slope. After 3 days of incubation at 37^0 C, a change from the original green colour to blue colour with streak of growth indicated a positive result (Cowan and Steel 1974).

3.7.7 Preparation of Methyl red and Voges Proskauer (MR-VP) Broth

MR – VP medium (oxoid) was prepared by dissolving 15 gm in 1 liter of distilled water, mixed well and 5ml each was dispensed into test tubes. These were sterilized by autoclaving at 121^0 C for 15 minutes. The *Aeromonas* isolates were inoculated into 5 ml test tubes containing MR-VP broth and incubated at 37^0 C for 2 – 4 days. The medium was then divided into two equal potions (McWhorter – Murlin and Hickman – Brenner, 1994).

3.7.8 Methyl – Red Test

To one portion 4 drops of 0.04 % methyl – red solution was added and mixed. A red colour is considered a positive result and a yellow colour indicates negative result (Cowan and Steel 1974).

3.7.9 Voges – Proskauer Reaction: Acetylmethyl Carbinol Production

To the second half of inoculated MR – VP broth, about 0.6 ml 5 % α-napthol solution and 0.2 ml 40 % potassium hydroxide solution (KOH) was added and shaken.

On observation after 15 minutes, a positive result was indicated by a strong red colour, while no change in colour gave a negative result (Cowan and Steel 1974).

3.7.10 Aesculin Hydrolysis

Bile aesculin (oxoid) plates incorporated with ferric salt were inoculated with *Aeromonas* isolates. Blackening in and around the bacterial growth indicated aesculin has been hydrolyzed. This is due to the reaction of the aglycone (6:7 dihydroxycoumarin) with the iron (Cottral, 1978).

3.7.11 Gelatin Hydrolysis (or Liquefaction)

Nutrient gelatin tubes were inoculated and incubated at 37^0 C for 14 days. At two–day intervals the tubes were taken out of the incubator and refrigerated at temperature of -4^0 C for two hours and then examined for liquefaction. A control tube of uninoculated medium was set in parallel. Liquefaction of the gelatin indicates that gelatin has been hydrolyzed (Cottral, 1978).

3.7.12 Carbohydrate Fermentation Test

This involved the preparation of peptone water and peptone water sugars fifteen (15) gram of peptone was dissolved in 1 litre of distilled water and 12.5 ml bromothymol blue was added as indicator. Bromothymol indicator was prepared by dissolving 0.1 gm in 2.5 ml of 0.1 mol/L (N/10) sodium hydroxide. The required quantity of sterile distilled water (47.5 ml) was added and mixed well.

One gram of the appropriate sugars (glucose, mannitol, sucrose, rhamnose, lactose, raffinose, salicin, mannose, dulcitol, sorbitol, galactose, and maltose) was dissolved in 9 ml of distilled water. This was added to the peptone water base, mixed thoroughly and 4 ml distributed into Bijou bottles with inverted Durham's tubes in some (for lactose and sucrose) and sterilized by autoclaving at 115^0 C for 20 minutes.

The *Aeromonas* suspected isolates were inoculated into the sterilized peptone water sugars, containing 0.2 % w/v bromothymol blue indicator and incubated at 37^0 C for 72 hours. Yellow (acid fermentation) indicated a positive result, while no change in colour (blue or green) indicated a negative result. Gas production was evidenced by the displacement of liquid in the Durham's tube in the medium.

3.7.13 Hemolysis of Sheep Red Blood Cells

All isolates were tested for hemolysis on 10 % sheep blood agar. The 10 % sheep blood agar was prepared by adding 10 ml of washed sheep blood to 90 ml of blood agar base based on standard methods (Altwegg, 1994). The organisms were picked from the nutrient agar slants and streaked onto the surface of the agar medium then incubated at 37^0 C for 24hrs. Production of β(beta) hemolysis was seen by the presence of clear zone around the point of bacterial growth. The α (alpha) hemolysis was evident with incomplete hemolysis with greenish or hazy outline around the organism, while λ(gamma) hemolysis was seen when there was no hemolysis at all (Sneath *et al*., 1986)

3.8 IDENTIFICATION OF VIRULENCE FACTORS AND GENETIC MARKERS

Selected biochemcially characterized isolates were grown on Brain Heart Infusion (BHI) broth overnight and transported in the broth medium in separate labelled test tubes to the Mary Hallaway Teaching Laboratory, Department of Biochemistry, ABU, Zaria. The identification of virulence factors and genetic markers included the following:

i. Extraction and quantification of soluble proteins.

ii. Isolation, purification and quantification of genomic DNA

iii. Agarose Gel Electrophoresis

iv. Restriction enzyme digestion using B*am* HI, E*co* R1 and S*al* 1.

The 26 strains of different species of *A. hydrophila, A. caviae, A. sobria* and *A. salmonicida* from different sources were subjected to molecular characterization.

3.9 PROTEIN ANALYSIS OF *AEROMONAS* SPECIES

The method of Biuret was used to quantify total protein in *Aeromonas* species (Nuto and Cheriton, 1972; Esievo and Saror, 1992).

The isolates were grown overnight in Brain Heart Infusion (BHI) broth for 37^0C for 24 hours. After incubation at 37^0 C, it was centrifuged at 10,000 g for 5 minutes using Harous labofuge. 0.5 ml of the supernatant was dispensed onto test tubes in duplicates. 2.0 ml of biuret reagent was added to the supernatant and incubated at room temperature for 30 minutes. Average of the two (duplicates) colorimetric readings was taken at 570 nm.

A blank was set in parallel and was prepared by adding 0.5 ml of distilled water to 2.0 ml biuret. The reagent was incubated at room temperature for 30 minutes and is used to zero the colorimeter.

A control tube was prepared by adding 0.5 ml of BHI (without organisms) and added to 2.0 ml of biuret reagent and the colorimeter reading taken at 570 nm The difference in colorimetric reading of broth culture without organisms was taken from the difference of colorimetric reading of broth culture with organisms.

A dendogram of average protein was extrapolated for the various *Aeromonas* species.

3.10 GEL ELECTROPHORESIS

3.10.1 Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS–PAGE) of *Aeromonas* proteins

This was carried out using the method of Laemmli (1970) and presented below.

a. Sample Preparation

Samples were prepared in separate test tubes containing *Aeromonas* 50 µl of loading buffer (made up of 2.0 ml glycerol, 2.0 ml of 10 % SDS, 0.25 mg bromophenol blue, 2.5 ml stacking gel buffer and 0.25 ml mercaptolethanol) was added and incubated at 90^0 C for 2 minutes.

b. Preparation of Running Gel (12 %) and Stacking Gel (4 %)

A mixture of 3.3 ml distilled water, 4.0 ml 30 % acrylamide mixture, 2.5 ml separating gel buffer pH 8.9, 0.1 ml 10 % SDS, 0.1 ml 10 % ammonium persulfulphate, and 0.01 ml TEMED was made and poured into assembled gel plates, leaving sufficient

space at the top for the stacking gel. 0.1 % SDS was gently overlayed on the gel mix. After polymerization, the overlay was removed and the surface of the separating gel was rinsed with water to remove any unpolymerized acrylamide.

The stacking gel was prepared by mixing the following; 1.4 ml distilled water, 0.33 ml 30 % acrylamide mixture, 0.25 ml stacking gel buffer pH 6.9, 0.02 ml 10 % APS, and 0.005 ml TEMED. This was used to fill the remaining space on the running gels and the comb inserted immediately.

c. Electrophoresis Run

After the stacking has been polymerized, the comb was removed, the wells rinsed to remove unpolymerized acrylamide.

Fourty microlitres of the prepared sample and marker protein were loaded into separate wells with the running buffer (glycerin/Tris buffer) poured into the buffer tank, the apparatus was coupled and connected to the power pack (a.d.c. supply). The power pack was switched on and operated at 25 mA, the current was increased at 50 mA when the samples were just entering the running gels.

The operation was stopped when the dye marker band was about 3mm to the end of the gels. The gels were removed and soaked in 12 % trichloroacetic acid (TCA) for 30 minutes to fix the proteins, and later transferred into Coomasie brilliant blue solution. After 12 hours, the gels were distained in a mixture of water; methanol; acetic acid (50:40:10). The protein bands became visible after rounds of distaining. The electrophoresis was repeated several times to ensure consistency of protein patterns.

d. Molecular Weight Determination

This was done as described using the formular of Webster and Campbell (1972). The following measurement taken were from the gels; gel length before staining, gel length after distaining, distance moved by dye marker and distance moved by protein band(s). The relative mobilities were calculated using the equation below;

$$\text{Mobility} = \frac{\text{Distance moved by band x gel length before staining}}{\text{Distance moved by dye marker x gel length after destaining}}$$

Since mobility α 1/ mol wt

Plot of log mol. Weights of marker against mobility was constructed and the molecular weight of the partially purified *Aeromonas* protein was extrapolated from the standard curve. Correlation coefficients was used as statistical methods to analyse the data generated from protein profiles of *Aeromonas* species.

3.11 PREPARATION OF *AEROMONAS* GENOMIC DNA

Total genomic DNA was extracted from harvested cultures using the Qiagen kit. 20 µl of proteinase K was dispensed into a 1.5 ml microcentrifuge tube, 200 µl of the separate organisms and 200 µl of the lysis buffer were added, vortexed and then incubated at 56^0 C. After 10 mins, 200 µl of absolute ethanol was added and vortexed.

The mixture was carefully loaded into a QLAMP spin column (in a 2 ml collection tube) and centrifuged. After 1 min the spin column was transferred into another collection tube and 500 µl buffer AW 1 is dispensed into it and centrifuged at 8000 rpm for 1 minute.

The spin column was again transferred to a clean collection tube and 500 µl buffer AW2 is dispensed into it and the centrifuged at 1400 rpm for 3 mins, after which the spin column was transferred into a 1.5 ml microfuge tube and 200 µl distilled water added, incubated for 1 min at room temperature, and then centrifuged at 8000 rpm for 1 min to elute the DNA.

a. Preparation of DNA Calibration Curve

Standard solution of Salmon sperm DNA was prepared (50, 100, 150, 200, and 250 µl/ml). The absorbance of each concentration was read at 260 nm, and the readings were plotted against the concentration values to obtain the standard curve.

b. DNA Purity Assessment

Absorbance of the extracted *Aeromonas* DNA was read at 260 nm and 280 nm, and the A260/A280 were calculated.

c. DNA Quantification

Absorbance of DNA at 260 nm was used to extrapolate for its concentration from the calibration curve.

3.12 AGAROSE GEL ELECTROPHORESIS OF *AEROMONAS* GENOMIC DNA

This involves the following procedures as described by Cokayne (1993).

a. Preparation of 1 % Agarose Gel

One gram of agarose was weighed in a beaker, 90 ml of water and 10 ml of 10 x Tris acetate – EDTA (TAE) buffer was transferred to it and mixed with a rod. The

agarose was dissolved by boiling in a microwave oven for 1min, after which 10 µl ethidium bromide was added and swired to mix.

The ends of the gel tray was sealed properly with tape while the dissolved (hot) agarose was allowed to cool to about 50^0 C. It was then poured into the sealed gel tray and the comb well inserted. After a solid gel formation, the tape and the comb were removed and the gel in the tray transferred to the buffer tank. IXTAE buffer was poured into the apparatus until it was 1 cm over the gel surface.

b. Loading the Samples

Five microlitres of loading dye (containing 1:1 of 5 % bromophenol blue and glycerol) was added to each 25 µl sample. Twenty microlitres of the stained samples were carefully dispensed into individual wells. In each run, 5 µl of marker ink was included in at least one well.

c. Running the Gel

The apparatus was coupled and connected to the power park which was set to run at 100 V. The DNA moved down to the positive terminal. The run was terminated when the dye front has traveled about 80 % of the gel length.

d. Visualizing DNA Bands

The gel was transferred into a UV transilluminator and photographed.

3.13 RESTRICTION ENDONUCLEASE DIGESTION OF *AEROMONAS* GENOMIC DNA USING B*am* H1, E*co* R1 AND S*al* 1

The enzymes E*co* R1, B*am* H1 and S*al* 1 were pre-tested on the 26 DNA samples and were determined to be the most appropriate enzymes for use as recommended by the suppliers.

Five microlitres (5 μl) of genomic DNA were dispensed into different 1.5 ml eppendorf tubes, followed by 39 μl of sterile distilled water, 5 μl of B*am* H1, E*co* R1 and S*al* 1 buffer and then 5 units each of B*am* H1, E*co* R1, S*al* 1 and Calf Intestinal Phosphatase (CIP). Each mixture was mixed and incubated at 37^0 C for 2 hours. To stop digestion, 5 μl of trace blue dye was added. After digestion, DNA fragments were separated by electrophoresis in 0.8 % agarose gel in 1XTAE buffer using 30 V for 15 hours. After 15 hours, electroporesis was terminated and the gels were visualized under UV transluminator and photographed (Cokayne, 1993).

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation of *Aeromonas* from Gut of Fishes, Poultry feces and Human Diarrhoeic stools

The bacteriological examination of feacal and stool samples for aeromonae was carried out in poultry, fishes and humans. Of the 1,160 samples tested, 195(16.8 %) yielded isolates while the rest were negative for isolates.

The 400 fishes examined comprised the following *Aeromonas* species 101 *Tilapia zillii,* 67 *Schilbe mystus,* 80 *Marcusenius senegalensis,* 67 *Clarias gariepinus,* 79 *synodontis filamentosus* and 6 *Alestes nurse*. *Aeromonas* species were recovered from 84 (21 %) samples (Table 4.1). *Tilapia zillii* and *Schilbe mystus* were more commonly encountered during the days of sampling in ABU dam while *Clarias gariepinus, Synodontis filamentosus, Alestes nurse* and *Marcusenius senegalensis* were commonly encountered in Zaria dam. The frequency of isolation of *Aeromonas* species from different species of fishes is contained in table 4.1. From the table, it was observed that *Tilapia zillii* yielded the highest number of isolates 24(23.76 %). There was no isolation of *Aeromonas* species from six of *Alestes nurse* species (Table 4.1).

The 540 poultry samples examined, yielded 82(15.18%) of the isolates (Table 4.2). *Aeromonas* species were recovered from 82 (15.18%) samples examined (Table 4.5). Brown harco (layers) were commonly encountered at the days of sampling in Zumunta farms, Area 'C', Zaria farms and Lape farms. Broilers (anak giant) were encountered at area 'F'. The percentage isolation of *Aeromonas* species from the different species of poultry examined. From the Table it was observed the 440 adult layers had the

highest prevanlence rate of 15.22 %. While broilers yielded 15% isolates. The overall frequency of isolation from the birds was found to be 82(15.18%).

Of the 220 stoll samples from diarrhoeic human, *Aeromonas* species were recovered from 29(13.18%) (Table 4.3). On the bases of age and sex, the result did show that more isolates (66.67%) were recovered from young males while 7.14% came from adult males. The result further revealed that young females yielded more (33.33%) isolates compared to adult females (8.33%). There was however, no association between sex and infection (OR = 1.20). There was however, age association between young and old.

Table 4.1 Species specific *Aeromonas* isolation rate from fishes

Fish species	Total number sampled	Total positive isolates	Percentage (%) Positive
Tilapia zillii	101	24	23.76
Schilbe mystus	67	11	18.33
Marcusenius senegalensis	80	17	21.25
Clarias gariepinus	67	14	20.89
Synodontis filamentosus	79	18	22.78
Alestes nurse	6	0	0.00
Total	**400**	**84**	**21.00**

Table 4.2 Isolation rate of *Aeromonas* from poultry in Zaria.

Poultry species	Total sampled	Total positive isolates	Percentage (%) Positive
Adult Broiler	100	15	15.00
Adult Layers	440	67	15.22
Total	**540**	**82**	**15.20**

Table 4.3 Prevalence of *Aeromonas* infection in relation to Age and Sex among the study population.

