CERTIFICATION

This is to certify that the Thesis titled "Prevalence and Characteristics of *Aeromonas* species and *Plesiomonas shigelloides* in Lagos" submitted to the School of Postgraduate Studies, University of Lagos for the award of the degree of Ph.D in Medical Microbiology, is a record of original research carried out by MR. SUNDAY ABRAHAM ALABI in the Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos.

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Date:- December, 1989

PREVALENCE AND CHARACTERISTICS OF AEROMONAS SPECIES AND PLESIOMONAS SHIGELLOIDES IN LAGOS

A THESIS

SUBMITTED IN FULFILMENT OF THE

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B Y

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PREVALENCE AND CHARACTERISTICS OF AEROMONAS SPECIES AND PLESIOMONAS SHIGELLOIDES IN LAGOS

B Y

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DEDICATION

This work is dedicated to the glory of

GOD ALMIGHTY

AND

MY LOVING WIFE

MRS. CECILIA FOLAKE ALABI

PREFACE

An association between diarrhoea and the presence of Aeromonas in faeces was first reported as early as 1937 by Miles and Halnan. Similarly, Plesiomonas has been associated with diarrhoea as far back as 1972 by Chatterjee and Neogy. In which case, the role of Aeromonas in diarrhoeal disease has been under investigation for about 50 years now and that of Plesiomonas for at least 17 years. Yet, a stage has not been reached when it can be unequivocally stated that Aeromonas or Plesiomonas causes diarrhoea.

Progress in recent years in the development of selective media for isolating these organisms, as well as increased awareness on the part of clinical microbiologists, have enhanced our knowledge of their environmental and geographical spread. Cases of gastrointestinal infection with Aeromonas or Plesiomonas have been reported from all parts of the world, with an indication that such infections can be water - or food - borne.

In most countries, the faecal carriage of Aeromonas or Plesiomonas is low, less than 1% and most isolates were from diarrhoea cases. However, in others such as Thailand, these organisms were found with equal frequency in diarrhoeal and healthy stools with the carriage rate being about 27%.

For the other established enteropathogens, particularly the Escherichia coli and Vibrio cholerae, the discovery of virulence factors such as enterotoxin production and enteroinvasiveness has led to a much better understanding of the pathophysiology of their infections. However, similar studies in Aeromonas or Plesiomonas appeared to further confuse the issue. Both heat-labile (LT) and heat-stable (ST) enterotoxins, cytotoxins, haemolysins, and invasive ability have been described in these organisms but their roles in enteric infections remain unclear.

Similarly, attempts have been made to correlate pathogenicity with species of Aeromonas and several workers have suggested that A. hydrophila and A. sobria are the pathogenic species, while A. caviae is not. However, others have produced clinical and epidemiological evidence that A. caviae may also cause diarrhoea and that absence of the accepted pathogenicity markers does not exclude the possibility of enteropathogenicity, as can be found in the classical enteropathogenic (EPEC) strains of E. coli.

As a matter of fact, there is hardly any other area of medical microbiology that contains so much contradictory evidence as found in the study of Aeromonas and Plesiomonas. The problem is further compounded by the inability of these organisms to adequately fulfil

the Koch's postulates. Do we then throw in the towel and accept defeat in the current efforts to unravel the mystery surrounding these organisms? What can we say of the situation in Nigeria regarding Aeromonas, Plesiomonas and acute diarrhoeal diseases? The answers to these questions and other interesting findings are presented in the account of this thesis that follows.

DECEMBER, 1989 S. A. ALABI

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My profound gratitude and sincere appreciation to my indefatigable supervisor, Professor Tolu Odugbemi, for all he has done to ensure the success of this work. His dedication, guidance, constructive criticism and rewarding advice throughout the course of this work are very much appreciated. I also want to specifically thank him for his special interest in me and members of my family, and for the use of his personal library.

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ABSTRACT

This study was carried out in two phases. The first phase involved the determination of the prevalence of *Aeromonas* species and *Plesiomonas shigelloides* in patients, with and without diarrhoea, reporting at the enteric laboratory of the Department of Medical Microbiology and Parasitology, Lagos University Teaching Hospital (LUTH), Idi-Araba, Lagos; over a 12-month period of October 1986 through September 1987. In the second phase the characteristics of isolates thus obtained were examined.

During the first phase of the study, a total of 2,350 faecal specimens from patients with diarrhoea were screened for the presence of *Aeromonas sp.* or *P. shigelloides*. Of this number, 53 (2.26%) were positive for *Aeromonas sp.* while 16 (0.68%) yielded *P. shigelloides*. On the other hand, of a total of 500 faecal specimens from patients without diarrhoea (controls) collected over the same period of time, only 2 (0.4%) were positive for *Aeromonas sp.*, while none of the specimens yielded *P. shigelloides*. In which case, both organisms were more frequently isolated from diarrhoeal than from control specimens. As a matter of fact, the difference in the isolation rates of *Aeromonas sp.* from patients with diarrhoea and controls was statistically significant ($p < 0.01$).

It is equally noteworthy that isolation rates of 2.26% Aeromonas sp. and 0.68% P. shigelloides compared favourably with those obtained for other established enteric pathogens in this study, such as the enteropathogenic Escherichia coli (EPEC, 2.85%), Shigella sp. (0.17%), Salmonella sp. (0.13%) and Y. enterocolitica (0.09%). Also, none of these enteropathogens was recovered in faecal specimens positive for Aeromonas sp. or P. shigelloides.

Further biochemical characterisation of the Aeromonas and Plesiomonas isolates revealed that of the 55 Aeromonas sp. 20 (36.4%) were A. hydrophila, 28 (50.9%) were A. caviae and 7 (12.7%) were A. sobria. It was also observed that of the numerous tests used in the characterisation, eleven simple ones, viz : the production of oxidase, catalase, hydrogen sulphide (H_2S) from Kligler's iron agar (KIA), pigmentation on nutrient agar, acid from glucose, xylose, dulcitol, adonitol, mannitol, inositol, and inability to grow in 6.5% NaCl; were highly reproducible. Based on these findings, a simple scheme was designed for the isolation and preliminary identification of Aeromonas sp. and P. shigelloides from faecal specimens.

Similarly, survival of Aeromonas sp. and P. shigelloides was measured in commonly used transport

media such as Cary-Blair, Stuart, Amies, phosphate bufferred saline, and alkaline peptone water (APW). Results obtained indicate that these organisms would survive in these media for at least one month. However, a modified form of APW containing 0.01% w/v FeSO4 appeared to be the most suitable for the long storage of these organisms.

In the same vein, a good number of Aeromonas sp. and P. shigelloides examined in this study produced haemolysins, heat-stable (ST) enterotoxin, or invasiveness; which are recognised virulence factors in the pathophysiology of acute diarrhoea. Specifically, about 70% of the Aeromonas sp. produced α or B-haemolysin, 20% produced ST and about 11% were invasive. Similarly, 25% of P. shigelloides produced ST and invasiveness while about 13% produced α - haemolysin. It is significant to note that all the isolates that produced virulence factors were isolated from patients with diarrhoea; thus indicating that these factors may have a role to play in diarrhoea associated with these organisms.

However, when strains producing virulence factors were fed orally to healthy adult mice, they failed to cause diarrhoea. It is noteworthy that these strains survived the gastric acid barrier in the stomach, and

were infact reisolated from faecal specimens of the animals. The possibilities are therefore that either these virulence factors were not elaborated *in vivo*, or that a number of host factors made mice unsuitable for experimental infection with *Aeromonas* or *Plesiomonas*.

The *in vitro* susceptibility pattern of *Aeromonas sp.* and *P. shigelloides* isolated in this study was determined against 15 selected antimicrobial agents. The results obtained have shown that these organisms are susceptible to most of the agents evaluated. Specifically, all the strains were susceptible to ofloxacin and at least 60($\geq$70%) strains were susceptible to gentamicin, carbenicillin, ceftriaxone, colistin, nalidixic acid, nitrofurantoin, chloramphenicol, tetracycline and co-trimoxazole. Also, less than 30% of the strains were susceptible to ampicillin.

Finally, *Aeromonas sp.* and *P. shigelloides* isolated during the course of this work were screened for the presence of plasmids. Interestingly enough, some of the isolates harboured plasmids with molecular weights ranging between 2.4×10^6 to 16.8×10^6 daltons. Of the 55 *Aeromonas sp.* examined, 12 (21.8%) harboured one or more plasmids. Similarly, a strain of *P. shigelloides*

possessed more than one plasmid.

Hence, the various findings reported in this study seem to suggest that *Aeromonas sp.* and *P. shigelloides* may have a significant role to play in acute diarrhoeal diseases in the Lagos environment. However, there is need for similar studies on a national scale to increase the present level of awareness; and for a better understanding of *Aeromonas* and *Plesiomonas* - associated diarrhoea in Nigeria.