Sex	**Total number sampled**	**Total positive isolates**	**Percentage (%) positive**
Male	82	13	16
Female	138	16	12
Total	**220**	**29**	**13.18**
X^2(Yates corrected) = 0.06	P = 0.696	OR = 1.20 (0.50< OR<2.90)	
Age (years)			
Adult males>12 years	70	5	7.14
Adult females>12 years	120	10	8.33
Young males<12 years	12	8	66.67
Young females<12 years	18	6	33.33

4.2 Biochemical Characterization of the *Aeromonas* species

All isolates that gave typical reaction in most or all the tests were considered to belong to the genus *Aeromonas.* The isolates were subjected to the various biochemical tests (Table 4.4). The isolates that were oxidase positive, catalase positive, gram negative, indole positive, motile, citrate positive, MR positive, gelatin positive and esculin positive were considered to belong to the genus *Aeromonas.* It was also observed that most strains of *A.hydrophila* fermented glucose, galatose, mannitol and sucrose (Table 4.4).

A.caviae isolates were also motile, gram negative, oxidase and catalase positive, H_2S negative, indole negative, urease positive and were both oxidative and fermentative on TSI, esculin positive, produce gas with glucose and negative to dulcitol, salicin, sucrose, rahamnose and galactose (Table 4.4).

A.sobria was oxidase and catalase positive, gram negative, VP negative and negative to esculin, indole, gelatin, salicin, sobitol and rhamnose (Table 4.4).

A.salmonicida isolates were oxidase and catalase positive, gram negative non-motile, indole and gelatin positive, and positive to the following sugars: salicin, rhamnose, galactose, sorbitol and sucrose (Table 4.4).

The Table 4.4 also dissipated biochemical tests which differentiates *Aeromonas* from other members of the *Enterobacteriaceae* and related genera such as *Plesiomonas*, *Vibrios* and *Pseudomonas* and also strains of a specie (biotype) were also determined from the biochemical test results.

Table 4.4 Biochemical Characterization of Isolates of *Aeromonas* from Fishs, Poultry and Human samples

Biochemical test	Number of typical *Aeromonas* species tested							
	n =82		n=37		n=22		n=54	
	A. hydrophila		*A. caviae*		*A. sobria*		*A. salmonicida*	
	No (+)	no (-)	No (+)	no (-)	no (+)	no (-)	no (+)	no (-)
Oxidase	82	-	37	-	22	-	54	-
Catalase	82	-	37	-	22	-	54	-
Gram	82	-	37	-	22	-	54	-
Motility	82	-	37	-	22	-	-	54
H_2S	82	-	37	-	22	-	-	54
Indole	82	-	37	-	22	-	-	54
Urease	42	40	20	17	20	2	52	2
Citrate	70	12	17	20	22	-	54	-
TSI	32	50	27	10	15	7	27	27
Esculin	70	12	17	20	7	15	20	34
MR	82	-	37	-	22	-	54	-
VP	8	74	6	31	2	20	-	54
Gelatin	80	2	37	-	22	-	54	-
Glucose and gas	42	40	15	22	5	17	30	24
Salicin	-	82	-	37	-	22	-	54
Sorbitol	10	72	-	37	-	22	-	54
Sucrose	30	52	-	37	-	22	-	54
Rhamnose	35	47	37	-	5	17	27	27
Galactose	60	22	37	-	22	-	30	24
Mannitol	50	32	-	37	5	17	34	20
Maltose	40	42	-	37	-	22	-	54
Raffinose	10	72	-	37	-	22	-	54
Lactose	20	62	7	30	10	12	27	27
Mannose	2	80	-	37	2	20	-	54
Dulcitol	-	82	-	37	-	22	4	50

Key:

+: Positive

- : Negative

n : Number of isolates tested

4.3 Prevalence and Distribution of *Aeromonas* species from Fishes, Poultry and Humans

Table 4.5 shows the distribution of the different species of *Aeromonas* from the four hundred fishes examined. *A. hydrophila* 47 (11.5%), *A. caviae* 6(1.50%), *A. sobria* 12(3.00%) and *A. salmonicida* 19(4.75%). A total of 84 isolates were recovered from fishes with an overall prevalence rate of 21 %. *A.hydrophila* (11.75 %) yielded the highest number of isolates compared to the 1.5% from *A.caviae*.

Table 4.6 shows the distribution of the different species of *Aeromonas* from the 540 poultry samples. On the bases of frequency of isolation, *A. hydroplila* 19(3.52 %), *A. sobria* 5(0.93 %), *A. caviae* 23(4.06 %) and *A salmonicida* 35(6.48 %). The prevalence rate of 15.18 % was recovered from poultry. More isolates were recovered from *A.salmonicida* (6.48 %) than *A.sobria* (0.93 %).

Table 4.7 contained the distribution of different species of *Aeromonas* from the 220 human stools. *A. hydrophila* 15(6.81 %), *A. sobria* 6(2.73 %) and *A. caviae* 8(3.64 %). On the whole 29 isolates were recovered with an overall prevalence rate of 13.18 %. With *A.hydrophila* as the most prevalent *Aeromonas* species (6.81 %) and *A.sobria* (2.73 %).

Table 4.8 shows the distributions of different species of *Aeromonas* in different species of fishes. *A. hyrophila* was the most prevalent specie 47(11.75 %). *Tilapia zillii* harbours the highest number of *Aeromonas* species 24(23.76 %).

Table 4.9 shows the distribution of different species of *Aeromonas* from broiler and layers. *A.salmonicida* was the most prevalent pathogen isolated 35(6.48 %). Layers had higher numbers of *Aeromonas* than boilers 67(15.22 %).

The distribution of different species of *Aeromonas* in different sexes and age groups of diarrhoeic patients revealed that *A.hydrophila* was the most prevalent human pathogen with prevalence rate of 8(66.67 %). *A.salmonicida* was not isolated from humans in Zaria. *A. sobria* were not isolated in adult males and young females (Table 4.10).

Distribution of *Aeromonas* species sampled in fishes, poultry and humans were compared. The prevalence rate was highest in fishes 21% followed by poultry (15.2 %) and humans (13.8 %). In poultry, *A.hydrophila* was the most prevalent species 82 (7.07 %) and *A.sobria* was the least prevalent species of *Aeromonas* 22 (1.90 %) isolated from the three different sources. The overall prevalence rate in fishes, poultry and humans was (16.8 %) (Table 4.11).

Fig. 4.1 shows frequency in which *Aeromonas* species were isolated from dams and markets. Comparison showed that *A.hydrophila* were the most prevalent *Aeromonas* species isolated from A.B.U. dam, while *A.salmonicida* was the lowest *Aeromonas* species in Zaria dam.

Frequency of isolation of *Aeromonas* species from the poultry farms did show that *A.caviae* was the most prevalent *Aeromonas* species in Zumunta farms, while there was equal prevalence of *A.sobria* and *A.hydrophila* in Area 'C' as seen in (Fig. 4.2).

Fig. 4.3 shows the frequency of isolation of *Aeromonas* species obtained from A.B.U. sick bay and A.B.U. Teaching Hospital. Comparison showed that *A.hydrophila* occurred most frequently in A.B.U. sick bay, while *A.caviae* was lowest in A.B.U. Teaching Hospital.

Table 4.5 Distribution of different *Aeromonas* species isolated from 400 fishes examined.

***Aeromonas* species**	**Number of isolates**	**% Prevalence**
A. hydrophila	47	11.75
A. caviae	6	1.50
A. sobria	12	3.00
A.salmonicida	19	4.75
Total	**84**	**21**

Table 4.6 Distribution of different *Aeromonas* species isolated from 540 poultry examined.

Species	Number of isolates	% prevalence
A. hydrophila	19	3.52
A sobria	5	0.93
A. caviae	23	4.26
A. salmonicida	35	6.48
Total	**82**	**15.18**

Table 4.7 Distribution of different *Aeromonas* species isolated from 220 humans examined.

Species	Number of isolation	% Prevalence
A. hydrophila	15	6.81
A.sobria	6	2.73
A. caviae	8	3.64
Total	**29**	**13.18**

Table 4.8 The distribution of different species of *Aeromonas* in species of fish.

Fish Species	Number of fish tested	Number of *Aeromonas* species isolated				Number and % isolated
		A. hydrophila	*A. caviae*	*A.sorbria*	*A. salmonicida*	
Tilapia zillii	101	17	4	0	3	24 (23.76)
Schilbe mystus	67	6	0	5	0	11 (16.41)
Marcusenius senegalensis	80	6	2	5	4	17 (21.25)
Clarias gariepinus	67	9	0	0	5	14 (20.89)
Synodontis filamentosus	79	9	0	2	7	18 (22.78)
Alestes nurse	6	0	0	0	0	0 (0.00)
Total (%)	**400**	**47 (11.75%)**	**6 (1.5%)**	**12 (3%)**	**19 (4.75%)**	**84 (21%)**

Key:
Percentage (%): Number of fish isolated of *Aeromonas* tested.

Table 4.9 The distribution of different species of *Aeromonas* in poultry.

Bird spp	Number of bird tested	*A. hydrophila*	*A. caviae*	*A. sobria*	*A.salmonicida*	Number and % isolation
Broiler	100	1	4	1	9	15 (15%)
Layers	440	18	19	4	26	67 (15.22%)
Total (%)	**540**	**19(3.51%)**	**23(4.25%)**	**5(0.92%)**	**35(6.48%)**	**82(15.18%)**

Key:
Percentage (%): number of poultry *Aeromonas* tested.

Table 4.10 The distribution of different species of *Aeromonas* in humans

Human groups	**Number of humans tested**	***A. hydrophila***	***A. sobria***	***A.salmonicida***	***A. caviae***	**No. and % Isolation**
Adult males	70	3	0	0	2	5 (7.14)
Adult females	120	7	1	0	2	10 (8.33)
Young males	12	2	4	0	2	8 (66.67)
Young females	18	4	0	0	2	6 (33.33)
Total (%)	**220**	**16(7.27%)**	**5(2.27%)**	**0.00%**	**8(3.63%)**	**29(13.18%)**

Key:
Percentage(%): number of humans *Aeromonas* tested

Table 4.11 Distribution of *Aeromonas* species in sampled fishes, poultry and humans

Human groups	Number of humans tested	*A. hydrophila*	*A. sobria*	*A. caviae*	*A. salmonicida*	Prevalence
Fishes	400	47	12	6	19	84 (21%)
Humans	220	16	5	8	0	29 (13.8%)
Poultry	540	19	5	23	35	82(15.2%)
Total	**1,160**	**82 (7.07%)**	**22 (1.90%)**	**37 (3.19%)**	**54 (4.66%)**	**195 (16.8%)**

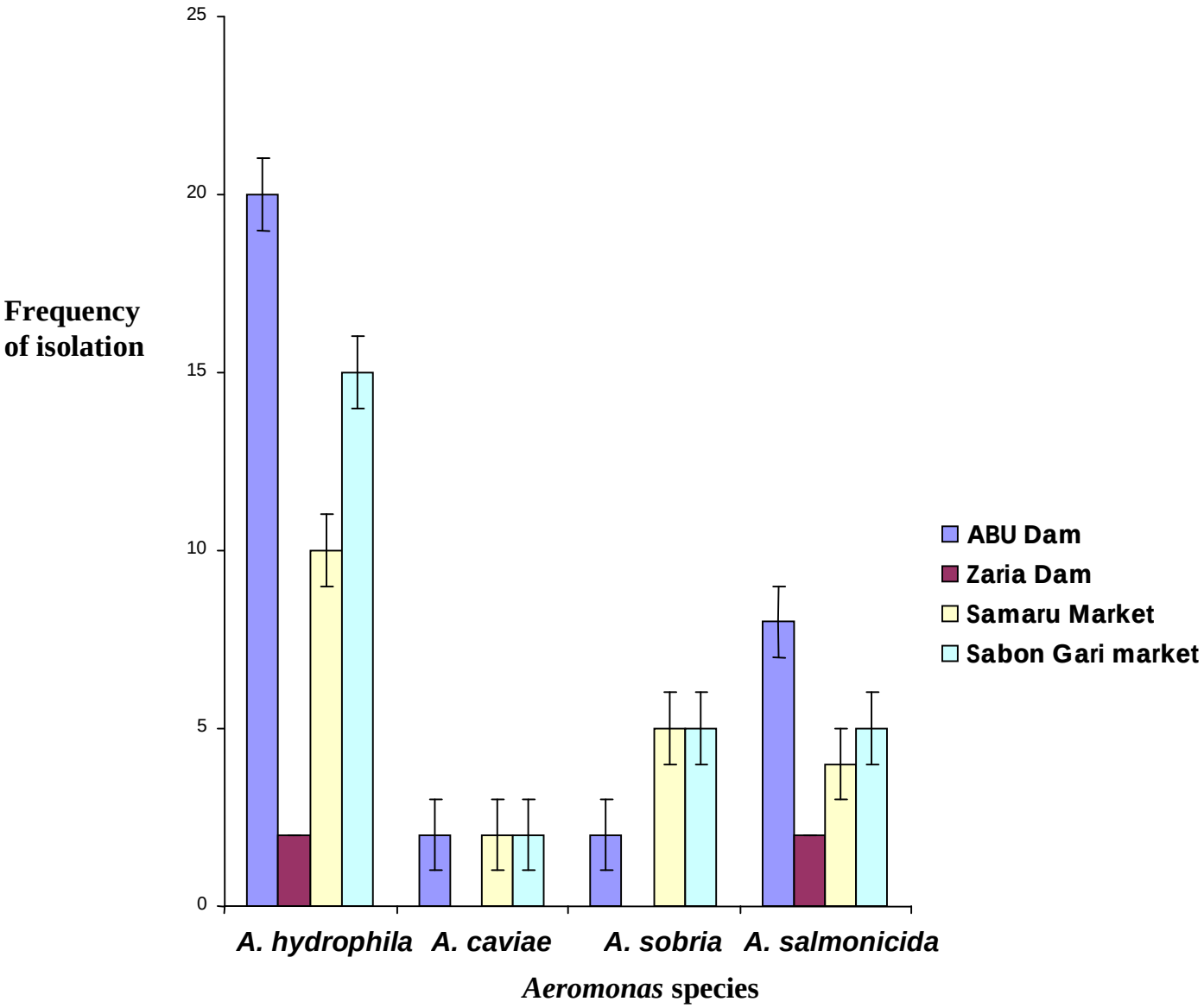

Fig. 4.1 Frequency of Isolation of *Aeromonas* species from fish obtained from dams and markets

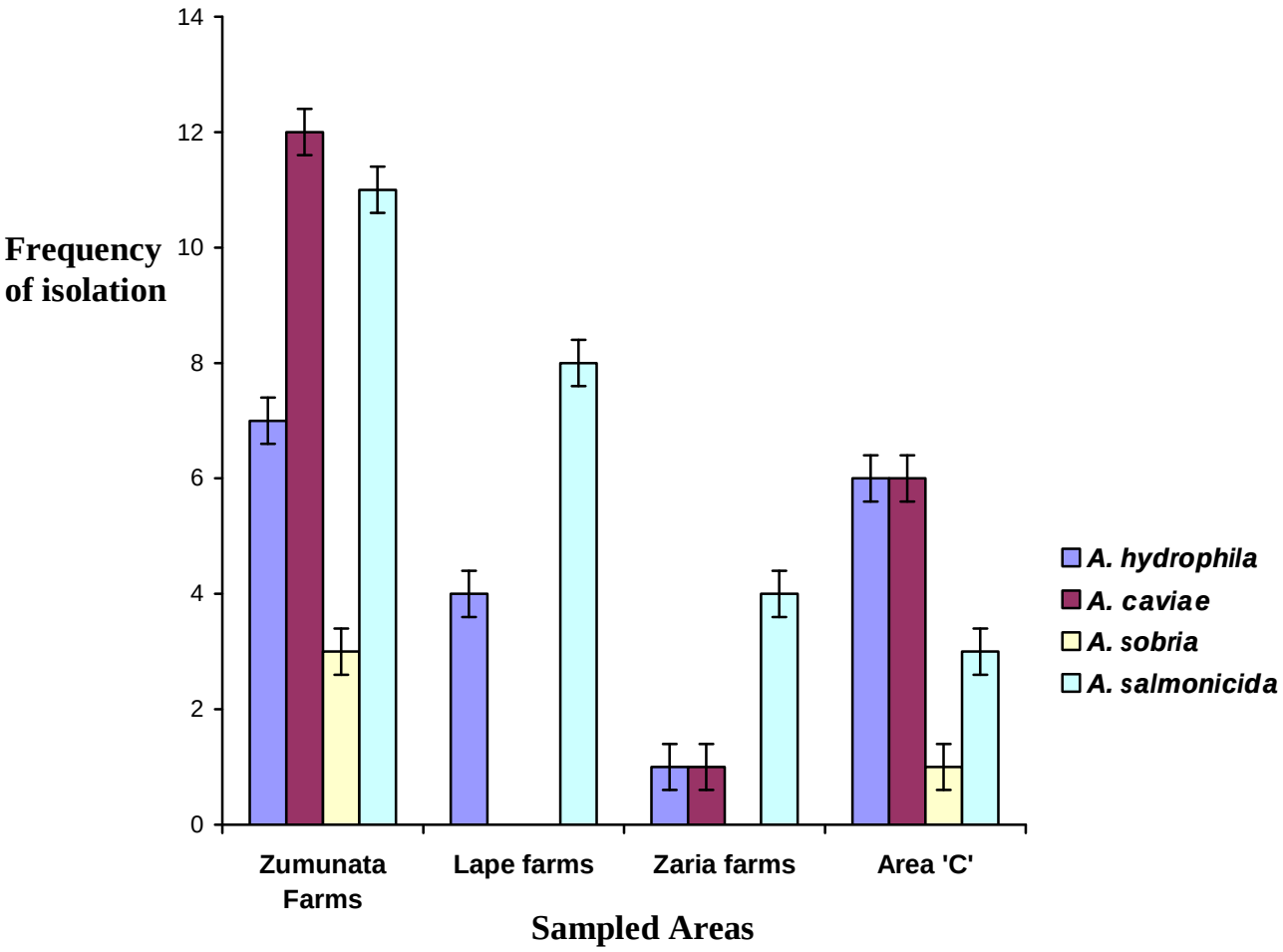

Fig.4.2 Frequency of isolation of *Aeromonas* species from Poultry obtained from commercial farms.

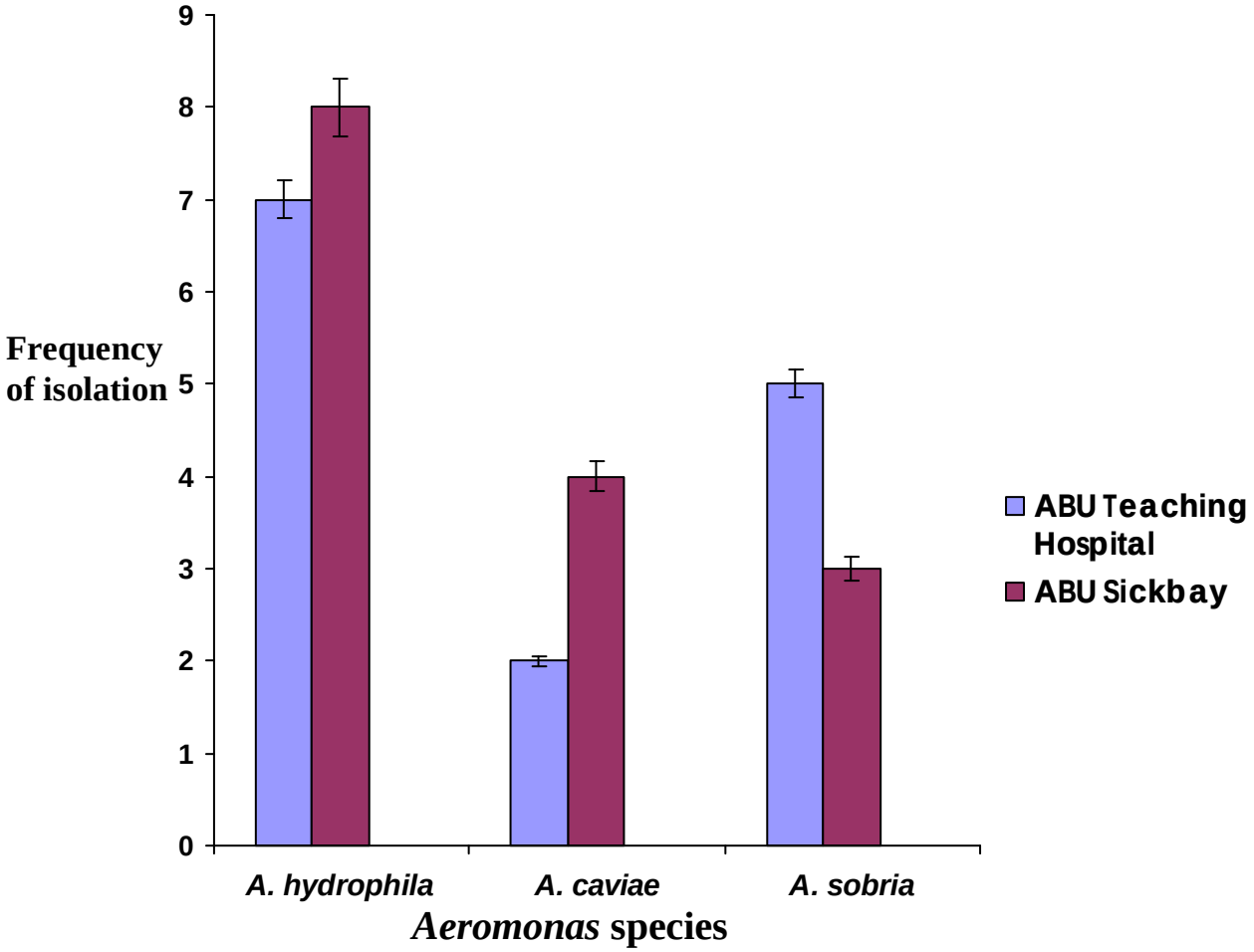

Fig. 4.3 Frequency of isolation of *Aeromonas* species from apparently normal human obtained from ABU sickbay and ABU Teaching Hospital in Zaria.

4.4 Hemolysin production in *Aeromonas* isolates on 10 % Sheep Blood

All the 195 *Aeromonas* isolates were tested for hemolysis using 10% sheep blood wich showed that 72 (36.92 %) were ß-hemolytic. *A.hydrophila* produced the highest amount of hemolysin compared to *A.sobria* (Fig. 4.4).

On species basis, *A hydrophila* 31(37.80 %) produced β -hemolysins, *A. caviae*, 14(37.83 %) produced β-hemolysins, out of 22 *A. sobria* 9(40.90 %) produced β-hemolysins, and *A.salmonicida* 18 (33.33 %) produced β-hemolysins.

Alpha hemolysis was observed in 10(5.13%) strains of *Aeromonas* and gamma hemolysis was in 113(57.95%) strains of *Aeromonas.*

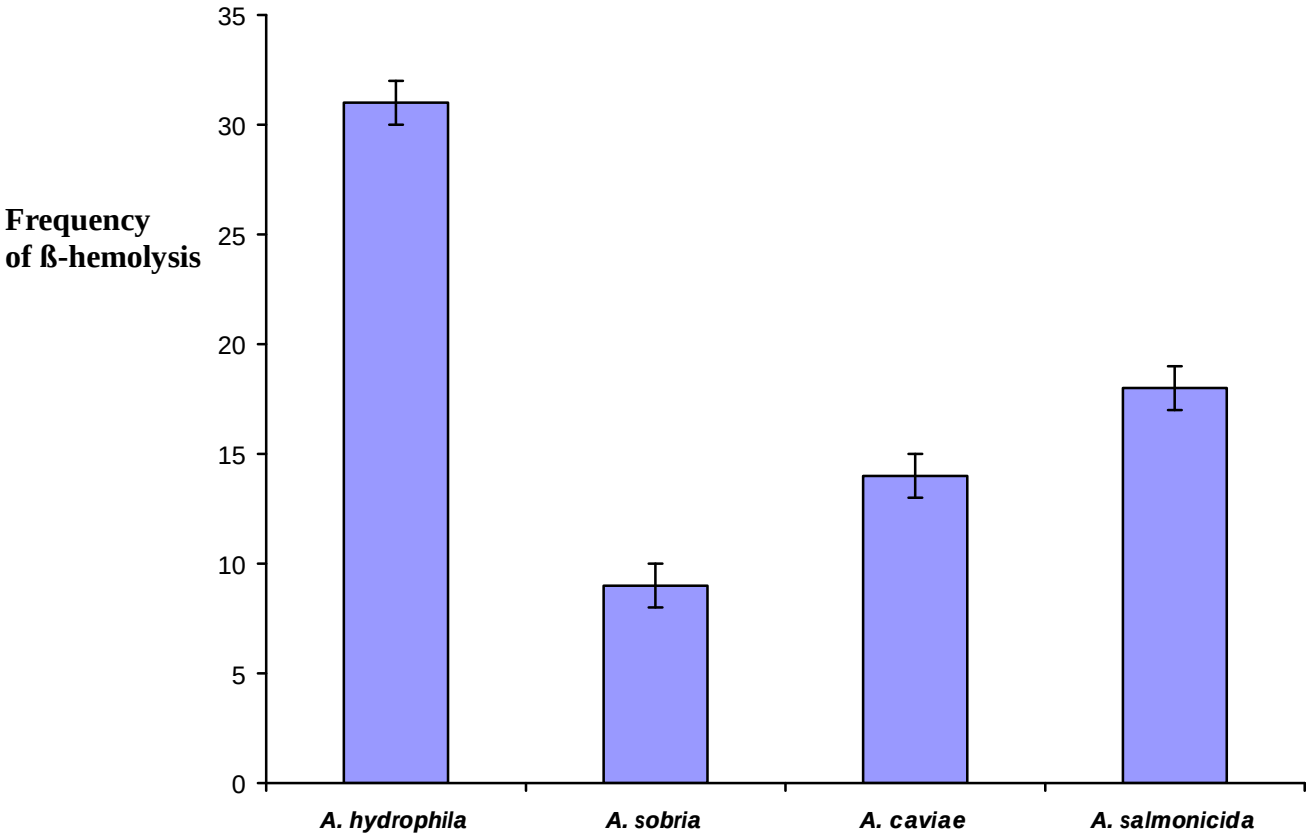

Fig. 4.4 Hemolytic patterns of *Aeromonas* species on 10% sheep blood tested from fishes, poultry and humans

4.5 Quantification of *Aeromonas* Proteins

The Fig. 4.5 shows the result of protein quantification of the various species of *Aeromonas.* Dendogram extrapolation of extracted and quantified *Aeromonas* protein by Biuret method revealed high protein concentration of 4.63 g from *A.sobria* from fish and 4.063 g of *A. hydrophila* from humans. No protein concentrations were recorded for *A.sobria* from poultry. Others had relatively lower *Aeromonas* protein concentration.

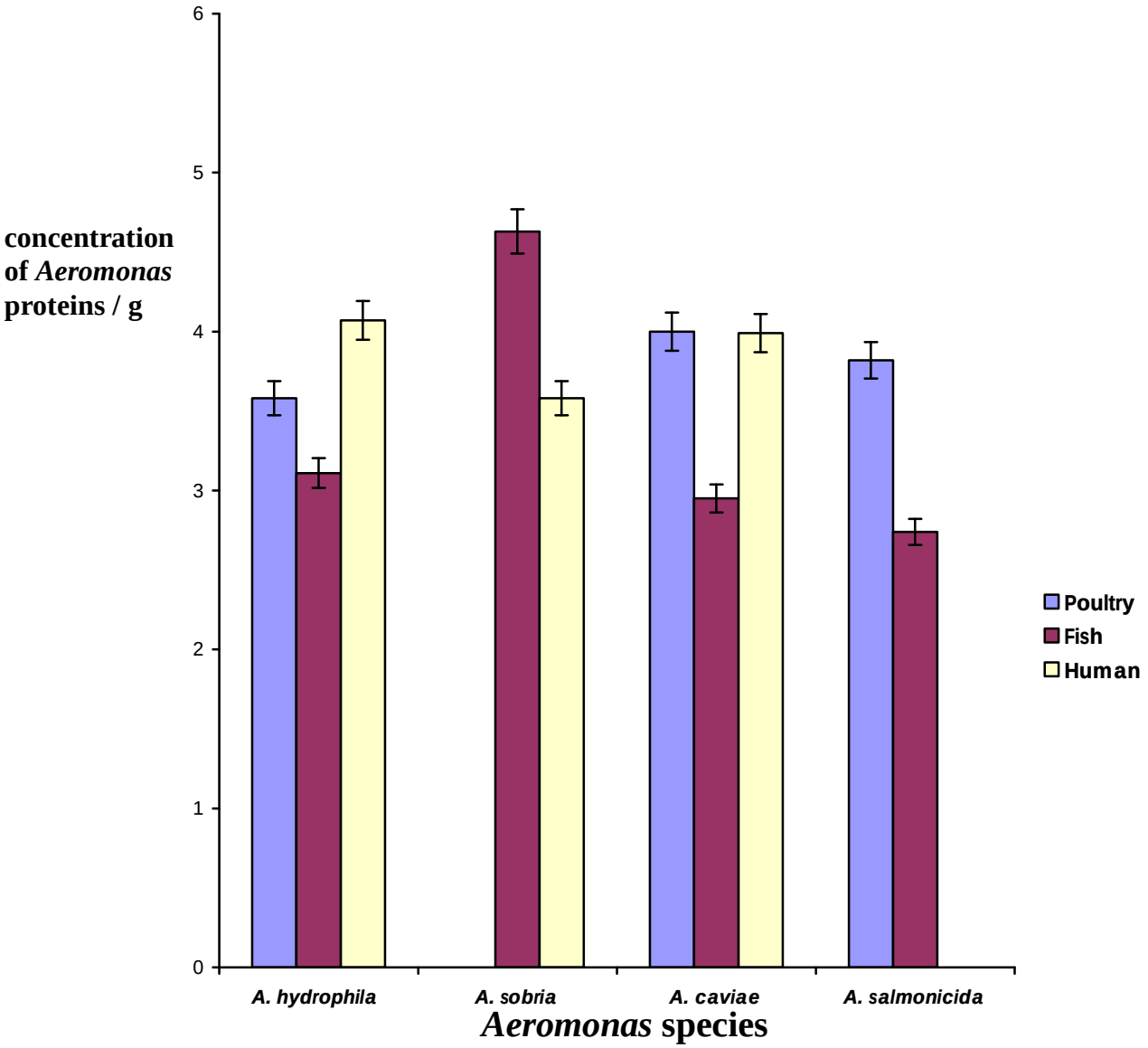

Fig. 4.5 Dendogram extrapolation of average proteins of species of *Aeromonas* isolated from fishes poultry and man

4.6 Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electropherograms were selected for use in *Aeromonas* species identification on the basis of the reproducibility of the protein patterns, the positions of the bands on the gels and the relative intensity of the bands.

Figs. 4.6-4.8 shows the band pattern of soluble proteins of *Aeromonas* species from fishes, poultry and man that revealed about 6 different types of toxins (proteins) and were visible on the gels when stained in Coomasie brilliant blue. On individual species, some were dimers, trimers and tetramers.

SDS-PAGE of the electrophoretic fingerprints yielded a unique elctropherogram in terms of position, intensity and number of bands revealed by the Coomasie staining. The protein bands of molecular weight between 35-73 kDa were recognized in gel electrophoresis.