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ABBREVIATIONS AND SYMBOLS USED IN THE THESIS

ABBREVIATION/SYMBOL	MEANING
DNA	Deoxyribonucleic acid
XDCA	Xylose desoxycholate citrate agar
ABA	Ampicillin blood agar
FeSO4	Ferrous sulphate
M - APW	Modified alkaline peptone water
DNase	Deoxyribonuclease
NA	Nutrient agar
SDS	Sodium dodecyl sulphate
EDTA	Ethylenediamine-tetra-acetic acid
$\bar{X}_s$	Mean survival rate
$\bar{X}_c$	Cumulative mean survival rate
X^2	Chi-square
$\sum$	Summation
n_r	Number of rows
n_c	Number of columns

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The genera *Aeromonas* and *Plesiomonas* consist of gram-negative, non-sporulating, oxidase positive, and facultative anaerobic rods which are naturally aquatic microorganisms, commonly isolated from water, soil, various cold and warm-blooded animals as well as humans. They are classified in the family *Vibrionaceae* on the basis of their morphology (gram-negative rods with predominantly polar flagella), facultative anaerobiosis, and guanine - plus - cytosine (GC) ratio (Popoff, 1984; Schubert, 1984).

Members of the two genera share many similarities and are often considered together. As a matter of fact, *Plesiomonas* strains were formerly placed in the genus *Aeromonas* as *Aeromonas shigelloides*. However, further biochemical and ecological characterisation revealed significant differences between the two to justify the establishment of a separate genus, *Plesiomonas* (Von, Graevenitz and Mensch, 1968; Von Graevenitz, 1985). At present, *Plesiomonas shigelloides* is the only recognised species in the genus *Plesiomonas* (Schubert, 1984), while the genus *Aeromonas* has four recognised species, *Aeromonas hydrophila*, *A. caviae*, *A. sobria*,

and A. salmonicida (Popoff, 1984). Recently, two new species, A. media and A. veronii have been proposed as additions to the genus Aeromonas (Allen, Austin and Colwell, 1983; Hickman-Brenner et al., 1987). It is however noteworthy to state here that taxonomy and classification of Aeromonas is far from being settled. In earlier reports and some recent ones, authors did not distinguish between Aeromonas species. In such cases, reports on A. hydrophila can be interpreted as encompassing both A. caviae and A. sobria (Davis, Kane and Garagusi, 1978; Hickman-Brenner et al., 1987; Kelly, Stroh and Jessop, 1988).

The first Aeromonas strain was described by Zimmermann in 1890, and the first Plesiomonas strain in 1947 by Ferguson and Henderson. These organisms largely escaped medical attention, since early researchers concentrated on strains from animals and from the environment which went under a variety of names (Pitarangsi et al., 1982; Ljungh and Wadstrom, 1985). These studies resulted in the early recognition of Aeromonas species as a pathogen in amphibians (Emerson and Norris, 1905; Shotts et al., 1972), reptiles (Marcus, 1971; Shotts et al., 1972), fish (Esch and Hazen, 1978; Haley, Davis and Hyde, 1967), snails (Mead, 1969), and cows (Wohlegemuth, Pierce and

Kirkbride, 1972).

Miller and Chapman (1976) observed that losses due to A. hydrophila in commercial and sport fishery may be substantial. An instance they gave was in 1973 when 37,500 fish died of A. hydrophila infections, over a single 13 - day period in a North Carolina lake in the United States of America. Several other authors have also suggested that the densities of Aeromonas in natural bodies of water may be an important contributing factor to epizootics in fish (Fliermans et al., 1977; Haley, Davis and Hyde, 1978; Shotts et al., 1972).

Miles and Halnan (1937) were the first to isolate Aeromonas strains from human faeces. The so-called Proteus melanovogenes was recovered from a patient with chronic colitis, but its presence was not considered clinically significant. Thus, Aeromonas was not considered a human pathogen until almost two decades later in 1954, when Hill, Caselitz and Moody described a case of fatal septicaemia in a 40 - year old Jamaican woman. The healthy female abruptly developed fulminant Aeromonas sepsis with metastatic necrotising myositis. Since then, Aeromonas has been reported to cause a variety of human infections, including cellulitis (McCracken and Barkely, 1972) wound infections (Hanson et al., 1977), bacterial endocarditis (Davis, Kane and

Caragusi, 1978), hepatobiliary infections (DeFronzo, Murray and Maddrey, 1973), septicaemia, especially in immuno-compromised individuals (Ketover, Young and Armstrong, 1973; Chopra et al., 1986), pneumonia and bacteremia (Reines and Cook, 1981), corneal ulcers (Feaster, Nisbet and Barber, 1978) and a fatal skin infection (Fulghum, Linton and Taplin, 1978).

A. hydrophila was first implicated in cases of severe gastroenteritis in 1961 in Colombia (Martinez-Silva, Guzman-Urrego and Caselitz, 1961). It was found to be the predominant organism in the aerobic flora of patients with diarrhoea, and was isolated in pure culture in a fatal case of enteritis in a newborn. Ever since, episodes of Aeromonas - associated diarrhoea or gastroenteritis have been reported from many countries of the world including the United States (Von Graevenitz and Mensch, 1968; Hazen et al., 1978; Agger, McCormick and Curwith, 1985; Ingram et al., 1987), Britain (Shread, Donovan and Lee, 1981; Millership, Curnow and Chattopadhyay, 1983), Sweden (Ljungh, Popoff and Wadstrom, 1977; Ljungh and Wadstrom, 1985), Canada (Trust and Chipman, 1979), Thailand (Pitarangsi et al., 1982), the Netherlands (Kuijper, Zanen and Peters, 1987), Indonesia (Gracey et al., 1982), Australia (Burke et al., 1983), Ethiopia (Waadstrom et al.,

1976) Bali (Santoso et al., 1986), and Nigeria (Agbonlahor et al., 1982; Agbonlahor, 1983).

In comparison to Aeromonas species, infections due to P. shigelloides have been less frequently reported in literature. However, P. shigelloides has been isolated from dogs, cats, and fresh-water fish (Tsukamoto et al., 1978; Arai et al., 1980). In humans, it has been implicated in cases of neonatal septicaemia and meningitis (Appelbaum et al., 1978; Dudley, Mays and Sale, 1982), post - operative (Splenectomy) infection (Curti, Lin and Szabo, 1985), septic arthritis (Gordon, Philpot and McGuire, 1983), an outbreak of hospital infection (Mellersh, Norman and Smith, 1984) and gastroenteritis, sepsis and osteomyelitis (Ingram, Morrison and Levitz, 1987). More recently, Nolte et al. (1988) reported a case of proctitis and fatal septicaemia in a 42 - year-old bisexual male at the Strong Memorial Hospital, Rochester, New York.

Similarly, P. shigelloides - associated diarrhoea have been reported in the United States (Holmberg and Farmer , 1984; Reinhardt and George, 1985), Britain (Mandal, Whale and Morson, 1982), Japan (Tsukamoto et al., 1978), Thailand (Echeverria et al., 1983), Bangladesh

(Huq and Islam, 1983), India (Chatterjee and Neogy, 1972; Sanyal, Saraswatchi and Sharma, 1980) and Zaire (Vandepitte, Makulu and Gatti, 1974).

The numerous reports from all parts of the globe on Aeromonas and Plesiomonas are clear evidences of increased interest in their study. This renewed interest also culminated in the 1st International Workshop on Aeromonas and Plesiomonas held from 5 - 6 September 1986 in Manchester, England. At that meeting, Von Graevenitz, a leading authority on Aeromonas and Plesiomonas studies, reported that the number of publications on these organisms between 1980 and 1985 was three times that of 1970 - 1975 (Von Graevenitz, 1986).

Despite the various reports on Aeromonas and Plesiomonas - associated diarrhoea, the organisms are still not universally accepted as enteropathogens. While some studies have ascribed aetiological roles to these organisms (Sanyal, Singh and Sen, 1975; Tsukamoto et al., 1978; Sanyal, Saraswathi and Sharma, 1980; Baman, 1980; Agbonlahor et al., 1982; Agbonlahor, 1983; Kipperman, Ephros and Lambdin, 1984; Ljungh and Wadstrom, 1985; Reinhardt and George, 1985a; Agger, McCormick and Gurwith, 1985; Moyer, 1987; Ingram, Morrison and Levitz, 1987; Huq and Islam, 1987); others have been unable to assign any clinical

significance to Aeromonas or Plesiomonas (Shread, Donovan and Lee, 1981; Pitarangsi et al., 1982; Millership, Curnow and Chattopadhyay, 1983; Morgan et al., 1985).

Concerted efforts over the years to uncover the mystery surrounding the role of these organisms in acute diarrhoeal diseases have focussed on improving their taxonomy, classification, isolation and identification, determination of potential virulence factors, antimicrobial susceptibility patterns, and lately plasmid analysis for a better understanding of their epidemiology. These are further reviewed to highlight the state of the art in the study of these biological "latecomers".