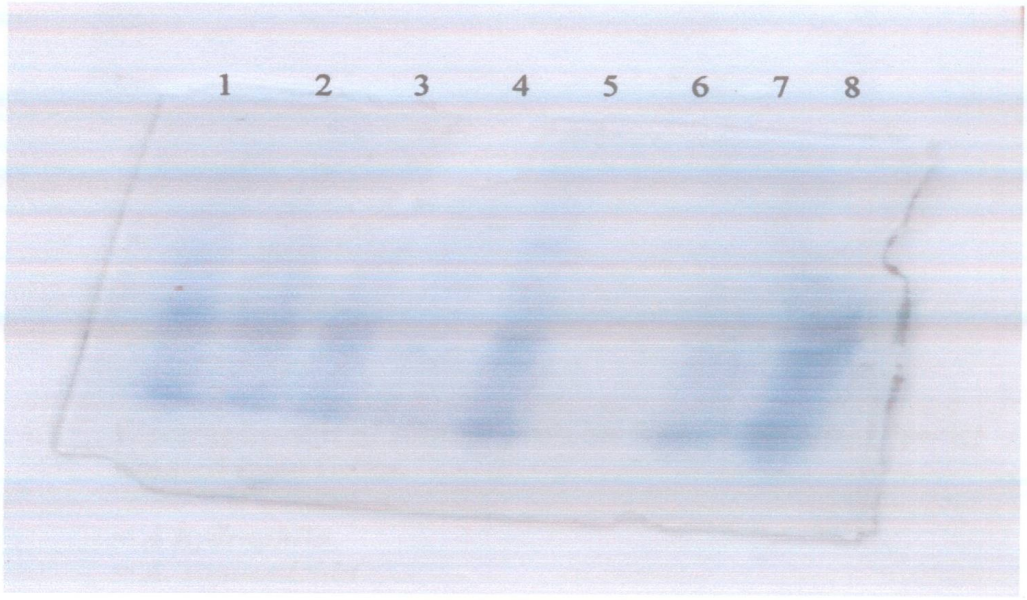

Fig: 4.6 **Electrophoresis of soluble proteins from *Aeromonas* species isolated from man.**

1, 5,8 = ***A. hydrophila***
2,3 = ***A.caviae***
4,6 = ***A.sobria***

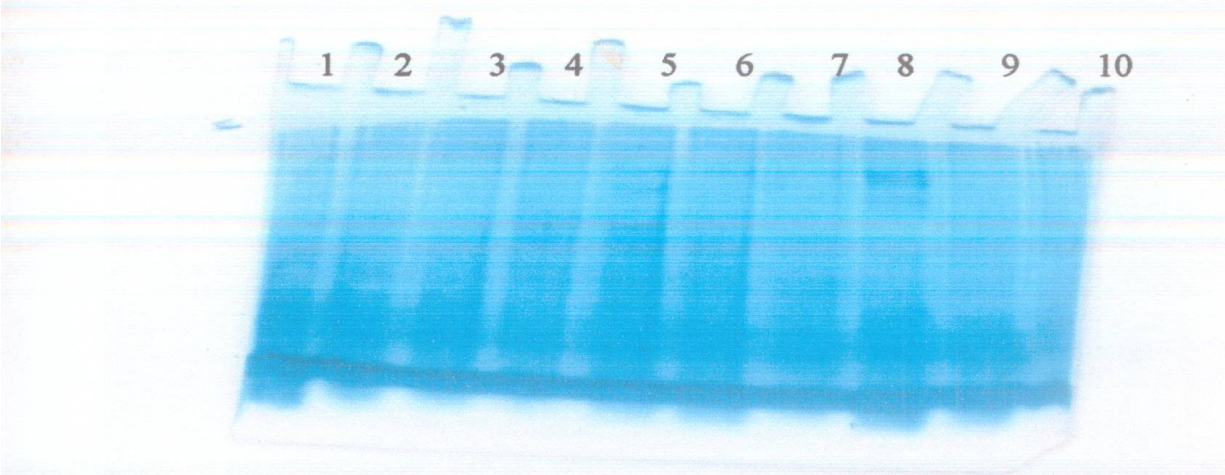

Fig : 4.7 **Electrophoresis of soluble proteins from *Aeromonas* species isolated from fishes.**

1, 5, 8	**= *A hydrophila***
2,3,10	**= *A. salmonicida***
6,9	**= *A.sobria***
7,4	**= *A.caviae***

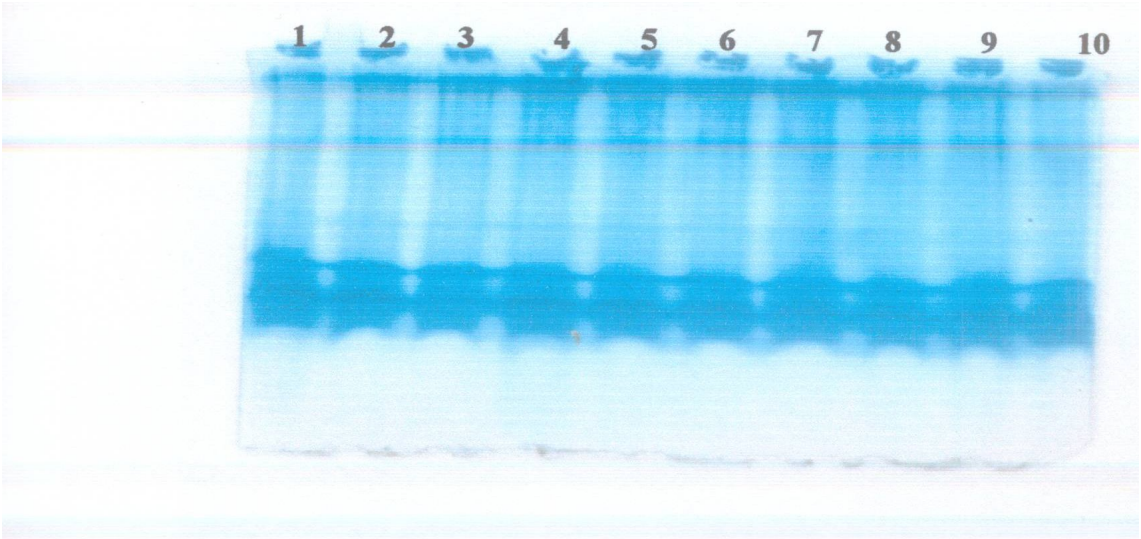

Fig: 4.8 **Electrophoresis of soluble proteins from *Aeromonas* species isolated from poultry**

1,2,10 = ***A.hydrophila***
3,4,9 = ***A.salmonicida***
5,6,7 = ***A.sobria***
8. = ***A.caviae***

4.7 Genomic DNA Analysis of *Aeromonas* species

Extraction of genomic DNA from *Aeromonas* species (*A.hydrophila, A.caviae, A.sobria* and *A.salmonicida*) using the Qiagen kit gave DNA yield of 450 and 425 µg/ml, 450 µg/ml and 375 µg/ml for the strains respectively (Table 4.12). The DNA extracts were of high purity as shown by the A260/A280 ratio of 1.6-1.8 (Table 4.13). A pure DNA extract should have A260/A280 of 1.6-1.8 (Zyskind and Bernstein, 1992). The DNA bands dissipated high molecular weight when purified by agarose gel electrcphoresis (Fig. 4.9).

Fig: 4.9 **The arrows above shows bands after Agarose Electrophoresis of Genomic DNA .**

Table 4.12 Quantification of *Aeromonas* Genomic DNA

Sample Number	Species of *Aeromonas*	A260	DNA concentration µg/ml
T 22	*A.hydrophila*	0.035	450
T 30	*A.caviae*	0.017	425
SM 23	*A.sobria*	0.016	450
RF 59	*A.salmonicida*	0.003	375
T3	*A.salmonicida*	0.012	425
CL 38	*A.hydrophila*	0.039	375
ZF 87	*A.caviae*	0.028	475
HTH 67	*A.caviae*	0.038	350
HTH 44	*A.caviae*	0.018	450
BC 29	*A.salmonicida*	0.007	425
HTH 41	*A.hydrophila*	0.018	425
CG 18	*A.hydrophila*	0.029	450
ACL38	*A.sobria*	0.016	475
HS 7	*A.hydrophila*	0.012	425
ZF 16	*A.hydrophila*	0.013	425
BC 11	*A.salmonicida*	0.010	450
SM 23	*A.sobria*	0.018	325
SM 12	*A.hydrophila*	0.007	475
CL 45	*A.hydrophila*	0.010	425
HS 2	*A.caviae*	0.019	425
HTH 25	*A.hydrophila*	0.018	425
CL 18	*A.hydrophila*	0.038	450
HTH 64	*A.hydrophila*	0.028	425
ZF 53	*A.hydrophila*	0.028	425
HS 37	*A.hydrophila*	0.016	475
ZF 26	*A.hydrophila*	0.038	325

Table 4.13 *Aeromonas* DNA Purity Assessments

Sample	A260	A280	260/280	Approx	Species of organism
CL 38	0.039	0.024	1.625	1.6	*A. hydrophila*
T3	0.012	0.008	1.757	1.6	*A. salmonicida*
ZF 87	0.028	0.017	1.647	1.8	*A. caviae*
HTH 67	0.038	0.030	1.266	1.3	*A. caviae*
HTH 44	0.018	0.100	1.800	1.8	*A. caviae*
MS 37	0.029	0.017	1.705	1.7	*A. sobria*
BC29	0.007	0.004	1.750	1.6	*A. salmonicida*
HTH41	0.018	0.011	1.636	1.6	*A. hydrophila*
CG18	0.029	0.017	1.705	1.7	*A. hydrophila*
T30	0.017	0.010	1.700	1.7	*A. caviae*
T22	0.035	0.020	1.750	1.8	*A. hydrophila*
ACL38	0.016	0.009	1.777	1.8	*A. sobria*
HS7	0.012	0.008	1.575	1.6	*A. hydrophila*
RF 59	0.018	0.014	1.636	1.6	*A. salmonicida*
ZF16	0.013	0.008	1.625	1.6	*A. hydrophila*
BC11	0.010	0.006	1.666	1.7	*A. salmonicida*
SM23	0.018	0.013	1.384	1.3	*A. sobria*
SM12	0.007	0.004	1.750	1.8	*A. hydrophila*
CL45	0.010	0.006	1.566	1.6	*A. hydrophila*
HS 2	0.019	0.012	1.583	1.6	*A. caviae*
HTH 25	0.018	0.011	1.636	1.6	*A. hydrophila*
CL18	0.038	0.022	1.727	1.7	*A. hydrophila*
HTH 64	0.028	0.018	1.555	1.6	*A. hydrophila*
ZF 53	0.028	0.017	1.647	1.6	*A. hydrophila*
HS 37	0.016	0.009	1.777	1.8	*A. hydrophila*
ZF 26	0.038	0.030	1.266	1.3	*A. hydrophila*

4.8 Analysis of *Aeromonas* Restriction Digests

Fig. 4.9 shows the Genomic DNA of *Aeromonas* species, the intensity of the bands enables us to confirm the quality of the gene.

Figs. 4.10- 4.12 shows the dissipation of several molecular fragments of *Aeromonas* genomic DNA Digested with E*co* R1, yielding fragments of molecular weight ranging 450 bp - 1.5 kb. Also restriction fragments generated via digestion with B*am* H1 yielded restriction fragments of molecular weight ranging from 950 bp and 450 bp. Digestion with S*al* 1 also showed fragments of molecular weight ranging 850 bp-450 bp.

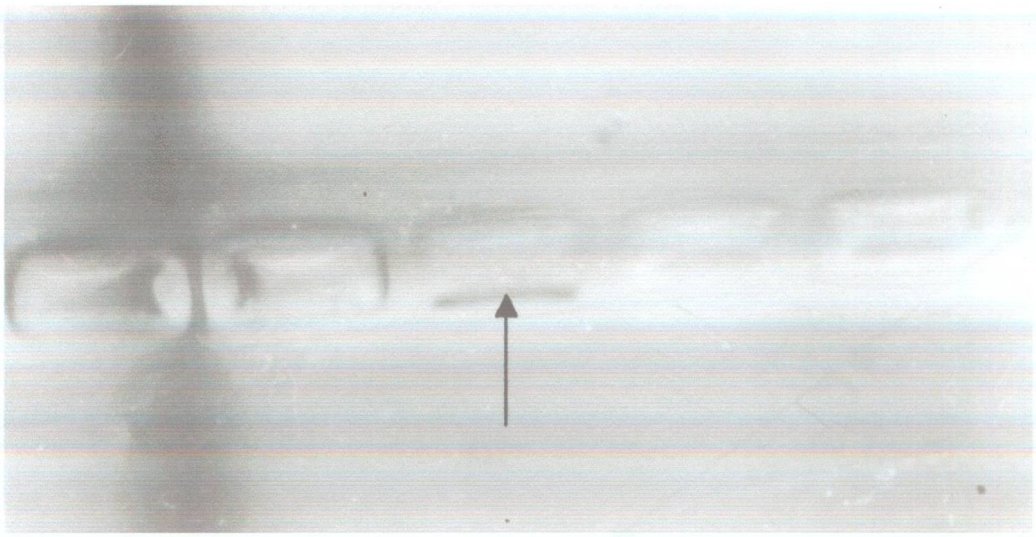

Fig: 4.10 The arrow shows the restriction fragment generated after DNA digestion with B*am*HI.

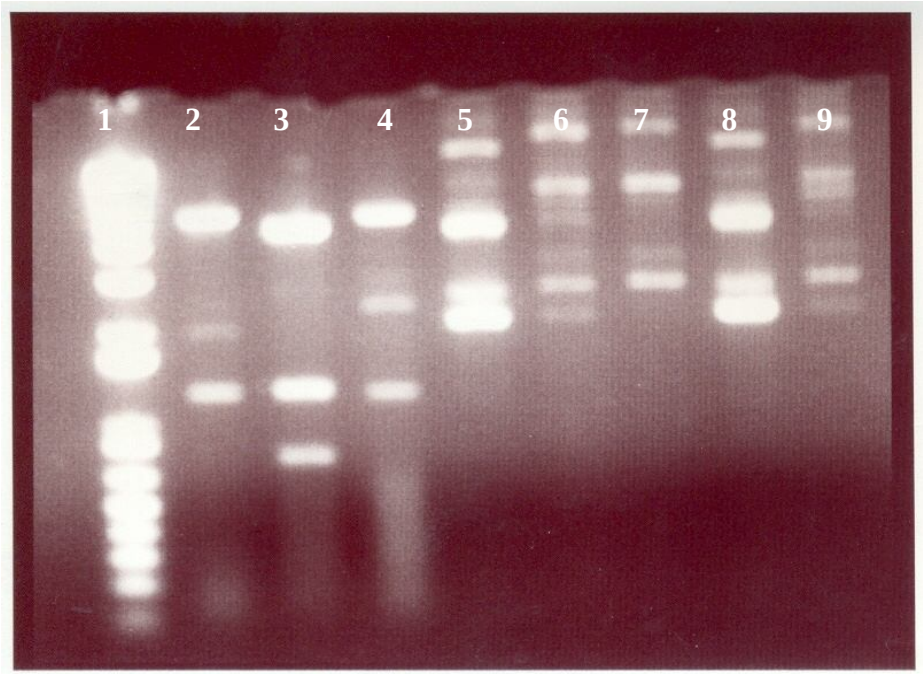

Fig. 4.11

Restriction fragments of *Aeromonas* species generated after digestion with E*co* R1

Key:
1 : Ladder
2 – 9: E*co* R1 restriction fragments

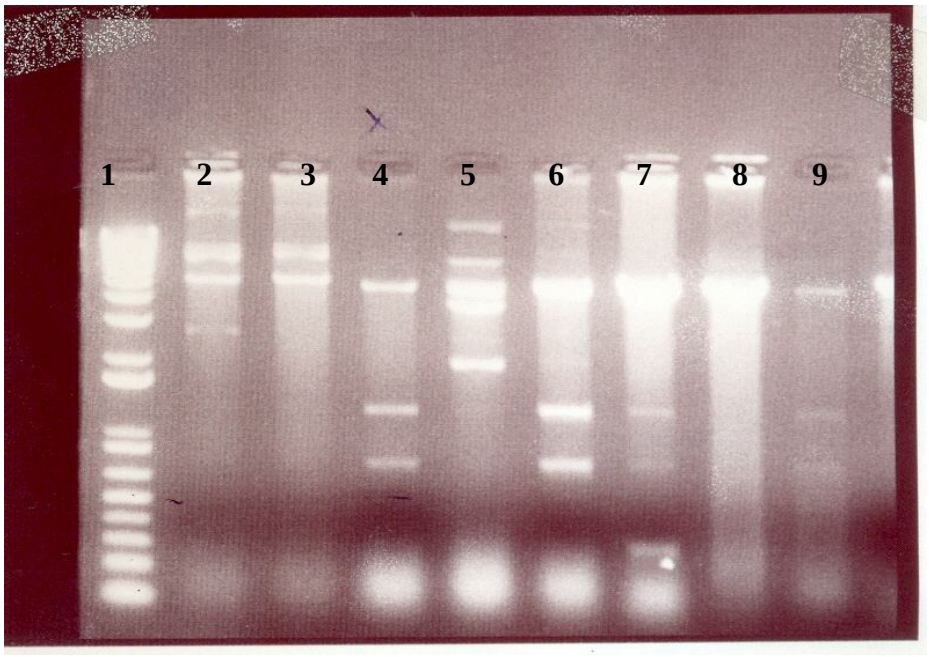

Fig. 4.12

Restriction fragments of *Aeromonas* species generated after digestion with B*am* H1 and S*al* 1

Key:
1 : Ladder
2 – 4: B*am* H1 restriction fragments
5 – 9: **S*al* 1 restriction fragments**

CHAPTER FIVE

5.0 DISCUSSION

The recovery of 195 strains of *Aeromonas* species from feacal samples indicates the suitability of this medium for field isolation of members of the genus *Aeromonas*. The research conducted also documented the occurrence of *A.hydrophila, A.caviae, A.sobria* and *A. salmonicida* in fishes, poultry and humans in the Zaria area.

There are several bases for a diagnosis of aeromoniasis, but the definitive one is isolation of the organism (Flyod, 2002). The identification of *Aeromonas* strains from fishes, poultry and humans as revealed by this study are therefore of public health significance. Most previous report on the investigation of aeromoniasis among fishes, poultry and human population were based on clinical signs and there have not been much effort to isolate and characterized aeromonae from fishes, poultry or human population in Nigeria (Thune *et al.,* 1982; Towner and Cockayne, 1993).

Review of literature from various parts of Nigeria have revealed that aeromoniasis is widely distributed in the country. There is a pattern of low, medium and high prevelance in specific areas and prevalence also varies among animal species in the same area (Abbey and Etang, 1988; Abbey *et al.,* 2004). The confirmatory diagnosis of aeromoniasis is based on isolation and identification of the organism from the infected host. The isolation and subsequent characterization of *Aeromonas* species from poultry, fish and human is therefore very significant.

The overall prevalence rate of 21 % of *Aeromonas* species found in fishes in this area was higher than the rate of 10.2 % found in fishes in Japan (Arai *et al*., 1980). It was also higher than the 3.8 % isolation rate reported by Kwaga *et al*. (1988) in Zaria.

Conversely, higher prevalence rate of isolation is a signal to public health authorities that aeromoniasis is on the increase. Appropriate measures need to be put in place to control and prevent this disease. The overall prevalence of 15.2 % found in poultry feces contrasts with Carnahan (2000) who isolated 12 % *Aeromonas* species from poultry feces in USA and the 2.5 % reported by Abbey *et al.* (2004). Our results clearly shows the existence possible seriousness of this disease in fish, poultry and humans in Zaria. The high prevalence of *Aeromonas* species reported in this study warns on public food safety problem in Nigeria. In most parts of Nigeria, people handle fish and poultry products with bare hands and sometimes with contaminated water which may promote the spread of the disease.

The overall prevalence rate of 13.8 % found in diarrhoeic patients is in agreement with the of 13.0 % reported by Janda *et al*. (1991) in Poland. It also corroborate well with the 13.2 % reported by Alvandi and Ananthan (2005). This suggests that *Aeromonas* infection might be food borne or water borne rather than air borne in origin. Lower incidence rates were observed in poultry and humans, thus suggesting that these samples may be transient hosts. Other factors responsible for the differences may include geographical location, seasons of the year, species of fishes, or poultry, health status of the individual and methods of isolation.

The knowledge and understanding of the prevailing species and biotypes of *Aeromonas* infecting fish, poultry and man are necessary to formulate appropriate policies and strategies for the control of aeromoniasis in a population.

Biotyping of *Aeromonas* species is generally regarded as an important epizootiological tool. Once a new infection has been confirmed in a flock, it is critical to

prevent further spread of the disease to other areas. It is generally important to determine by epidemiological trace-back analysis where infection originated, how it is spread and what measures needed to curtail the spread of the disease from the primary source. Wherever possible, trace-back is confirmed by comparison of the outbreak strain with isolates obtained from primary source (Janda *et al*., 1991; Bricker *et al*., 2000).

From the present results, it was observed that none of the fish species, poultry or humans was found to harbour two or more biotypes both of either *A.hydrophila, A. caviae, A.sobria* or *A. salmonicida*. This compares favourably with Van Damme and Vandepitte, (1980); Kwaga *et al.,* (1988); Abbey and Etang, (1988) as their reports revealed that one fish habours two biotypes of *A.hydrophila*. The fact that only one biotype of *Aeromonas* was encountered in this study does not indicate that these species and biotypes are representatives of Nigeria. They can be said to be representative of the areas which thay were obtained. It is therefore suggested that similar studies should be undertaken to cover larger areas of Nigeria. Such studies may identify more species and biotypes and add to knowledge of the epidemiology of aeromoniasis in Nigeria. It is also important to establish prevalence and distribution of aeromoniasis in our country.

The distribution of different *Aeromonas* isolates in some fish species tested showed, that *A.hydrophila* was the most prevalent at 11.75 % followed by *A.salmonicida A.caviae*, and *A.sobria* with prevalence rates of 4.75 %, 3.00 % and 1.50 % respectively.. The high prevalence of *A.hydrophila* is in agreement with previous workers (Austin and Allen-Austin, 1985, Santose *et al.,* 1988; Palumbo *et al*., 1989; Ocholi, 1991; Olanike, 1999 and Yakubu *et al.,* 2005). The prevalence rates they obtained were; 11.65 % *A.hydrophila,* 4.70 % *A.salmonicida,* 2.99 % *A.caviae* and 1.50 % *A.sobria.*

Interestingly, the different fish species examined showed variations in prevalence of infection with *Aeromonas* species. The prevalent rates in the different species were *Tilapia zillii* (23.76 %), *Synodontis filamentosus*, (22.78 %), *Marcusenius segnegalensis* (21.25 %), *Clarias gariepinus* (20.89 %), *Schilbe mystus* (16.01 %), while no isolation was made from *Alestes nurse*. Zero prevalence encountered in *Alestes nurse* might be due to the small sample size taken from such fishes. Because of the involvement of these organisms (*A.hydrophila, A.caviae* and *A.sobria*) in human infections (Van-Damme and Vandepitte, 1980; Kwaga *et al*., 1988) reported that their presence in fish might be a potential source of human infection especially where fish are not properly handled before consumption. The prevalence of *A.salmonicida* has been associated with fish spoilage and fish diseases or aeromoniasis outbreaks in cultured ponds (Krieg and Holt, 1984 and Floyd, 2002).

The distribution of the *Aeromonas* species from the two poultry species tested showed that *A. salmonicida* was most prevalent (6.48 %) followed by *A. caviae* 4.26 %, *A. hydrophila,* 3.52 % and *A.sobria* 0.93 % respectively. The higher prevalence of *A.salmonicida* in poultry is in contrast with reports of previous workers (Hinton *et al.*, 2003) who isolated 0.8 % *A.salmonicida* in United States of America. Interestingly, the different poultry species examined showed variations in prevalence of infection with *Aeromonas* species. The prevalence rates in different species were broilers (15 %) and layers (15.22 %). The factors responsible for this difference in poultry might be due to season of the year, system of production, type of feeds given to birds, nature of water in terms of hygiene and certain other environmental factors.

The distribution of different species of *Aeromonas* species in humans indicates that prevalence rate was highest for *A. hydrophila* (6.81 %) followed by *A.caviae* (3.64 %) and *A. sobria* (2.73 %). This agrees with prevalence of *A. hydrophila* (6.1 %) *A. caviae* (3.1 %) and *A. sobria* of (2.2 %) reported by Janda and Brenden (1987). *A.salmonicida* was not isolated from humans in Zaria. This can be attributed to its host specificity, moreover, *A.salmonicida* is a non-zoonotic pathogen (Krieg and Holt, 1984; Baron and Finegold, 1990). From the results of this study, there is an association between age and the rate of *Aeromonas* infection ($x^2 = 6.01$, df = 2, $P<0.05$). Young males <12 years have the highest rate of infection (66.67%) followed by young females (33.33%). Adult males and adult females had lower infection of 7.14% and 8.33% respectively. This conforms to the works of Abbey *et al*. (1988) in Nigeria. The fact that *Aeromonas* infections is more common in young might be attributed to the fact that children have lower immunity than the elderly and more likely to be infected by virulent strains of *Aeromonas*. Children at this young age have uncontrollable vices and may be exposed to niches and surfaces that are easily contaminated. The habit of indiscriminate picking up and putting objects in the mouth also encourages transmission of the bacteria.

Since *A.hydrophila*, *A. sobria* and *A. caviae* are implicated in human infection, Van Dame and Vandepitte (1980); Kwaga *et al.* (1988); Mailafia, (2003); and Yakubu *et al.* (2005), the prevalence in fish and poultry might be a potential source of human infection especially where fishes and poultry are not properly cooked or handled before consumption. This shows that these products may be possible vehicle for the dissemination of food-borne *Aeromonas* gastroenteritis (Sha *et al.*, 2002). The high prevalence of *A. salmonicida* may also be associated with reported fish and poultry

disease (aeromoniasis) outbreaks in cultured ponds and in poultry farms (Krieg and Holt, 1984; Calnek *et al.,* 1997, Floyd, 2002 and Esteve *et al*., 2003).

The presence of motile *Aeromonas* species in humans might indicate horizontal transmission of infection from fish or poultry to man. The incorporation or mixing of poultry manure and fish meal and inclusion of fish in poultry diet or feeding fish with contaminated water might be a potential source of cross infection with *Aeromonas* species (Floyd, 2002). Humans could also handle with their bare hands both fish and poultry products which might be a potential source of contamination.

Kwaga *et al.* (1988) used tetrathionate broth as enrichment followed by plating on Xylose Lysine Desoxycholate (XLD) and had isolation rate of *A.hydrophila* to be 3.8 %. Olanike (1999) and Yakubu *et al.,* (2005) used starch-ampicilin as plating medium obtained isolation rate of 47 % and 48 % receptively. In the present study, tetrathionate broth was used as enrichment followed by plating on macConkeys agar gave an over all prevalence rate (in fishes poultry and man) 16.8 %. The isolation rate of *Aeromonas* species in this study using tetrathionate broth was higher than isolation rate when other aforementioned enrichment were used. This will lead to the suggestion that *Aeromonas* species will best be isolated using tetrathionate broth. The lower prevalence rate reported by Kwaga *et al.* (1988) between April and May entails low prevalence at the time the research was conducted. It will appear that the prevalence of *Aeromonas* in Zaria is on the rise, therefore appropriate control measures is needed.

The general biochemical profile in this study was indicated by quantitative phenotypic reactions which were typical for the genus *Aeromonas* and the organisms showed mesophilic behaviour of being able to grow at 37^0 C. This agrees with the

findings of Gomez-leon *et al.* (2005) whose isolate(s) showed mesophilic behaviour on MacConkey agar.

Motility test yielded results for motile *Aeromonas* group, and very variable in *A. salmonicida*. Production of indole was also used to separate *A. hydrophila* from *A. caviae* and *A. sobria* (Figueras *et al.*, 2000; Olayinka *et al.* 2004). However, this properties were crucial in distinguishing species of motile and non motile *Aeromonas*. Another feature used to distinguish *A. caviae* and *A. sobria* was esculin hydrolysis in which *A. caviae* produce esculin and *A. sobria* did not produce esculin, therefore the production of the brown diffusible pigment, is lacking in other species (Wicklund and Dalsgaard, 1988). However, this is in agreement with Austin *et al.* (1998). It was observed by Dalsgaard *et al.* (1998) who found *A. caviae* to produce a brown diffusible pigment. Reaction on TSI and citrate utilization were tested on the strains of different species of motile *Aeromonas* and were positive (Palumbo *et al.*, 1989; Pavan *et al.*, 2000; Alam *et al.*, 2006).

Other biochemical tests such as methyl red and vogues proskauer test, gelatin liquefaction, sucrose fermentation, grams staining, oxidase, catalase were all confirmatory biochemical tests that were used to confirm the genus *Aeromonas* (Huys *et al.*, 2001). An extensive revision of the biochemical tests proposed by different authors to differentiate the species of the *Aeromonas* complex revealed the existence of many contradictory data, such as acid production for sobitol, salicin and sucrose. While most other species were alkaline on rhamnose, galactose, mannitol, maltose and raffinose (Abott *et al.*, 1992). These tests are therefore useful to distinguish these four species of *Aeromonas* (Kaznowski, 1998; Ullman *et al.*, 2005). The results of this study mostly agree with the results of Abbott *et al.*, (1992) and indicated that *A. salmonicida* is salicin,

sorbitol, sucrose, maltose and raffinose negative while the *A. hydrophila* group shows varying degrees of possitivity to sugars (Abott *et al.,* 1992).

β-hemolysin production was highest in *A.sobria* and is in agreement with the findings of Bath *et al*. (1974) and is also in agreement with the reports of Ibrahim *et al.* (1996) in Saudi Arabia, who found some isolates of *A. hydrophila* to produce 100 % β –hemolysins. Okrend *et al.* (1987) found β–hemolysins rate of 93 % in isolates from raw poultry. Hence, the proportion of β –hemolytic strains reported in this study was low compared to previous study. The factors responsible for this differences may lie in the strains and species differences within the genus *Aeromonas.*

The results of this study implies that majority of the *Aeromonas* species tested in this environment produced hemolysins. Our findings is agreement with the virulence properties and hemolysin production by *Aeromonas* species isolated from fish (Santose *et al*., 1988). It also confirms the assertion by Chopra *et al*. (1993); Rahman *et al*. (2002) that *Aeromonas* species are known to produce cytolytic, cytotoxic, heat stable and heat labile enterotoxigenic gene. The gene is known to have a multivirulence property and is involved in lethality, hemolysis and enterotoxigenicity. Hence, β-hemolysis is a good model for *in vitro* hemolysin detection with excellent reproducibility (Mosimabale, 1980; Carmen *et al.,* 2004; Duplessis *et al.,* 2006).

The average amount of protein concentration per gram of cells was determined for the 26 isolates of *Aeromonas* species using spectrophotometric analysis. Dendogram was extrapolated and shows the total amount of protein and the output of protein expression

and possible nucleotide sequence of the *Aeromonas* species involved in pathogenicity. It could be deduced that the highest protein concentration was in *Aeromonas sobria* from fish, this may be indicated for a particular fish disease or other pathological lesions caused by *A. sobria* as reported by Cipriano and Bullock (2001). Moreso, quantity is a reflection of synthesis of proteins and this proteins are expressed by the DNA which could entail that there may be mutation leading to renewed synthesis of new proteins to cope with the adverse effects of the environment. The lower protein in *A. salmonicida* in fish may be associated with lower virulence and may be responsible for the lower outbreaks of fish diseases in some of our cultured ponds (Okpokwasili and Ogbulie, 2001; Bechet and Blondeau, 2003). Moreso, common fish carriers of *A.salmonicida* such as *Salmo salar* are scarce in our environmental water which could serve as definitive host that may aid dissemination of *Aeromonas salmonicida* (furunculosis).