Taxonomy and Classification:

Like other recently recognised enteric pathogens such as Campylobacter and Yersinia, taxonomy and classification of Aeromonas and Plesiomonas have been undergoing modifications and changes as new facts about them are uncovered. These organisms have gone through a variety of names since the first strain of Aeromonas was described by Zimmermann towards the end of the last century and that of Plesiomonas by Ferguson and Henderson in 1947.

The first human isolate of Aeromonas was designated Proteus melanovogenes by Miles and Halnan in 1937, while Hill, Caselitz and Moody (1954) established human pathogenicity of Aeromonas in strains they called Vibrio jamaicensis. Such was the confusion in the nomenclature of Aeromonas until the early sixties when Schubert (1960), Eddy (1960), and Ewing et al. (1961) performed further biochemical and ecological characterisation of Aeromonas strains. These first major attempts at classification established two species of the genus Aeromonas - A. hydrophila group and A. salmonicida. Distinguishing characteristics between the two species are that, unlike the A. hydrophila group, A. salmonicida is non-motile, grows only below 37°C and hence non-pathogenic for man.

Further improvement on the classification of Aeromonas was made by Schubert (1974) in the 8th edition of the Bergey's Manual of Determinative Bacteriology. He divided the A. hydrophila group into two major species and five subspecies which are A. hydrophila subsp. hydrophila, A. hydrophila subsp. anaerogenes, A. hydrophila subsp. proteolytica, A. punctata subsp. punctata, and A. punctata subsp. caviae. Subsequently, A. hydrophila subsp. proteolytica was found to be a halophilic vibrio and has now been removed from the genus

Aeromonas and is now classified as Vibrio proteolyticus (Hickman-Brenner et al., 1987).

On the basis of a numerical taxonomic study of 68 strains of Aeromonas by Popoff and Veron (1976) and a subsequent DNA homology study as well as phenotypic similarities, Popoff et al. (1981) subdivided the A. hydrophila group into three species - A. hydrophila, A. sobria and A. caviae. This classification was used in the latest edition of the Bergey's Manual of Systematic Bacteriology (Popoff, 1984). Though this classification scheme by Popoff is generally accepted for practical purposes, taxonomic problems still persist within the genus Aeromonas. For instance, Allen, Austin and Colwell (1983) proposed a new aquatic species, A. media which is non-motile like A. salmonicida and has not been found in human specimens. More recently, Hickman-Brenner et al. (1987) proposed yet another species, A. veronii to represent the ornithine decarboxylase positive strains of Aeromonas. The species name, veronii, was in honour of M. M. Veron, a French microbiologist who has contributed greatly to the development of the genera Vibrio and Aeromonas, and who coined the family name Vibrionaceae in 1965.

Commenting further on the unsettled species of the genus Aeromonas, Hickman-Brenner et al. (1987) observed

that two basic subdivisions of the genus can be defined based on phenotypic differences and pathogenicity. The first group is non-motile, does not grow at 35 to 37°C and is pathggenic for fish. This is the psychrophilic group that is known as A. salmonicida. The second group grows at 35 to 37°C, is usually motile, and has been isolated from human clinical specimens and other sources. This is the mesophilic group. In literature, this group has been variously referred to as Aeromonas hydrophila sensu lato (Sakazaki and Balows, 1981), the A. hydrophila group (Hickman-Brenner et al., 1987), or the A. hydrophila complex (Statner, Jones, and George, 1988). This probably explains why most reports on human Aeromonas isolates in the past were mainly on A. hydrophila. There is no doubt that some of the strains referred to as A. hydrophila may be A. sobria or A. caviae.

At present, it would appear that the only generally accepted species in the genus Aeromonas is A. salmonicida, the aetiological agent of furunculosis in salmonid fish, which has never been implicated in human disease. For the mesophilic group, while most authors have preference for the classification scheme of Popoff (1984) recognising three species of A. hydrophila,

A. caviae, and A. sobria; others like Hickman-Brenner et al. (1987) prefer referring to them as the A. hydrophila group.

On the other hand, P. shigelloides is the only species in the genus Plesiomonas and this has made the nomenclature and classification less confusing. Members of the genus share many similarities with Aeromonas sp and were often considered together. As a matter of fact, the generic name Plesiomonas was derived from the Greek word for neighbour to demonstrate its relationship with Aeromonas (McNeeley et al., 1984). Similarly, some members of the genus Plesiomonas share antigenic properties with Shigella strains, hence the species name shigelloides (Von Graevenitz, 1985). Over the years, P. shigelloides has undergone taxonomic changes and has been variously known as C27 (Ferguson and Henderson, 1947), Pseudomonas shigelloides (Bader, 1954), Ps. michigani (Sakazaki et al., 1959), Aeromonas shigelloides (Ewing, Hugh, and Johnson, 1961), Fergusonia shigelloides (Sebald and Veron, 1963) and Vibrio shigelloides (Hendrie, Shewen and Veron, 1971).

Isolation and Identification:

The increasing frequency of isolation of Aeromonas species and P. shigelloides from clinical specimens can

be attributed in part to improved diagnostic procedures, as well as the increased awareness by clinical microbiologists of unusual Gram-negative bacterial infections. Both organisms are not nutritionally exacting and as such they can be grown on most common enteric media such as MacConkey agar (MAC) and desoxycholate citrate agar (DCA). On such enteric media, they may appear as lactose - fermenters, late lactose-fermenters or non lactose fermenters. Therefore, they have been mistaken in the past for Escherichia coli, non-pigmented Serratia marcescens and other Enterobacteriaceae (Trust and Chipman, 1979; Von Graevenitz, 1985).

Being facultative anaerobes, incubation is usually in air at 37°C for 18 to 24 hours. Popoff (1984) recommended 28°C as the optimal growth temperature for motile Aeromonas strains. However, Statner, Jones and George (1988) specifically examined the effect of incubation temperature on growth and soluble protein profiles of motile Aeromonas strains,and reported that optimal growth temperature for this group of organisms is not necessarily 28°C. Their results even showed that better growth, manifested by a shorter lag phase and higher rate of log-phase growth, can occur at 37°C.

Though this finding has not been investigated for *P. shigelloides*, the situation may not be different from that observed in *Aeromonas* strains.

In the last two decades, a variety of differential and selective media have been described for the enhanced isolation of *Aeromonas sp.* and *P. shigelloides* from clinical specimens. These include deoxyribonuclease test agar supplemented with toluidine blue and ampicillin (Von Graevenitz and Zinterhofer, 1970), inositol-brilliant green-bile salts agar (Schubert, 1977), pril-xylose-ampicillin agar (Rogol *et al.*, 1979), MacConkey agar with trehalose instead of lactose (Kaper *et al.*, 1981), xylose - desoxycholate - citrate agar (Shread, Donovan and Lee, 1981), blood agar supplemented with 10 - 30 ug/ml of ampicillin (Gracey, Burke and Robinson, 1982; Millership and Chattopadhyay, 1984; Mishra *et al.*, 1987) and cefsulodin - irgasan - novobiocin agar (Altorfer *et al.*, 1985).

A number of these media have been evaluated for their sensitivity and specificity to select *Aeromonas sp.* and *P. shigelloides* from faecal specimens. Von Graevenitz and Bucher (1983) evaluated nine solid and two liquid media, and recommended four media including xylose

desoxycholate citrate agar (XDCA) and inositol-brilliant green-bile salts agar (IBBA) for Aeromonas sp. and P. shigelloides. They also recommended alkaline peptone water (APW) as enrichment broth for both organisms.

Similarly, Kay, Guerrero and Sack (1985) recommended the use of sheep blood agar with 10 ug of ampicillin per ml (ABA - 10), preceded by an overnight enrichment in APW for Aeromonas sp.. However, Mishra et al. (1987) recommended blood agar with 30 ug/ml (ABA - 30) as being more selective for Aeromonas strains, although this will preclude the isolation of small numbers of ampicillin sensitive strains.

More recently, Kelly, Stroh and Jessop (1988) compared 4 differential media for the isolation of Aeromonas sp. from stool specimens. Results of their study suggested that blood agar with 20 ug/ml of ampicillin (ABA - 20) is superior to the other media, but optimal recovery of the organism would require the use of more than one medium. They also discovered that at least 10% of their isolates would have been missed if B-haemolysis is used to screen their (ABA - 20) plates for Aeromonas strains.

In the past, it was believed that a selective medium may not be necessary for the isolation of Aeromonas sp. and P. shigelloides from human faeces and that routine

enteric media would suffice. However, there are recent reports that carbohydrates present in enteric agars may have some inhibitory effect on these organisms, presumably because of by-products of carbohydrate metabolism (Millership & Chattopadhyay, 1984; Robinson et al., 1984). Many authors now recommend the use of blood agar with ampicillin in conjuction with XDCA or IBBA, for optimal recovery of these organisms from clinical specimens.