The presence of moderately high amount of protein in *A.hydrophila* from man may be responsible for the source of common gastrointestinal ailments and diarrhea associated problems in humans. Thus further attesting to the likelihood of the proteins playing a crucial role in pathogenicity. Appropriate measures need to be put in place to control and destroy these organisms. Lower concentrations of the proteins may be associated with lower pathogenicity recorded in some areas.

It is interesting to note that other related strains of *Aeromonas* species that are yet to yield to precise taxonomy could be quiet distinguishable by the electrophoretic method (Mastin and Rhodes, 1978; Ikediobi and Igboanusi 1983; Ainsworth *et al.*, 2006).

The species of motile *Aeromonas* were similarly identified on the basis of the positions, density, and number of stable bands in the electrophoretic patterns of their soluble proteins. The presence or absence of a major dense band in *A.hydrophila*, *A sobria* and *A. caviae* coupled with constancy of the bands in all, could form the basis of tentative classification of this species (Ugochukwu *et al.*, 1977).

The first group will consist of those containing both the band and a minor dense band while the second group would comprise those in which the major band has disappeared but still containing the second band. The second group of *Aeromonas* would be looked as site-specific hybrids resulting from crossing two species of *Aeromonas hydrophila*. Desborough and Paloquin (1966) while working with potatoes, have compared protein patterns from site-specific hybrids with those from an artificial mixture of their two parental protein extracts and established two main differences between hybrids and parents.

Electropherogram obtained from the four *Aermonas* species was not in agreement with that obtained by Luzzatto and Buttistuzzi (1985) and Soule *et al.* (2005). We have found two major bands and two minor bands on the SDS- PAGE disc gel, which was also dissimilar to that obtained by Nuto and Cheriton (1972) who obtained a single band with a predominance of dimmers and tetramers, our SDS-PAGE results showed that most *Aeromonas* species exists as trimers. This experimental value suggests a possibility of two subunits with the same molecular weight and structure moving as a single band and two other subunits that are not alike and thus responsible for the molecular differences within the bands on the disc electrophoretic gel.

The results of the protein bands indicated that the protein profiles of *A.hydrophila* were different from that of *A.caviae* and vice versa, other species might be looked as site specific hybrids. This study reinforced the theory that *Aeromonas* species establishes a highly heterogeneous group, isolates from similar or identical phenotypes could have similar or identical protein fingerprints. This forms a basis of taxonomic classification of the genus *Aeromonas* (Millership, 1996). The presence and position of the single dense band in *A. hydrophila, A.sobria* and *A.caviae* and its absence in *A. salmonicida* could form a partial basis for distinguishing among these species.

The molecular weight of the *Aeromonas* proteins obtained in this study compares favourably with 73 kDa obtained by Cipriano and Bullock (2001). The value is also higher than the molecular weight of 10-22 kDa obtained on similar organisms (Graf, 2002). Gavin *et al.* (2002) attributed higher molecular weight of *Aeromonas* species to the presence of lateral flagelins and the aberrant migration which was thought to result from post translational modification through glycosylation.

Analysis of SDS-PAGE protein patterns reinforced by the up-graded, unweighted mean average (UPGMA) grouping system provided a good characterization of *A. caviae* stains as homogeneous group of microorganisms, possessing significant difference from the other two mesophilic *Aeromonas.* This is in good agreement with other biochemical and enzymatic tests (Ali *et al.,* 1996; Ainsworth *et al.*,2006).

Aeromonas protein profiles clearly showed two groups, with correlation coefficient (cc) of 0.70, which in our experiment was not an acceptable value for the standard of assigning two strains to the same species. Strains biochemically identified as *A. hydrophila* showed cc of 0.64, which was equally not acceptable for species

designation. Inter-species comparison highlighted this heterogeneity, showing two mixed subgroups, both containing strains that were assigned to *A.sobria* and *A.hydrophila* species on the bond of biochemical features (Ainsworth *et al.,* 2006).

The purity assessed of *Aeromonas* DNA indicated about 24 of the 26 *Aeromonas* species (92.30 %) were pure DNA extracts. Genomic DNA extracts identified were of high purity as indicated by A260/A280. This is therefore an indication of the high efficiency of the Qiagen kit, since even the standard salmon sperm DNA has A260/A280 of 1.86.

It was found that genomic DNA extracted after AGE was common and similar amongst most organisms isolated from fish, poultry and man. Fecal *Aeromonas,* and their profiles also assisted in the discrimination of particular strains belonging to the same species. For example, the four isolates related to *Aeromonas hydrophila* could be differentiated on the basis of their distinctive genomic profiles. Although the exact nature of the Poultry *Aeromonas* genomic DNA remains cryptic, in other *Aeromonas* isolates, the presence of genomic DNA has frequently been associated with antibiotic resistance determinants and the production of toxins and antibiotic compounds (Barbosa *et al*., 2005). The occurrence of genomic DNA could also be seen as an indicator of genetic stability and potential for genetic transfer in these species. This is important as gut ecosystems are thought to act as superiors of resistant bacteria and resistance genes that can be passed on to humans through food chain (Barbosa *et al*., 2005).

The genomic DNA of *Aeromonas* isolates were subjected to restriction digestion using rare-cutter restriction enzymes showed that the linear DNA molecules generated in

the B*am* HI digestion of chromosomal DNA was separated according to size in an agarose gel. Electrophoresed DNA was further confirmed by digesting the genomic DNA with B*am* H1, which leads to the dissociation of the gene from the plasmid leading to the multiple bands seen in the various wells, corresponding 450 bp and 1.5 kb of gene and vectors.

The work has shown the dissipation of several molecular fragments of *Aeromonas* genomic DNA digested with E*co* R1, B*am* H1 and S*al* 1. These were specially designed restriction endonucleases that cleaves the *Aeromonas* DNA at rare-ends (GC ' AT or 5^1-GC ' GGCCGC-3^1). This reinforced the fact that restriction enzymes could cleave off gene from their respective plasmids. These fragments could be used as a basis for classification and molecular identification of *Aeromonas* species.

Following *in-vitro* digestion of our isolated chromosomal DNA with restriction endonuclease (B*am* H1) which recognizes specific sequences which cuts the DNA into a number of fragments (Chan, 1979; Oslen *et al.*, 1993). The restriction enzymes were known to specially cut *Aeromonas* genomic DNA at rare-ends such as GC and AT terminals with the B*am* H1, E*co* R1 and S*al* 1 cuting the bacteria genome at only few sites resulting to large restriction fragments. The resulting DNA fragments are large and were separated readily by the agarose gel electrophoresis. This is in agreement with the work of Chan, 1979; Oslen *et al.* (1993) who generated about 450 bp pair DNA using B*am* H1 in Germany. Restriction endonucleases cleaves at different patterns and evolve up to 200 different fragments of different sizes. The differences on the sizes and nature of the fragment depend on the type of microorganisms, nature of the restriction enzymes, and nature of the separating gel, current used and molecular size of the DNA.

The array of the different fragments generated by the restriction enzymes shows the potential of molecular characterization of *Aeromonas.* This would especially be suitable at determining evolutionary diversions wherever mutation occurs.

CHAPTER SIX

7.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- The objectives of this research have been greatly achieved. We have isolated and characterized various species of *Aeromonas* from feces of fishes, poultry and human diarrhoeic stools using bacteriological culture methods of identification.
- The results clearly demonstrates the occurrence of the pathogenic *Aeromonas* species such as *A. hydrophila, A. caviae, A. sobria* and *A. salmonicida* in the Zaria area. *A. hydrophila* was the commonest biotype in fish and man while *A. salmonicida* was the commonest biotype in poultry.
- The prevalence and distribution of various species of *Aeromonas* from poultry, fishes and man were determined. There is possibility of cross transmission of *Aeromonas* species between fishes, poultry and man.
- The study has demonstrated the presence of virulent factors from the isolates via production of ß-hemolysins on 10 % sheep red blood cells. Soluble proteins responsible for virulence were extracted and quantified from some of the various species of *Aeromonas* by spectrophotometric (Biuret) method. SDS-PAGE revealed common electrophoretic patterns of most species of *Aeromonas* toxins, through production of minor and major bands which were dense on the gels.
- The genomic DNA from the several *Aeromonas* species was extracted, purified, quantified and digested with B*am* H1, E*co* R1 and S*al* 1 restriction endonucleases to produce several restriction fragments ranging between 450 bp – 1.5 kb. This may

form basis for classification and epidemiological trace-back analysis of members of the genus, species and strains of *Aeromonas.*

- The significance of this finding is provision of restriction fragments crucial for epidemiological trace-back analysis to locate the true source of infection in fish, poultry and humans. However, the organisms may possess many virulent determinants which are associated with pathogenicity.

6.2 Recommendations

In view of the economic and public health importance of this disease, there is need for further studies on the identification of *Aeromonas* species from other sources to reveal the true extent and control strategies of this disease in our environment.

These findings should stimulate further interest in *Aeromonas* research, so as to evolve better control strategies such as those listed below.

1. Detailed epidemiological surveys are desirable to investigate other cases and associated risk factors of aeromoniasis amongst dogs, pigs, cattle, horses wildlife, zoo, aquatic and laboratory animals in other parts of Nigeria.
2. The public should be educated about the dangers of pathogenic *Aeromonas* species, moreover protective clothings such as handgloves, masks e.t.c. should be used when handling fish or poultry products.
3. Proper cleaning and disinfection of fishponds and poultry farms or other vehicles or prophylactic measures to prevent vertical or horizontal dissemination of possible *Aeromonas* contaminants.

4. Development of safe inhibitors and antibodies against the enzymes that will be used as vaccine to confer immunity and curb this organisms in man and animals.
5. Cloning, sequencing of the restriction fragments, assembly of the whole genome and evaluation of gene function.
6. Amplification of the specific genes coded by the restriction fragments for a particular enzymes such as sialidasis, phosphomonoesterases or other toxins or proteins of other pathological significance.
7. Expression of *Aeromonas* genes or proteins in competent cells such as E.*coli*. This may be useful in Veterinary Medicine, Agriculture, Biotechnology and other industries.
8. Evaluate the immune response of mice, guinea pigs and Nigerian fishes, poultry or other animal species to *Aeromonas* and to produce effective *Aeromonas* vaccine for use in Nigeria.
9. Establishment of Central Aeromoniasis Laboratory (CAL) that is adequately equipped with facilities and well trained staff to undertake *Aeromonas* reference works in veterinary and human hospitals.
10. To institute an effective functional surveillance programme for monitoring, and campaign for control and eradication of aeromoniasis in Nigeria.

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APPENDICES

Appendix I: Phenotypic Differentiation of Different Subspecies of *Aeromonas salmonicida*

Characteristic	*A. salmonicida*	*A. achromogenes*	*A.masoucida*	*A.smithia*
Indole production	-	+	+	-
Methyl red	+	+	+	-
Vogues proskauer	-	-	+	-
H_2S production	-	-	+	+
Lysine decarboxylase	d	d	d	-
Arginine dihydrolase	+	+	+	[-]
D-Glucose acid	+	+	+	[+]
D-Glucose gas	+	-	+	[+]
L-Arabinose acid	+	-	+	
D-Galactose	+	+	+	-
Glycerol acid	d	d	d	[-]
Maltose acid	+	+	+	-
D-Mannitol acid	+	-	+	-
Sucrose acid	-	+	+	d
Trehalose acid	+	+	+	-
Esculin hydrolysis	+	-	+	-
Lipase (corn oil)	+	+	+	-
Ornithine dehydrogenase	d	d	d	+
Brown pigment	+	-	-	-

Key:
+ = 90% or more strains are positive
- = 90% or ore strains are negative
[-] = 10% or more strains are positive
d = 11-75% or more strains are positive
[+] = 76-89% or more strains are positive

Source: (Holt *et al.*, 1984; Borrel *et al.*, 1998).

Appendix II: Phenotypic Differentiation of Different Species of *Aeromonas*

Characteristics	***A.hydrophila***	***A.caviae***	***A.sobria***	***A.salmonicida***
Motility	+	+	+	-
Gram staining	-	-	-	-
Oxidase test	+	+	+	+
Catalase test	+	+	+	+
Esculin hydrolysis	+	+	-	d
Indole production	+	-	-	d
Fermentation of glucose, galactose, mannitol	d	d	d	d

Key:
[+] = Positive
[-] = Negative
[d] = Variable (depending on the strains).

Source: Cruickshank *et al*. (1975).

Appendix III: Some Biochemical Characteristics that Differentiate Species of *Aeromonas* Isolated

Characteristics	***A. hydrophila***	***A. caviae***	***A. sobria***	***A. salmonicida***
Motility	+	+	+	-
Gram stain	-	-	-	-
Oxidase test	+	+	+	+
Catalase	+	+	+	+
Esculin hydrolysis	+	+	-	d
Indole production	+	-	-	d
Fermentation of glucose, mannitol and galactose	d	d	d	d
MR	+	+	+	+
VP	-	-	-	-

Key:
(-) : Negative
(+): Positive
(d): Variable

Source: Cruickshank *et al*. (1975)

Appendix IV: Biochemical Characteristics which Differentiate *Aeromonas* from other Members of the *Enterobacteriaceae* and Related Genera

Characteristics	***E. coli***	***Pseudomonas***	***Aeromonas***
Oxidase test	-	+	+
Glucose (gas production)	+	-	+

Key:
(-) : Negative
(+): Positive

Source: Cruickshank *et al*. (1975).

Appendix V: Differentiation of *Aeromonas* and *Plesiomonas*

Characteristics	***Aeromonas***	***Plesiomonas***
Gelatin liquefaction	+	-
Sucrose fermentation (gas production)	+	-

Key:
(-) : Negative
(+): Positive

Source: Cruickshank *et al*. (1975).

Appendix VI: Data Showing Frequency of Isolation of *Aeromonas* Species from Fish obtained from Dams and Markets

Source	**No. Examined**	***A. hydrophila***	***A. caviae***	***A. sobria***	***A. salmonicida***
ABU Dam	144	20 (13.45)	2 (1.2)	2 (1.2)	8 (5.29)
Zaria Dam	49	2 (4.08)	-	-	2 (4.08)
Samaru Market	104	10 (10.41)	2 (2.08)	5 (5.20)	4 (4.16)
Sabon Gari Market	103	15 (14.56)	2 (1.9)	5 (4.80)	5 (4.50)
Total	**400**	**47(11.75%)**	**6(1.5%)**	**12(3%)**	**19(4.75%)**

Appendix VII: Data Showing Frequency of Isolation of *Aeromonas* Species from Poultry obtained from Commercial Farms.

Source	**No. Examined**	***A. hydrophila***	***A. caviae***	***A. sobria***	***A. salmonicida***
Zumunta farms	130	7 (5.38)	12 (9.23)	3 (2.30)	11 (8.46)
Lape farms	100	4 (4.00)	-	-	8 (8.00)
Zaria farms	100	1 (1.00)	1 (1.00)	-	4 (4.00)
Area 'C'	110	6 (5.45)	6 (5.45)	1 (0.91)	3 (2.72)
Area 'F'	100	1 (1.00)	4 (4.00)	1 (1.00)	9 (9.00)
Total	**540**	**19(3.51%)**	**23(4.25%)**	**5(0.92%)**	**35(6.48%)**

Appendix VIII: Data Showing Frequency of Isolation of *Aeromonas* Species from Humans obtained from ABU Sickbay and ABU Teaching Hospital in Zaria.

Source	**No. Examined**	***A. hydrophila***	***A. caviae***	***A. sobria***
ABU Teaching Hospital	120	7 (5.83)	2 (1.67)	5 (4.11)
ABU Sickbay	100	8 (8.00)	4 (4.00)	3 (4.00)
Total	**220**	**15(6.81%)**	**6(2.72%)**	**8(3.63%)**

Appendix IX: Data showing Pattern of *Aeromonas* Species on 10% Sheep blood tested from Fishes, Poultry and Humans

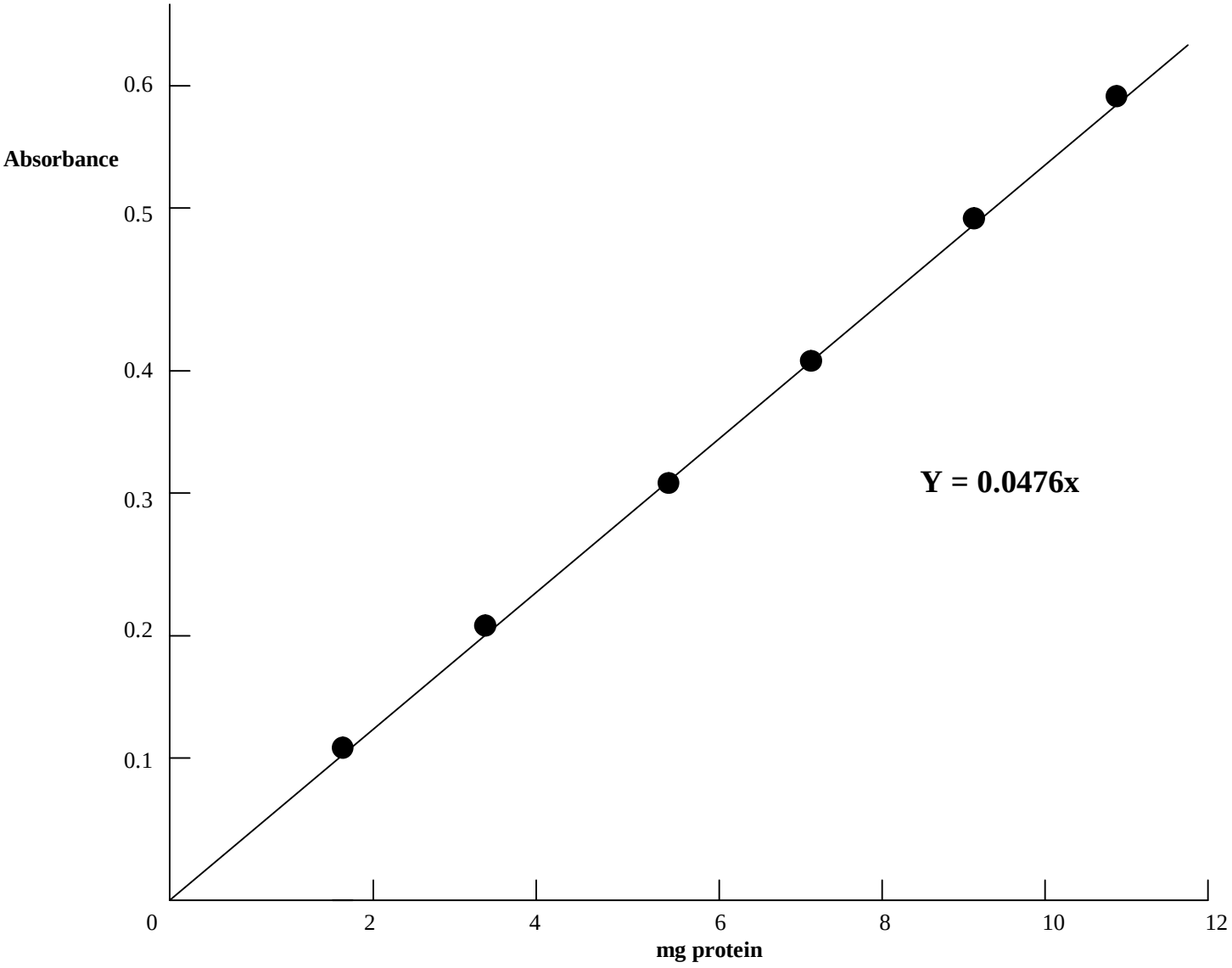
Species	No. Tested	β-hemolytic No (%)	Non β- hemolytic No (%)
A. hydrophila	82	31 (37.80)	51 (62.19)
A. sobria	22	9 (40.90)	13 (59.09)
A. caviae	37	14 (37.83)	23 (62.16)
A. salmonicida	54	18 (33.33)	36 (66.66)
Total (%)	**195**	**72 (36.92%)**	**123 (63.07%)**

Appendix: X

Various Isolates of *Aeromonas* Species Subjected to Molecular Studies

S/no	Sample	Organism
1	Cl38	*A. hydrophila*
2	T3	*A. salmonicida*
3	ZF87	*A. caviae*
4	HTH67	*A. caviae*
5	HTH44	*A. caviae*
6	MS37	*A. sobria*
7	BC29	*A. salmonicida*
8	HTH41	*A. hydrophila*
9	CG18	*A. hydrophila*
10	T30	*A. caviae*
11	T22	*A. hydrophila*
12	ACL38	*A. sobria*
13	HS7	*A. hydrophila*
14	RF59	*A. salmonicida*
15	ZF16	*A. hydrophila*
16	BC11	*A. salmonicida*
17	SM23	*A. sobria*
18	SM12	*A. hydrophila*
19	CM45	*A. hydrophila*
20	HS2	*A. caviae*
21	HTH25	*A. hydrophila*
22	CL18	*A. hydrophila*
23	HTH64	*A. hydrophila*
24	ZF53	*A. hydrophila*
25	HS37	*A. hydrophila*
26	ZF26	*A. hydrophila*

Appendix XI:

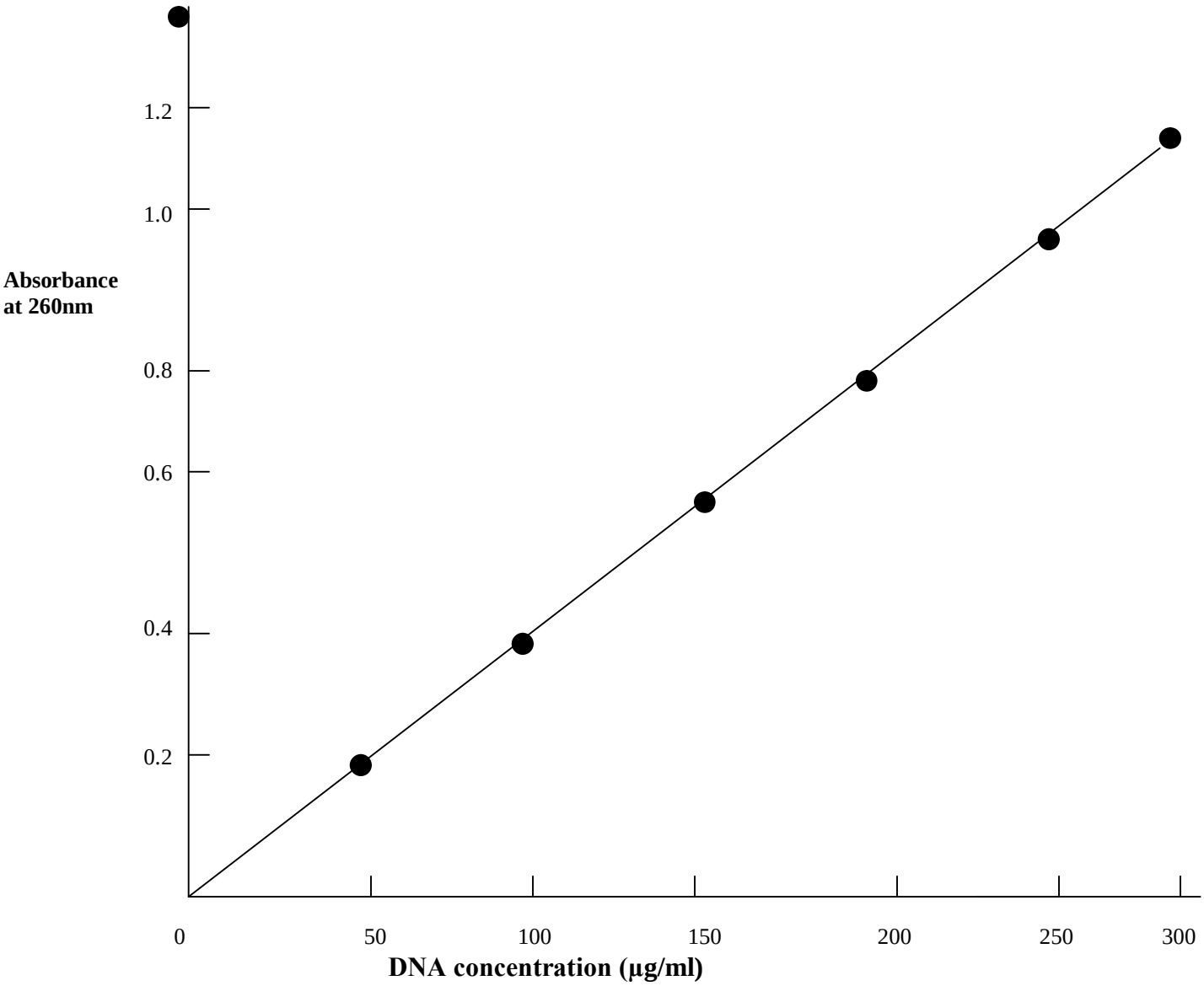


Standard Curve for Protein

Appendix XII: Data Showing the Average Protein of Species of *Aeromonas*.

Source	*A. hydrophila*	*A. sobria*	*A. caviae*	*A. salmonicida*
Poultry	3.58	-	4.00	3.82
Fish	3.11	4.63	2.95	2.74
Human	4.07	3.58	3.99	-

Appendix XIII:

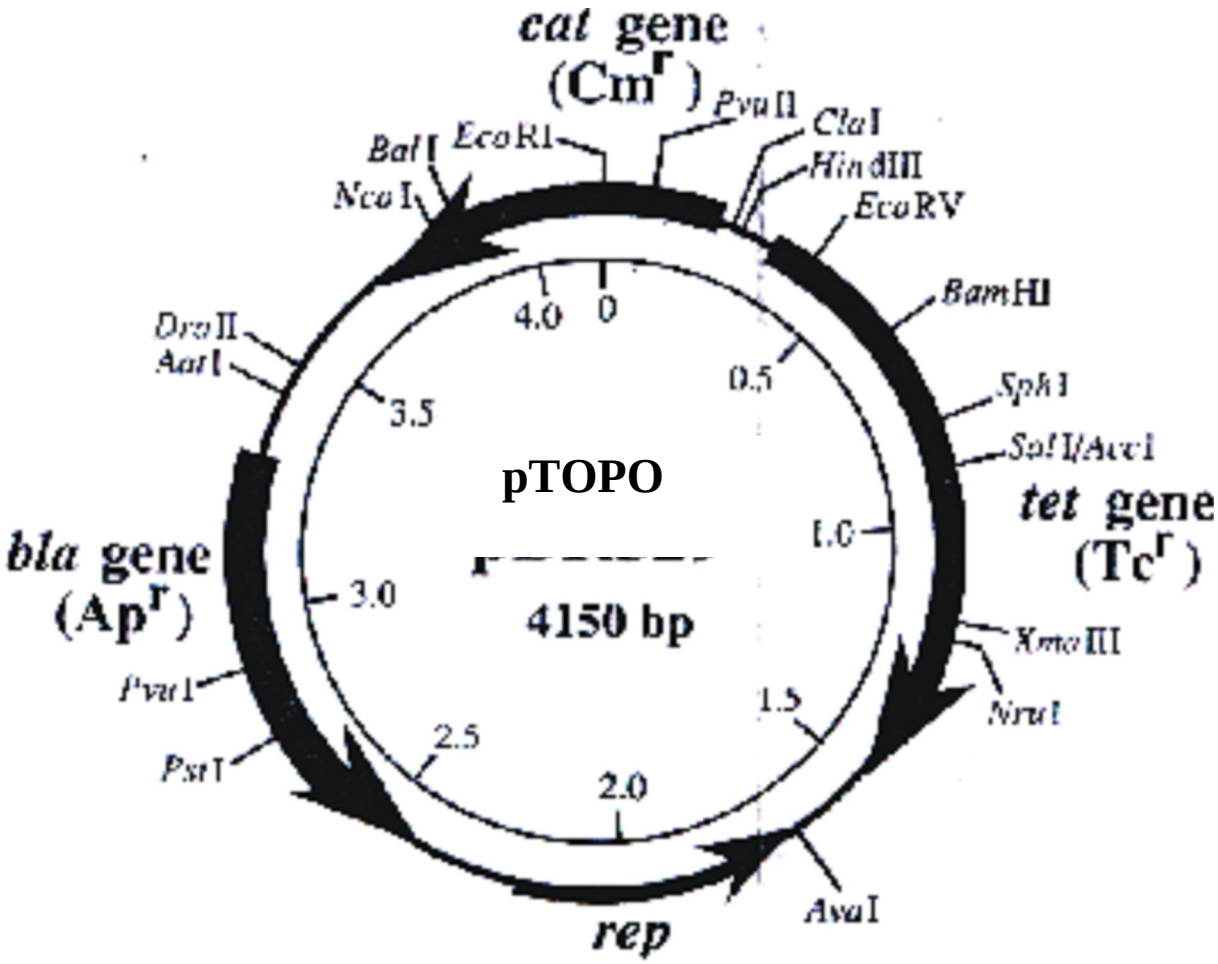


Calibration Curve for DNA

Appendix XIV:

Genetic and Restriction Enzyme Map of Plasmid

pTOPO



Source: Zyskind and Bernstein, (1992).

Appendix XV: Preparation of Stock Solutions.

1.1 Acrylamide/Bis (30 %)

73 ml of double distilled water (dd H_2 0) was added to the bottle containing 30 g of premixed and preweighed acrylamide/ bis containing 29.22 g Acrylamide and 0.78 g Bis. This gave a final volume of 100 ml. for high quality electrophoresis of native proteins, the solution was filter-sterilized. This was however not necessary if used in casein substrate gets for the separation of proteases. The solution was stored in a dark bottle at 4^o C. It had a shelve life of 30 days in these storage conditions.

1.2 TRIS (5 M, PH 8.8) for resolving gels

28.171 g Tris (ultra pure) was dissolved in 50 ml of dd H_2 O in a 150 ml beaker. The PH was adjusted with concentrated Hcl to 8.8. The mixture was made up to a final volume of 100 ml with dd H_2 O and stored at 0-4^o C.

1.3 TRIS (1.0 M, PH 6.8) stacking gels

2.11 g of Tris (ultra pure) was dissolved in 50ml of dd H_2O in a 150 ml beaker. The PH was adjusted with concentrated HCl to 6.8 The mixture was made up to a final volume of 100ml with ddH_2 O and stored at 0-4^0 C.

1.4 Sodium Dodecyle Sulphate (SDS) (12 %)

12.0 g of SDS was weighed into a 150 ml beaker and dd H_2O was added to make up a final volume of 100 ml. The solution was stored room temperature.

1.5 Resolving Gel Solution

casein (0.01 % (w/v) of the final volume of the resoling gel solution) was first weighed into a 25 ml beaker. Volumes of the following solutions were added:

30 % acrylamide/bis ……. 4.0 ml

Tris PH 8.8 ……...4.48 ml

dd H_2O ……...3.48 ml

12 % SDS ……. 90 ml

12 % Ammonium persulphate (APS)……90 ml

APS, also known as cracking buffer for the cracking sound it makes when dissolving in water, was always freshly prepared. It was only when the resolving gel solution was about to be poured into the gel plates that 6 ml of TEMED was added. since these gave a final volume of 9 ml, 0.01 % wlv casein was 0.0091 g. The resulting gel produced from this recipe was 12 % acrylamide

1.6 Stacking Gel Solution

Casein was not included in the preparation of stacking gel solutions. Volumes of the following were taken:

30 % acrylamide/Bis…….1.5 ml

Tris PH 6.8 ……...1.25 ml

dd H_2 0 ……..7.2 ml

10 % SDS ……..100 ml

10 % APS (freshly prepared) ……50 ml

TEMED ……..10 ml

The stacking gel solution was poured over the resolving gel immediately after TEMED was added.