Like other enteric bacterial pathogens, identification of Aeromonas sp. and P. shigelloides is based primarily on morphological and biochemical characteristics. On blood agar (BA), many strains of Aeromonas show a large zone of α- or B-haemolysis though nonhaemolytic strains do occur (Von Graevenitz, 1985). The colonies are small (1 to 3mm), smooth, convex, and haemolytic ones may become dark green after 3 - 5 days (Gilardi, Bottone and Birnbaum, 1970). On enteric differential media, majority are non lactose - fermenters but a few are lactose - fermenters. In contrast to Aeromonas, P. shigelloides are usually not B-haemolytic on BA and generally do not ferment lactose on enteric agars (Von Graevenitz, 1985).

On XDCA, both organisms do not ferment xylose and are therefore colourless whereas most members of the

Enterobacteriaceae and other xylose fermenters are pink, often with a surrounding pink halo. On brilliant green-bile salts agar (BBG), all coliforms appear as colourless colonies and Aeromonas sp. and P. shigelloides are distinguished by their rapid oxidase reaction. The oxidase test is extremely useful in the separation of the Vibrionaceae, from the larger family of Enterobacteriaceae (Von Graevenitz, 1985).

On Kligler iron agar (KIA) or triple sugar iron agar (TSI), an alkaline slant over an acid and gas butt is observed, indole is usually produced from tryptophane which helps to separate Aeromonas sp. and P. shigelloides from pseudomonads, alcaligenes and flavobacteria (Von Graevenitz, 1985).

In summary, isolation of Gram-negative, oxidase positive, motile rods, giving a fermentative reaction in TSI or KIA suggests Aeromonas or P. shigelloides. Their fermentative ability distinguishes them from the pseudomonads. Their ability to grow in the presence of vibriostatic compound 2, 4 - diamino - 6, 7 - disopropylpteridine phosphate (0/129), as well as their inability to grow in 6.5% NaCl broth separates them from the genus Vibrio. Furthermore, Aeromonas sp. can be separated from P. shigelloides by the former's production of DNase, its lack of ornithine decarboxylase, and its fermentation of mannitol but not inositol. Recently,

Hickman-Brenner *et al.* (1987) reported some ornithine decarboxylase producing strains for which the name *Aeromonas veronii* has been proposed.

Epidemiology:

Aeromonas and *Plesiomonas* are natural inhabitants of both fresh and salt water which is thought to be the source of the infecting organisms (Hazen *et al.*, 1978; Ljungh and Wadstrom, 1985). They are commonly isolated from fish and other invertebrates living in these waters, from soil, and food. They are established as primary pathogens of cold-blooded animals, particularly fish (Miller and Chapman, 1976). In humans, they have been associated with a wide spectrum of diseases, including water-associated soft tissue infections, septicaemia, food poisoning and diarrhoea particularly in immuno-compromised hosts (Davis, Kane and Garagusi, 1978; Agbonlahor *et al.*, 1982; Ljungh and Wadstrom, 1985; Nolte *et al.*, 1988).

The incidence of healthy carriers of *Aeromonas* varies, reflecting the number of bacteria contaminating the drinking water. As such, in Australia and Europe the incidence is less than 1% (Lautrop, 1961; Gracey *et al.*, 1982). In Thailand however, Echeverria *et al.* (1981) isolated *Aeromonas* from all drinking-water jars and

74% of the canals in the study area. However, among the United States of America (USA) Peace Corps volunteers in Thailand, with less prior exposure to Aeromonas, the incidence was low (Echeverria et al., 1981). Furthermore, Aeromonas species have been reported to survive chlorinated water (Le Chevallier et al., 1982) and can multiply at temperatures below 8°C (Schubert, 1981). In a related study, Gray (1984) isolated Aeromonas from drinking water and from 11.8% of livestock (horses, cows, sheep and pigs) consuming this water.

Generally, the number of healthy faecal carriers of P. shigelloides has been reported to be very low. Arai et al. (1980) found Plesiomonas in the faeces of only 3 (< 0.01%) of 38,454 subjects in Japan. Also, Pauckova and Fukalova (1968) found the organism in only 15 (0.14%) of 10,643 faecal specimens in Czechoslovakia. However, Pitarangsi et al. (1982) surprisingly isolated Plesiomonas from the faeces of 25 (5.5%) of 451 subjects without diarrhoea in Thailand.

Though well-defined water - or food-borne outbreaks of gastrointestinal infection caused by Aeromonas or Plesiomonas are not commonly reported, a few reports point to these routes as most probable mode of transmission. Tsukamoto et al. (1978) reported two water-borne epidemics in Japan in which P. shigelloides were isolated

from patients, tap-water and ponds; and no other bacterial enteropathogen was identified. Similarly, Rutala et al. (1982) suggested that an oyster - associated outbreak of diarrhoeal disease in North Carolina was possibly caused by P. shigelloides. In Zaire, diarrhoea possibly related to consumption of P. shigelloides contaminated fresh - water fish has been reported (Van Damme and Vanderpitte, 1980).

In what is probably the first documented case in Nigeria, Agbonlahor et al. (1982) reported a case of food poisoning due to A. hydrophila in a 64 - year old man seen at the University of Jos Teaching Hospital on the 7th of July, 1980. The man presented with sudden onset of acute diarrhoea and abdominal pains some 20 hours after a meal of fried prefrozen edible land snails (Achatina sp.). His stool culture yielded a heavy growth of A. hydrophila and no other enteric pathogen was isolated. Similarly, in a retrospective study, A. hydrophila was recovered from oysters associated with gastroenteritis in Louisiana, USA. The oysters were earlier found to be negative for Salmonella, Vibrio parahaemolyticus and diarrhoeatic shellfish poison (Wekell et al., 1986).

On the seasonality of Aeromonas and Plesiomonas - associated diarrhoea, higher isolation rates have been reported for these organisms during the wet months of

the year (Ljungh and Wadstrom, 1985, Von Graevenitz, 1985). In another related study at the Infectious Disease Section, Michigan State University, USA, Agger, McCormick and Gurwith (1985) reported that diarrhoeal cases due to A. hydrophila were detected 1.5 times more often during the summer months than the winter months, and most occurred in children less than 2 years of age. Recently, Aeromonas has been implicated as a cause of travellers' diarrhoea (Rahman and Willoughby, 1980; Gracey et al., 1984; Echeverria et al., 1984).

Clinical Features:

The clinical manifestation of Aeromonas and Plesiomonas - associated enteric infections range from acute watery diarrhoea sometimes with fever, to acute or chronic dysentery suggesting the involvement of both the small and large intestine (Ljungh and Wadstrom, 1985; Von Graevenitz, 1985). Most episodes are usually self - limiting but may present quite severe symptoms. It is likely that surface characteristics determine the level of adhesion in the gut and that the production of toxins and enzymes determine the severity and kind of manifestation (Ljungh and Wadstrom, 1985). As in other

enteric infections, host factors also play a role in the eventual outcome of an attack.

Furthermore, there appeared to be geographical differences in the symptoms accompanying Aeromonas - related enteric infections. In North America most patients present with acute watery diarrhoea whereas in Australia as many as 20% of the patients present with dysentery or colitis (Gracey et al., 1982). Common symptoms include fever above 38°C, abdominal cramps and diarrhoea of more than 10 days duration (Agger et al., 1985; George et al., 1985). In a case report of Aeromonas food poisoning in Nigeria by Agbonlahor et al. (1982), the 64-year old patient had sudden onset of acute diarrhoea and abdominal pains. He was also afebrile, did not vomit and was mildly dehydrated.

On the other hand, Plesiomonas - associated gastroenteritis may present as a mild self-limiting illness or as a mucoid, bloody diarrhoea with polymorphonuclear leucocytes found on faecal smears (Reinhardt and George, 1985a; Holmberg et al., 1986). Rolston and Hopfer (1984) have noted that patients with gastrointestinal malignant neoplasms may be more susceptible than normal persons to Plesiomonas - associated diarrhoea and that such cases may be relatively severe. Recently, Holmberg et al. (1986) reviewed enteric infections caused by Plesiomonas and

reported that gastrointestinal symptoms and fever usually occur within 48 h of ingestion of contaminated food or water, and last an average of 11 days.

Although, most patients with Aeromonas or Plesiomonas associated enteric infections recover without complications, extraintestinal infections and other complications do occur particularly in immunocompromised hosts. Septicaemia, meningitis, cellulitis and septic arthritis are common systemic complications of enteritis due to Aeromonas and Plesiomonas (Reinhardt and George, 1985a; Agger, McCormick and Gurwith, 1985). Recently, Ingram, Morrison and Levitz (1987) reported a case of gastroenteritis, sepsis, and osteomyelitis caused by P. shigelloides in an immunocompetent host.

Several studies have reported that the incidence of Aeromonas and Plesiomonas - associated enteritis was higher in the summer or wet months of the year (Agger, McCormick and Gurwith, 1985; Reinhardt and George, 1985a). Similarly, enteritis due to these organisms affect all age groups but the very young (children under 3 years) and the fairly old (>60 years) and above are more susceptible (Agbonlahor et al., 1982; Reinhardt and George, 1985a; Sack, Lanata and Kay, 1987).

Virulence Factors:

Attempts to demonstrate enteropathogenicity of Aeromonas sp. and P. shigelloides have resulted in the identification of virulence characteristics, which are recognised as being important in the pathophysiology of diarrhoea caused by established pathogens such as Vibrio cholerae, Escherichia coli and Shigella species (Cleary and Pickering, 1986, Prado et al., 1986).

Several earlier studies have shown that Aeromonas sp. produce a variety of biologically active extracellular substances which are potential virulence factors. These include enzymes (Von Graevenitz, 1985), haemolysins (Wretlind, Mollby and Wadstrom, 1971), cytotoxins (Donta and Haddow, 1978), enterotoxins (Sanyal, Singh and Sen, 1975; Asao et al., 1984), proteases (Dahle, 1971) and surface adhesins (Atkinson and Trust, 1980). On the other hand, most of the few studies to demonstrate virulence factors in P. shigelloides have not been successful (Sanyal, Singh and Sen, 1975; Pitarangsi et al., 1982).

Haemolysins

Many bacteria are able to lyse erythrocytes, based on the activity of a most heterogenous group of toxins, the haemolysins. Majority of human isolates of Aeromonas

are haemolytic, and many of their extraintestinal clinical manifestations are accompanied by soft tissue necrosis indicating that haemolysin is a virulence factor. The presence of two distinct haemolysins in Aeromonas sp. was first suggested by Caselitz (1966) and later studied by others (Wadstrom et al., 1976; Boulanger et al., 1977; Ljungh, Wretlind and Mollby, 1981).

The most potent of the haemolysins, B-haemolysin or aerolysin, has been purified and characterised (Bernheimer and Avigad, 1974; Ljungh, Wretlind and Mollby, 1981). It is a heat-labile protein with molecular weight of 50 - 51,000 daltons. It was found to be cytotoxic with a broad cell specificity and binds rapidly to cell membranes also at $0^{o}C$. In addition, it is dermonecrotic in rabbit skin and lethal to rabbits and mice (Ljungh, Wretlind and Mollby, 1981). In the rabbit intestinal loop (RIL) test, it causes accumulation of small amounts of sanguinolent fluid with high calcium and albumin content, indicating leakage of intracellular substances due to membrane damage (Ljungh and Krenovi, 1982).

Aeromonas strains also produce a second haemolysin, α - haemolysin, which is a weak haemolytic agent that causes incomplete haemolysis of ox erythrocytes with an enzymatic kinetics (Ljungh, Wretlind and Mollby, 1981).

It also induces the release of large molecular weight markers in radioactively labelled fibroblasts concomitant with rounding of the cells and fading of the nuclei (Thelestam and Ljungh, 1981). However, its role in pathogenicity remains to be elucidated.

Recently, the production by A. hydrophila of another haemolysin called "Asao" toxin was demonstrated by Asao and his colleagues in Japan (Asao et al., 1984). The haemolysin has enterotoxic and cytotoxic effects, and a molecular weight of 48,000 to 50,000 daltons. In polyacrylamide gel electrophoresis at pH 4.0 and sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis, the haemolysin migrated as a single band. The purified haemolysin caused fluid accumulation in infant mouse intestines and rabbit ligated ileal loops, and was cytotoxic to Vero cells. However, these biological activities were lost after heating at 56°C for 5 minutes, indicating that it is a heat-labile protein. Furthermore, the production of the "Asao" toxin and a cholera reactive factor by strains of A. hydrophila was also demonstrated by Chopra et al. (1986).

Enterotoxins

Enteropathogenicity in strains of Aeromonas and Plesiomonas was first demonstrated in India by Sanyal, Singh and Sen (1975) by injecting whole cell cultures in

rabbit intestinal loop (RIL) tests. They observed that A. hydrophila was enteropathogenic, causing a fluid accumulation of 1.0 - 2.0 ml per cm of gut length. They also noted that gut reaction could be produced with an inoculum as low as 10^4 viable bacteria. On the other hand, P. shigelloides did not cause a significant gut reaction and their rates of multiplication in the gut was much lower than that of A. hydrophila.

Furthermore, the presence of an extracellular heat-labile protein enterotoxin in A. hydrophila strains was shown independently by Wadstrom, Ljungh and Wretlind (1976) in Sweden, and Annapurna and Sanyal (1977) in India. The enterotoxin was produced in various complex media and appeared in the culture medium during the late logarithmic growth phase (Ljungh, Wretlind and Mollby, 1981). Subsequently, some enterotoxin production has since been confirmed by other workers in strains isolated from humans and animals (Pitarangsi et al., 1982; Boulanger, Lallier, and Cousineau, 1977; Dobrescu, 1978; Agbonlahor, 1983; Chopra et al., 1986).

On further characterisation, Ljungh and Kronevi (1982) reported that A. hydrophila enterotoxin causes fluid accumulation in rabbit, rat and mouse intestinal loops without mucosa damage. The onset of fluid accumulation in rabbit intestine was rapid, as with

E. coli heat-stable (ST) toxin, and maximal after 6 - 10 hours. Also analysis of accumulated fluid showed no significant difference between fluids accumulated in response to cholera toxin (CT), *E. coli* LT and *Aeromonas* enterotoxin with respect to electrolyte and protein content. These data then suggest *Aeromonas* enterotoxin as a cytotonic enterotoxin which induces fluid secretion at least partially via the adenylate cyclic adenosine monophosphate (cAMP) pathway (Keusch and Donta, 1975).

Evidence that *A. hydrophila* may also produce a heat-stable (ST) enterotoxin was provided by Boulanger, Lallier and Cousineau (1977). They reported fluid secretion induced by heated (100°C) samples of *A. hydrophila* culture filtrate. They further reported that fluid secretion was inhibited in rats immunised with cholera toxin, thus indicating a similarity between the two toxins. More recently, Chakraborty *et al.* (1984) cloned an enterotoxin which caused a positive suckling mouse test but negative haemolysin tests in *E. coli*. They thus provided conclusive evidence of a cytotonic — and not a cytotoxic enterotoxin produced by *A. hydrophila*.

Several attempts to demonstrate enteropathogenicity or enterotoxigenicity of *P. shigelloides* have not been successful. However, Sanyal, Saraswatchi and Sharma (1980)

reported distension of rabbit intestinal loops with live cells as well as culture filtrates of 13 strains of *P. shigelloides*. The suckling mouse test was also reported positive with the strains. As with *E. coli* ST, onset of fluid accumulation in rabbit intestine was early and the activity was stable at 100°C. The culture filtrates also caused lysis or dislodgement of adrenal YI cells and CHO cells.

Invasiveness

Some patients with *Aeromonas* and *Plesiomonas* intestinal infection have been reported to present with dysentery (Rahman and Willoughby, 1980; Mandal *et al.*, 1982; Taylor *et al.*, 1986). Ingram *et al.* (1987) reported a case of gastroenteritis, sepsis, and osteomyelitis caused by *P. shigelloides* in an immuno-competent host. More recently, Nolte *et al.* (1988) also reported proctitis and fatal septicaemia caused by *P. shigelloides* in a 42-year-old bisexual man. These are pointers to the fact that these organisms may be invasive.

Few experiments to demonstrate invasiveness (Sereny, 1955) using strains of *A. hydrophila* and *P. shigelloides* have been negative (Ljungh and Krenqvi, 1982; Sanyal *et al.*, 1980). However, on the basis of histopathological studies, Pitarangsi *et al.*

(1982) stated that Aeromonas can be invasive. These findings were later confirmed by Lawson, Burke and Chang (1985) who showed invasion of HEp-2 cells by faecal isolates of A. hydrophila.

Furthermore, at least one heat-stable (100°C for 30 mins) and one heat-labile protease have been described in Aeromonas (Riddle, Graham and Amborski, 1981; Buckley et al., 1981). The heat-stable protease has been reported to show an antigenic relationship to a protease produced by strains of V. cholerae (Dahle and Sandvik, 1971). Similarly, both enterotoxigenic and non-enterotoxigenic Aeromonas strains have been reported to produce a variety of surface haemagglutinins which are putative candidates for intestinal colonization factors (adhesins), enabling the organism to establish itself in the small bowel (Atkinson and Trust, 1980). Furthermore, Freer et al. (1978) also reported hydrophobic cell surface properties, haemagglutinating activity and fimbrae or fimbrae-like structures as common features in enterotoxigenic Aeromonas isolates.

Antimicrobial Susceptibility Pattern:

The discovery of penicillin in 1928 by Fleming, and the sulphonamides in the 1930s marked the beginning of the modern era of antimicrobial therapy. Subsequently,

the judicious and widespread use of antimicrobial agents has altered the importance of many human diseases and change the patterns of medical care. Diseases such as bacterial endocarditis, miliary tuberculosis and cryptococcal meningitis, formerly regarded as always fatal, are now curable.