1.7 Running Buffer

Tris (3.03 g, ultra pure), 14.42 g of glycine and 1.0 g of SDS were weighed into a 1 liter conical flask and dd H_2 O was added to make up the finalvolume of 1.0 l. The buffer was stored at room temperature.

1.8 Coomassie Blue Stain

Comassie brilliant blue powder (1.0 g) was weighed into a 1.0 l conical flask containing 450 ml methanol. To this, 100 ml Acetic acid and 450ml dd H_2O were respectively added to make up the final volume of 1.0 l and stored at room temperature.

1.9 Solution for destaining Coomassie Blue Stained gels

Acetic acid (70 ml) and 200 ml of methanol were measured into a 1.0 l conical flask. This was made to a final volume of with 730 ml dd H_2O and stored at room temperature.

1.10 Phosphate Buffered Saline (PBS) solution

To prepare 1 liter of PBS solution, the following were weighed into a 1.0 l conical flask:

NaCI …….8.0 g

KCI ……...0.2 g

Na_2 HPO_4. 12 H_2O…….2.9 g

KH_2PO_4 ……0.2 g

To this mixture, dd H_2O was added to a final volume of 1.0 l. The resulting solution is expected to have a pH of 7.0-7.5. However, PBS tablets from Sigma were used. In this case, each tablet was dissolved in 200 ml of dd H_2O. This gave a solution containing 0.01M phosphate buffer, 0.0027 m Kcl, 0.137 m NaCI and a PH of 7.4 at 25^{o} C. Therefore to obtain 1.0 l of PBS solution, 5 PBS tablets were used. The solution was stored at 0.5^{0} C.

1.11 Triton X -100 (2.5 %) in PBS

To prepare 1 liter, 25 ml of Triton X – 100 into a 1.0 l conical flask. 975 ml of PBS solution was added and stirred until completely dissolved.

1.12 Sample Buffer

Small amounts of sample buffer were usually required. Therefore not much of the stock solution was prepared at a time. To Prepare 25 ml of sample buffer, the following quantities were taken:

Tris (ultra pure) ……0.3775 g

Glycerol ……5.0 ml

These were dissolved with 8.75 ml of dd H_2O and the PH was adjusted to 6.75 with conc. HCI after which the following were added:

SDS ……1.0 g

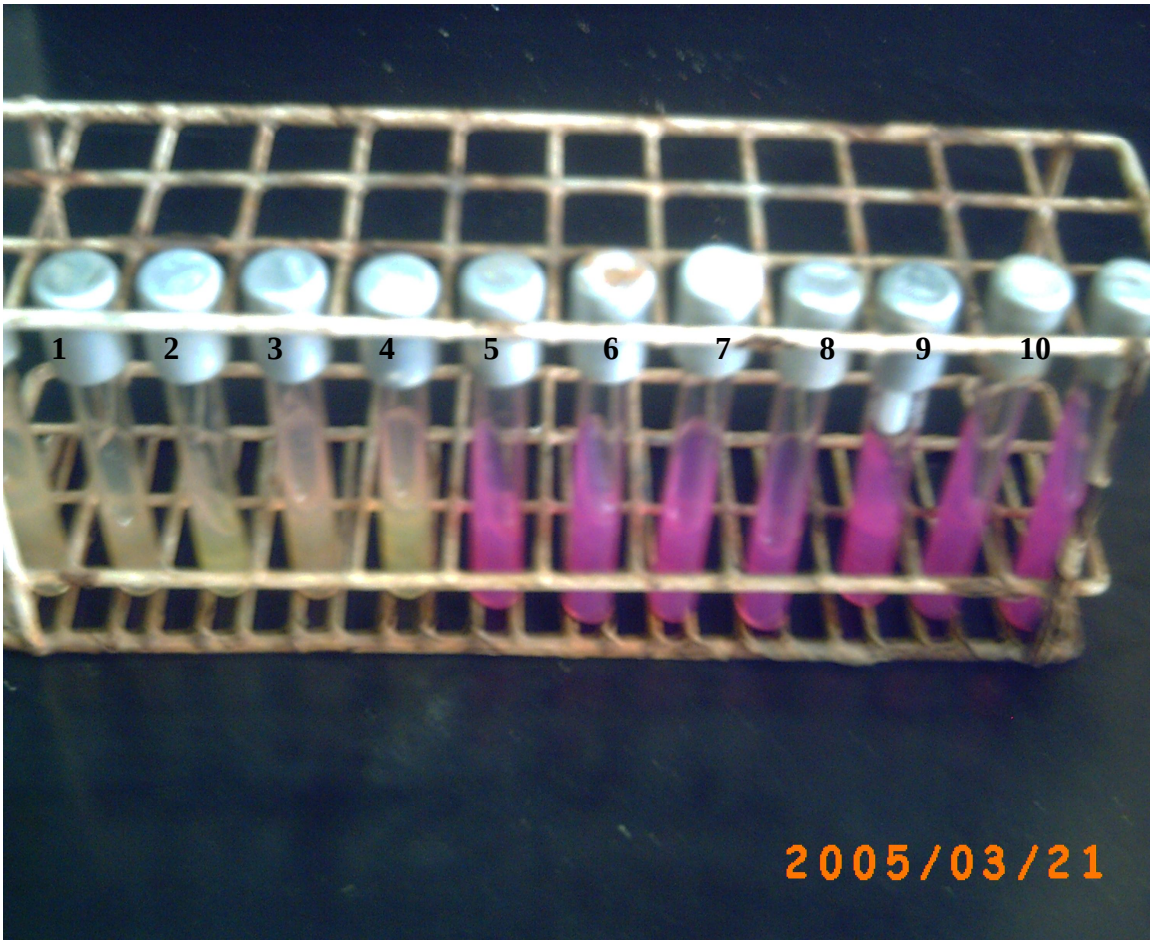
2-Mercptoethanol ……..2.5 ml

Bromophenol blue…….0.5 mg

This was made up to a final volume of 25 ml with dd H_2O. The solution was stable forat least I month stored at -20^{o} C.

Appendix XVI:

Dissipation of Biochemical Tests showing Reaction on Urea Agar by *Aeromonas* Species.



Key:
Tubes 1-4 = Urea negative
Tubes 5-10 = Urea positive

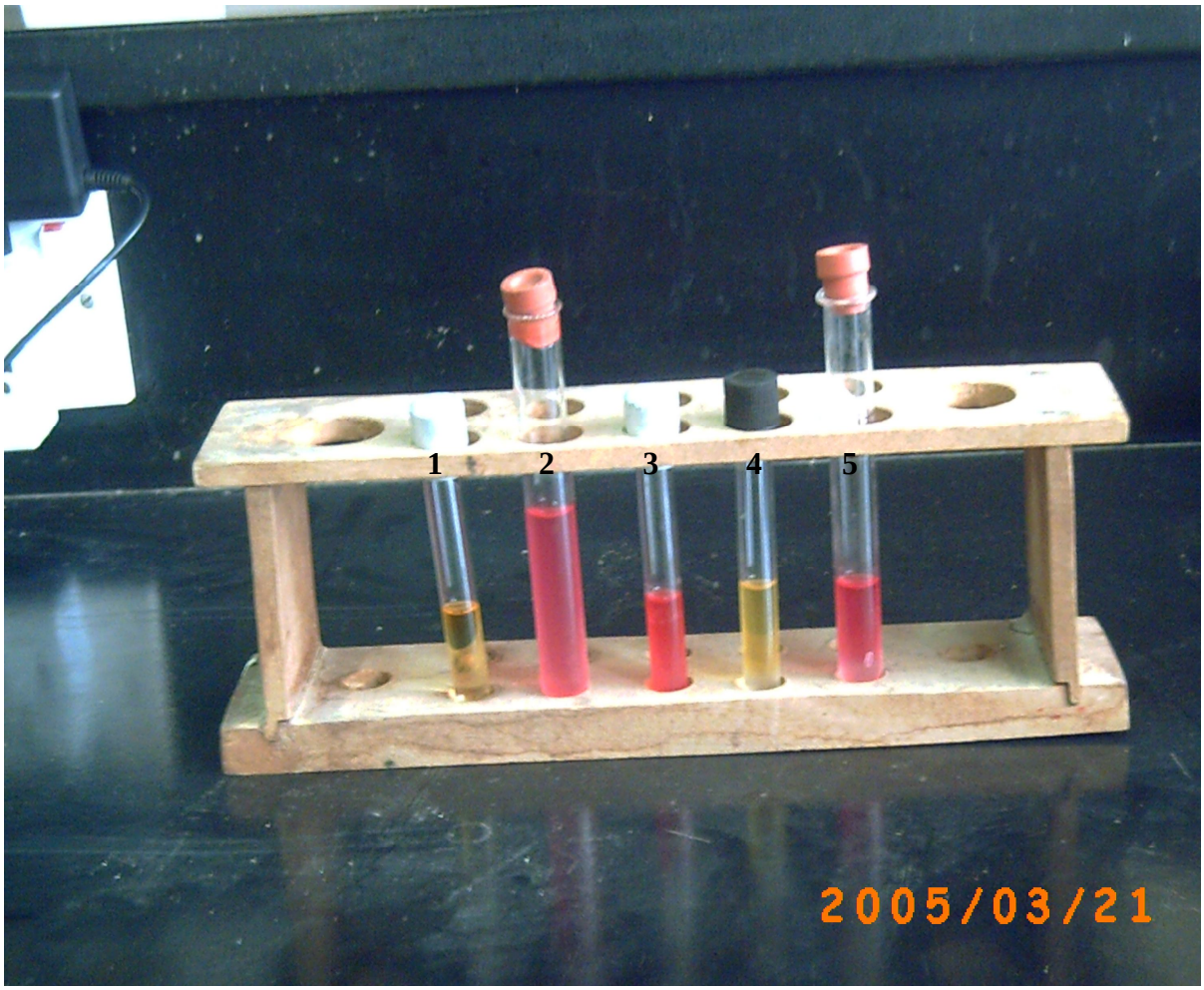
Appendix XVII:

Dissipation of Biochemical Tests showing Reaction of *Aeromonas* on Triple Sugar Iron (TSI)

Key:
Tubes1-4 &7 = alkaline / acid
Tubes 5-6 =Gas

Appendix XVIII:

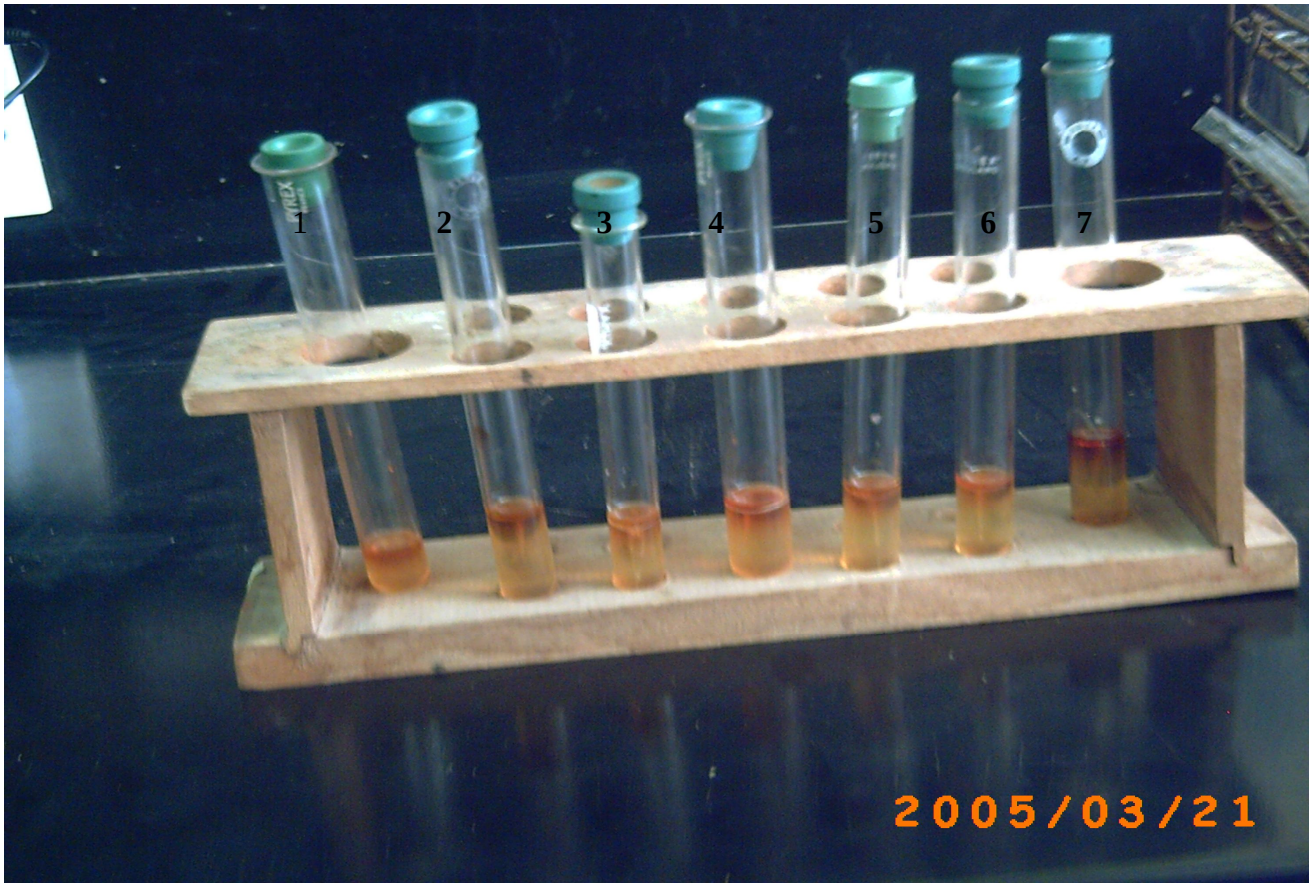
Dissipation of Biochemical Tests showing Reaction of *Aeromonas* on Methyl Red and Voges-Proskauer (MRVP).



Key:
Tubes 2,3&5 = MRVP +ve
Tubes 1 & 4 = MRVP -ve

Appendix XIX:

Dissipation of Biochemical Tests showing Reation of *Aeromonas* on SIM Agar



Key:
Tubes 1-7 = Indole +ve

Appendix XX: Molecular Weight Estimation

Molecular weight estimation using ovalbumin 44xDa and trypsinogen 24KDa marker protein standard.

Relative mobility = $\frac{\text{Distance moved by band x gel length before staining}}{\text{Distance moved by dye x gel length after staining}}$

RMW	**Log_{10} RMW**	**rM**
44 KDa	1.64	0.26
24 Da	1.38	0.34
-	-	0.38

RMW = Relative Molecular Weight

r M = Relative Mobility

0.38 = Relative mobility of *Aeromonas* proteins

rmt α ^{1}/mwt, rm = K/mwt, Rmmwt = K

rmt mwtt – rmap mwtap

rmt = Relative molecular weight of trypsinogen = 0.34

mwtt = Molecular weight of trypsinogen = 37 KDa

rmap = Relative mobility of *Aeromonas* protein = 0.38

mwtap = Mole weight of *Aeromonas* protein =

mwtap = $\frac{0.34 \times 24}{0.38}$ = 21 KDa

This method was used to estimate molecular weight of *Aeromonas* species.