Most cases of acute enteritis caused by Aeromonas and Plesiomonas are self-limiting and require only symptomatic treatment and rehydration. However, antimicrobial treatment is indicated in patients with extra-intestinal complications, those immunocompromised and those with prolonged enteric infection. Both organisms have been reported to be usually sensitive (in vitro) to chloramphenicol, tetracycline, polymyxins, nitrofurantoin, nalidixic acid, colistin, and aminoglycosides (Overman, 1980; Washington, 1972; Von Graevenitz, 1985). On the other hand, both are said to be universally resistant to ampicillin and penicillin (Trust and Chipman, 1979), though a strain producing an inducible B-lactamase capable of hydrolysing oxacillin and methicillin has been reported in Aeromonas (Sawai et al., 1978). More recently, Bakken et al. (1988) reported B-lactamase resistance in Aeromonas sp., caused by inducible B-lactamases active against penicillins, cephalosporins, and carbapenems.

Based on their susceptibility to a number of antimicrobial agents, Reinhardt and George (1985b) reported that agents that might be useful for the treatment of enteric infections due to Aeromonas or Plesiomonas include tetracycline, chloramphenicol, trimethoprim - sulphamethoxazole and the quinolones. However, like other enteropathogens, it is suggested that antimicrobial susceptibility of an isolate be determined before treatment is initiated.

Screening for Plasmids in Aeromonas and Plesiomonas:

Plasmids are extrachromosomal deoxyribonucleic acids (DNA) capable of stable autonomous replication. Their recognition came in the early 1950s soon after the discovery of conjugation in the bacterium Escherichia coli by Joshua Lederberg (1952). While they are not essential for the survival of bacteria, plasmids may code for a wide variety of genetic determinants which permit their bacterial hosts to survive better in an adverse environment, or to compete better with other microorganisms occupying the same ecological niche.

Plasmids are initially recognised by the phenotypic properties or biological functions they confer on a cell. These include resistance to antimicrobial agents (Datta, 1962; Olukoya et al., 1988a & b; Rotimi, Emina and

Eke, 1983), some metal ions (Novic, 1967; Smith, 1967) and colicins (Fredericq, 1958). Other plasmid mediated properties that have been documented include virulence (Zink et al., 1978; Gotuzzo et al., 1987), enterotoxin production (Smith and Halls, 1968), ability to hydrolyse certain compounds (Ishiguro et al., 1979), and adherence (Nandadasa et al., 1981). Recently, plasmid profile analysis has become a very useful tool in the epidemiological studies of bacterial infections (Odugbemi et al.,1983a & b; Tenover et al., 1985; Nakamura et al., 1986).

Presently, very little is known on the prevalence of plasmids in Aeromonas or Plesiomonas. However, Shotts et al. (1976) has reported plasmid-mediated antibiotic resistance in A. hydrophila isolated from fish, while Olsen and Wright (1971) reported that A. salmonicida can be the recipient of resistance plasmids from Enterobacteriaceae and the pseudomonads. More recently, P. shigelloides isolated from case of proctitis and fatal septicaemia in an immunocompetent host, was shown to possess a 100-megadaltons plasmid. These findings have emphasised the need for screening of clinical isolates of these organisms for possible plasmid-mediated characters.

Nigerian Isolates of Aeromonas and Plesiomonas:

Obi and Nzeako (1980) were the first to report isolation of A. hydrophila along with Salmonella and Shigella species from about 40% of 39 edible land snails (Achatina sp.) in the Eastern part of Nigeria. They also reported that 58% of their isolates were enterotoxigenic. However, the first report of an Aeromonas - associated food poisoning in Nigeria was by Agbonlahor et al. (1982) in a 64-year-old male seen at the University of Jos Teaching Hospital on 7th July, 1980. The patient presented with sudden onset of diarrhoea and abdominal pains, and reportedly had a meal of fried prefrozen edible land snails some 20 hours before reporting. His stool culture yielded a heavy growth of A. hydrophila and no other enteric pathogens was isolated. Also, the A. hydrophila isolate was enterotoxigenic and B-lactamase positive.

Furthermore, in another prospective study between October 1979 and March 1981 at the Lagos University Teaching Hospital, Agbonlahor (1983) reported the isolation of A. hydrophila from the stools of six patients with diarrhoea. Also, all the isolates were B-lactamase positive, while five were haemolytic and cytotoxic, thus suggesting clinical significance of the isolates.

While one appreciates the efforts of these pioneers, information presently available on Aeromonas and Plesiomonas - associated diarrhoea in Nigeria is far from being satisfactory. The organisms are not routinely sought in clinical specimens probably due to lack of understanding of isolation techniques or mere assumption that they are not clinically significant based on reports from other parts of the world. This situation is quite unacceptable and hence, there is an urgent need for further studies of local isolates to understand their epidemiology and possible roles in acute diarrhoeal diseases.

Objectives of the study

1. To establish the prevalence of Aeromonas sp. and P. shigelloides in faecal specimens of patients, with or without diarrhoea, submitted to the enteric laboratory of the Lagos University Teaching Hospital (LUTH), Idi-Araba, Lagos, Nigeria.

2. To examine the biochemical characteristics of the isolates, with a view to propose a simple scheme for their isolation and preliminary identification from faecal specimens.

3. To examine the survival of the isolates in transport media commonly used in the enteric laboratories.

4. To examine the isolates for the production of haemolysins, heat-stable (ST) enterotoxin, and invasiveness, which are potential virulence factors.

5. To examine the enteropathogenicity of isolates possessing potential virulence factors in healthy adult mice.

6. To determine the <u>in vitro</u> susceptibility pattern of isolates to some antimicrobial agents.

7. To screen the isolates for the presence of plasmids.

CHAPTER 2

PREVALENCE OF AEROMONAS SP. AND P. SHIGELLOIDES IN PATIENTS WITH AND WITHOUT DIARRHOEA IN LUTH, LAGOS, NIGERIA

INTRODUCTION

Several reports on the isolation of *Aeromonas sp.* and *P. shigelloides* from faecal specimens of patients in different parts of the world indicate that these organisms may be capable of causing acute diarrhoeal diseases (Bulger and Sherris 1966; Cumberbatch *et al.*, 1979; Agbonlahor, 1983; Agger, McComick and Gurwith, 1985; Rutala *et al.*, 1982; Ljungh and Wadstrom, 1985; Arai *et al.*, 1980; Reinhardt and George, 1985a). Diarrhoea associated with these organisms are reported to be usually mild and self-limiting, though a more severe form that was acute colitis or a cholera-like illness have been documented (Ljungh and Wadstrom, 1985; Huq and Islam, 1983; Agger, McCormick and Gurwith, 1985).

On the other hand, some investigators have not been able to ascribe any aetiological role to either of these organisms based on their isolation rates from patients with and without diarrhoea (Morgan *et al.*, 1985; Figura *et al.*, 1986). For instance, in a study in Thailand by Pitarangsi *et al.* (1982), both *Aeromonas sp.* and *P. shigelloides* were isolated with similar frequencies from individuals with and without diarrhoea, suggesting that both organisms may be part of the normal human intestinal flora.

Hence, the significance of Aeromonas sp. and P. shigelloides as agents of diarrhoea is still controversial. Their clinical significance has not been proven conclusively, and is subject of current research efforts worldwide. It is a paradox however that most of these researches are going on in laboratories in the developed countries, while very little is being done in the developing countries where acute diarrhoeal diseases are more endemic.

In Nigeria, very little information is available on Aeromonas and Plesiomonas - associated diarrhoea. However, Agbonlahor (1983) emphasized the opportunistic pathogenic role of six Aeromonas isolates from patients with acute diarrhoea in Lagos. Though normal subjects were not included as controls in the study, the result was unique in that it emphasized the seemingly important role of Aeromonas as an opportunistic pathogen in the Lagos environment.

Despite this report by Agbonlahor (1983) and the recent universal upsurge in research activities on Aeromonas and Plesiomonas - associated diarrhoea, the organisms are still not commonly sought from routine stool cultures in Nigeria, and diarrhoea that may be due to them are probably grossly under-estimated. There is an urgent need therefore to update available information on these organisms, as well as identify

new ones that may be peculiar to our environment.

This chapter determines the prevalence of Aeromonas sp. and P. shigelloides in patients with and without diarrhoea seen at the Lagos University Teaching Hospital, Lagos, Nigeria.

MATERIALS AND METHODS

Patients and Source of Isolates:

The study included 2,350 patients with diarrhoea and 500 patients without diarrhoea (controls) who submitted faecal specimens for culture at the enteric laboratory of the Department of Medical Microbiology and Parasitology, Lagos University Teaching Hospital (LUTH), Idi-Araba, Lagos; from October 1986 through September 1987. Diarrhoea in this study was defined as three or more loose stools per day within two days of collection of faecal specimen. Patients in the control group had no diarrhoea within two weeks preceding specimen collection. Other data obtained on each patient include age, sex, history and clinical features such as abdominal pain, anorexia and weight loss.

Lagos is a cosmopolitan city and Federal capital of Nigeria. It has an estimated population of about

Non-xylose fermenting colonies on XDCA were then tested for oxidase production (Kovacs, 1956), and oxidase positive colonies were subcultured onto nutrient agar (Oxoid Ltd, Wade Road, Basingstoke, Hampshire), for further characterisation. Similarly, ABA plates were flooded with 1% sodium dimethyl-T-phenylenediamine monohydrochloride (oxidase reagent), and oxidase positive colonies were quickly picked for subculture to ensure that viability was not lost. Growth in APW after 18 - 24 h incubation were subcultured onto XDCA and ABA, and cultures obtained were tested for oxidase as stated earlier.

Oxidase positive colonies that were Gram-negatives, and not typical of Pseudomonas aeruginosa by pigmentation, odour were screened on Kligler's iron agar (KIA) and motility indole urea (MIU) medium, for the production of hydrogen sulphide, gas, indole and urease enzyme, as well as motility and utilisation of glucose and lactose. Further confirmation of isolates as Aeromonas sp. or P. shigelloides was by the criteria outlined by Von Graevenitz (1985) in the Manual of Clinical Microbiology, based on earlier characterisation of Aeromonas by Popoff (1984) and Plesiomonas by Schubert (1984) in the Bergey's Manual of Systematic Bacteriology (N. R. Krieg and J. G. Holt (ed.), Vol. 1).

Isolation and Identification of other bacterial pathogens:

Fresh faecal samples were inoculated onto MacConkey agar (MAC), desoxycholate citrate agar (DCA), thiosulphate - citrate - bile salts - sucrose agar (TCBS), and blood agar base (BA) containing 5% sheep blood. Selenite F broth was used as an enrichment for Salmonella and Shigella while APW was used for V. cholerae. Also, cold enrichment at 4°C in phosphate buffered saline (pH 7.3) was used for Yersinia enterocolitica isolation. Inoculated plates, except those for Y. enterocolitica, were incubated aerobically at 37°C for 18 - 24 h. The Y. enterocolitica plates were incubated aerobically at room temperature (22 - 25°C) for 24 - 48 h. On primary isolation, suspicious colonies were screened using KIA and MIU. Further identification of isolates was by standard biochemical tests as described by Cowan and Steel (1974), and Kelly, Brenner and Farmer (1985). Serological confirmation of identified isolates was done by slide agglutination using Wellcome antisera (Wellcome Reagents Ltd, Wellcome Research Laboratories, Beckenham, England). For economic reasons, culture of stools for Campylobacter was not done.

Statistical Analysis:

Statistical analysis of results was done using the chi - square test (Colton, 1974).

Control Strains:

Positive control strains of *Aeromonas sp.* and *P. shigelloides* included in this study were kindly provided by Dr. D. M. Jones of the Public Health Laboratory, Withington Hospital, Manchester, England.

RESULTS

The isolation rates of bacterial enteric pathogens from 2,350 faecal samples of patients with diarrhoea, during the 1-year period from October 1986 through September 1987 were shown in Table 1. *Aeromonas sp.* was isolated from 53 (2.26%), *P. shigelloides* from 16 (0.68%), *Shigella sp.* from 4 (0.17%), *Salmonella sp.* from 3 (0.13%) and *Y. enterocolitica* from 2 (0.09%) of faecal specimens examined. Enteropathogenic *E. coli* (EPEC) was isolated from 39 (2.85%) of 1,368 specimens examined while *V. cholerae* was not isolated from any of the specimens. Therefore, in this study, EPEC was the most frequently isolated enteropathogen, closely followed by *Aeromonas sp.* and *P. shigelloides*. Other established enteropathogens like *Shigella*, *Salmonella* and *Yersinia* were less frequently encountered than *Aeromonas sp.* or *P. shigelloides*.

Table 2 shows the prevalence of *Aeromonas sp.* and *P. shigelloides* in faecal specimens of patients with or without diarrhoea (controls) examined during the study.

Aeromonas sp. was isolated from 53 (2.26%) of 2,350 patients with diarrhoea and from 2 (0.4%) of 500 controls (χ^2 = 7.32, $p < 0.01$). On the other hand, P. shigelloides was isolated from 16 (0.68%) of corresponding number of patients with diarrhoea and none of the controls (χ^2 = 3.42, $p > 0.05$).

In this study, characteristics of Aeromonas sp. and P. shigelloides - associated diarrhoea were not significantly different from those of other known bacterial enteric pathogens. Clinical features observed in Aeromonas - associated diarrhoea include abdominal pains in 3 (5.7%), anorexia in 4 (7.6%), weight loss in 3 (5.7%) and vomiting in 5 (9.4%) of 53 patients (Table 3). Similarly, there was abdominal pains in 2 (2.5%), anorexia in 2 (17.5%), weight loss in 1 (6.5%) and vomiting in 1 (6.3%) of 16 P. shigelloides - asseciated diarrhoea (Table 3).

The distribution of cases of Aeromonas sp. and P. shigelloides - associated diarrhoea according to age and sex of patients is shown in Table 4 and Figures 1 and 2. There were 20 (37.7%) Aeromonas sp. related episodes in males and 33 (62.3%) in females. On the other hand, more Plesiomonas - associated diarrhoea occurred in males, 10 (62.5%), than in females, 6 (37.5%). However, the difference in sex distribution of isolates was not statistically significant (χ^2 = 3.5, $p > 0.05$).

Hence, Aeromonas sp. and P. shigelloides - associated diarrhoea are not sex related. Diarrhoea due to Aeromonas sp. or P. shigelloides appeared to affect all age groups (Table 4). However, 23 (43.4%) of Aeromonas sp. - associated diarrhoea occurred in children under 5 years, while 7 (13.2%) of such episodes occurred in patients over 70 years old. Similarly, 3 (18.8%) of P. shigelloides - associated diarrhoea occurred in children under 5 years, while 5 (31.3%) of such episodes occurred in patients over 70 years old.

Figure 3 shows the monthly isolation of Aeromonas sp. during the study. Majority of the isolates were obtained during the wet months of July 10 (18.9%), August 11 (20.8%) and October 12 (22.6%). However, fewer isolates were recovered during the dry months, with no isolates in November and December. This pattern of distribution is indicative of a water-related infection. Also, monthly isolation of P. shigelloides (Fig. 4) is similar to that of Aeromonas sp. with peaks in September (25%) and October (18.8%). P. shigelloides was not isolated in the months of December, February, April and May, which form major part of the dry season in Nigeria.

COMMENT

This prospective 12-month study has shown that *Aeromonas sp.* and *P. shigelloides* are found in association with diarrhoea more often than in control patients in our environment. Although mere isolation of an organism from a diseased condition is not enough to confer pathogenicity on such an organism, the results are suggestive of aetiological roles for both *Aeromonas sp.* and *P. shigelloides* in this environment. Also, isolation rates of 2.26% *Aeromonas sp.* and 0.68% *P. shigelloides* from patients with diarrhoea, compared favourably with those of other recognised enteric pathogens obtained in this study. Although *Campylobacter* was not included in this study, its isolation rates in similar studies in our environment range between 5.2 to 10.7% (Odugbemi *et al.*, 1987).

Furthermore, *Aeromonas sp.* were significantly more frequently isolated from patients with diarrhoea than from controls ($\chi^2 = 7.32$, $p < 0.01$). Though the difference in isolation rates of *P. shigelloides* from patients with diarrhoea and controls was not statistically significant ($\chi^2 = 3.42$, $p > 0.05$), there were more isolates from the diarrhoea group. It is equally noteworthy that the figures obtained for *Aeromonas sp.* are in agreement with preliminary findings in a related study by Eko and Utsalo

at the Medical Microbiology and Parasitology Department, University of Calabar in which Aeromonas sp. were frequently isolated from patients with gastroenteritis (2.5%) than from control patients (1.0%) (Personal communication).

Workers in other parts of the world have also shown an isolation rate of less than 1% for Aeromonas sp. in normal subjects in several large screening studies (Lautrop, 1961; Catsaras and Buttiaux, 1965; Paukova and Fukalova, 1968; Burke et al., 1983). Also, Arai et al. (1980) found Plesiomonas in the faeces of only 3 (0.01%) of 38,454 normal subjects examined in Japan. However, Pitarangsi et al. (1982) found no difference in the isolation of Aeromonas sp. from normal citizens of Thailand and those with diarrhoea. Such a finding may indicate a high incidence of chronic carriage in Thailand.

Aeromonas sp. and P. shigelloides - associated diarrhoea affects all age groups, particularly children under 5 years of age (Table 4, Figs. 1 & 2). In an earlier report in Lagos by Agbonlahor (1983), two out of six Aeromonas isolates from acute diarrhoea cases were from children. In the present study, more Aeromonas sp. were isolated from females than males, while the reverse

was the case for P. shigelloides. However, the difference was not statistically significant to say that the diseases are sex - related ($\chi^2 = 3.5$, $p > 0.05$).

Other characteristics of Aeromonas and Plesiomonas - associated diarrhoea observed in this study include abdominal pain, anorexia, weight loss and vomiting. These clinical features were not distinct from those of diarrhoea due to other established enteric pathogens. Furthermore, most of the Aeromonas sp. and P. shigelloides strains were isolated during the warm and wet months of the year (Figs. 3 & 4). Several other workers have stated that the association of these organisms with water may be responsible for the apparent seasonality in the incidence of diarrhoea associated with them (Burke et al., 1983; George et al., 1985; Reinhardt and George, 1985a; Agger, McCormick and Gurwith, 1985).

Both Aeromonas sp. and P. shigelloides have been reported to cause a more severe form of diarrhoea in immunocompromised-hosts (Ljungh and Wadstrom, 1985; Von Graevenitz, 1985), thus suggesting that the organisms may be opportunistic pathogens. In this study, clinical conditions that might have predisposed to Aeromonas - associated diarrhoea were less frequent, and these were peptic ulcer in 3 (5.7%) and Kwashiorkor in 1 (1.9%) of our patients. Similarly, there was only a case each of

diabetes mellitus and kwashiorkor in a total of 16 patients with Plesiomonas - associated diarrhoea.

In conclusion, it has been widely reported that Aeromonas sp. and P. shigelloides are not part of the normal human intestinal flora (Davis, Kane and Garagusi, 1978; Reinhardt and George, 1985). Results of this study have also shown that these organisms are found in association with diarrhoea more often than in controls, in the Lagos environment. Characteristics of diarrhoea observed are also similar to those due to classical enteropathogens. However, while these findings are strongly suggestive of potential enteropathogens, there is need to further characterise the local isolates for clinical significance in Nigeria.

Table 1: Isolation Rates of Some Bacterial Enteropathogens From Stools of Patients With Diarrhoea Seen at the Enteric Laboratory of LUTH, between October 1986 and September 1987

Bacteria	No. of Stools Examined	No. of Positives	% of Positives
Enteropathogenic *E. coli*	1,368	39	2.85
Aeromonas sp.	2,350	53	2.26
P. shigelloides	2,350	16	0.68
Shigella sp.	2,350	4	0.17
Salmonella sp.	2,350	3	0.13
Yersinia enterocolitica	2,350	2	0.09
Vibrio cholerae	2,350	0	0

Table 2: Prevalence of Aeromonas sp. and P. shigelloides in Stools of Patients With and Without Diarrhoea Seen at the Enteric Laboratory of LUTH, between October 1986 and September 1987

	Aeromonas sp.		P. shigelloides	
	Diarrhoea	Non-Diarrhoea	Diarrhoea	Non-Diarrhoea
No. of Stools Examined	2,350	500	2,350	500
No. of Positives	53	2	16	0
Isolation Rates	2.85	0.4	0.68	0
	($\chi^2 = 7.32$, $p < 0.01$)		($\chi^2 = 3.42$, $p > 0.05$)	

Table 3: Clinical Features Observed in Aeromonas sp. and P. shigelloides - associated Diarrhoea in LUTH

Clinical features	No. (%) of positives in 53 Aeromonas-associated diarrhoea	No. (%) of positives in 16 P. shigelloides-associated diarrhoea
Abdominal pain	3(5.7%)	2(12.5%)
Anorexia	4(7.6%)	2(12.5%)
Weight loss	3(5.7%)	1(6.5%)
Vomiting	5(9.4%)	1(6.5%)

Table 4: Distribution of Cases of Aeromonas sp. and P. shigelloides - associated Diarrhoea by Age and Sex

Age in Years	Aeromonas sp.			P. shigelloides		
	Male	Female	Total	Male	Female	Total
0 - 5	8	15	23	2	1	3
6 - 10	2	4	6	1	0	1
11 - 20	1	3	4	1	2	3
21-- 30	3	4	7	2	0	2
31 - 40	0	1	1	1	0	1
41 - 50	1	0	1	1	0	1
51 - 60	0	1	1	0	0	0
61 - 70	1	2	3	0	0	0
71 - 80	1	3	4	1	0	1
81 - 90	1	0	1	0	1	1
91 - 100	2	0	2	1	2	3
Total (%)	20(37.7%)	33(62.3%)	53(100)	10(62.5%)	6(37.5%)	16(100)

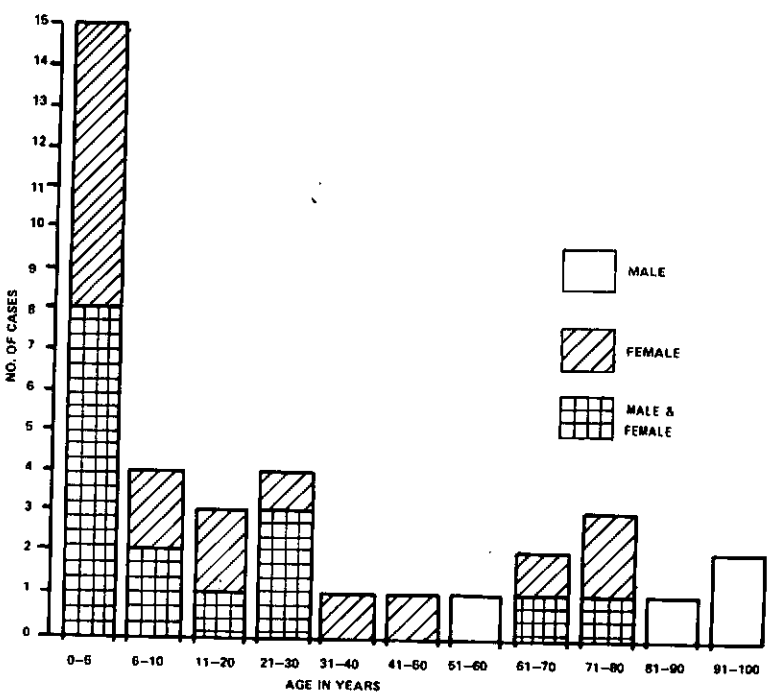


FIG. 1: DISTRIBUTION OF CASES OF *AEROMONAS* – ASSOCIATED DIARRHOEA BY AGE AND SEX.

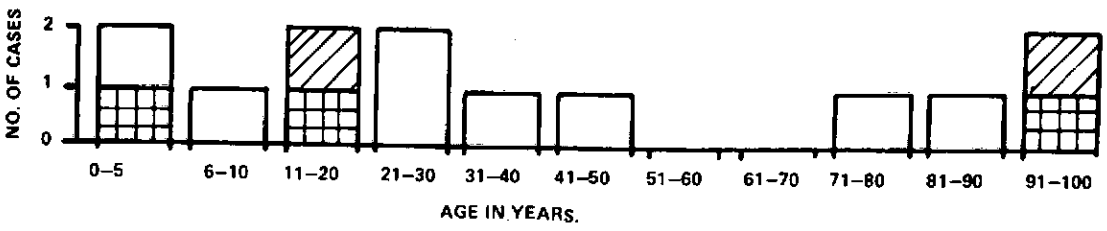


FIG. 2: DISTRIBUTION OF CASES OF *P. SHIGELLOIDES* – ASSOCIATED DIARRHOEA BY AGE AND SEX

MALE FEMALE MALE & FEMALE

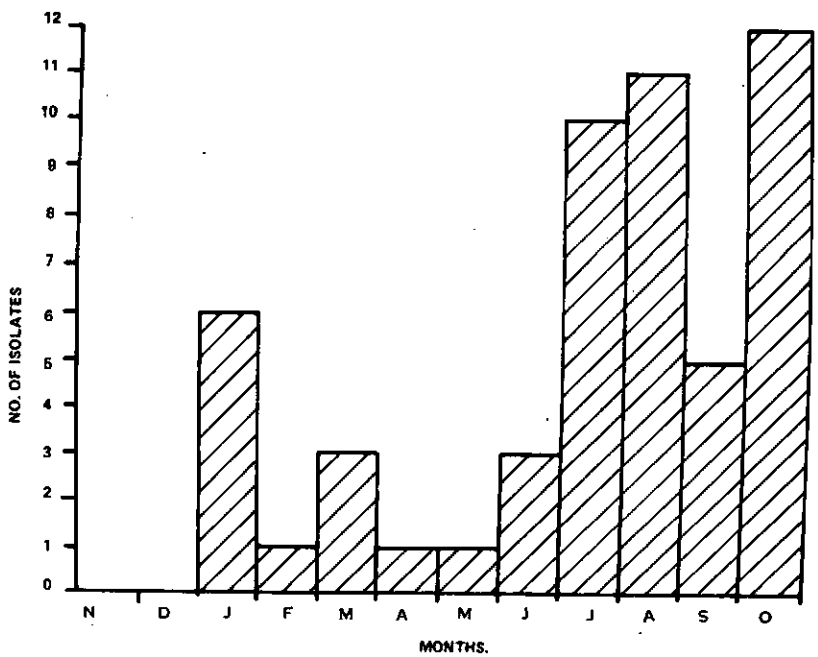


FIG. 3: MONTHLY ISOLATION OF *AEROMONAS SPP* FROM 53 PATIENTS WITH DIARRHOEA SHOWING SEASONALITY IN THE DISTRIBUTION OF ISOLATES.

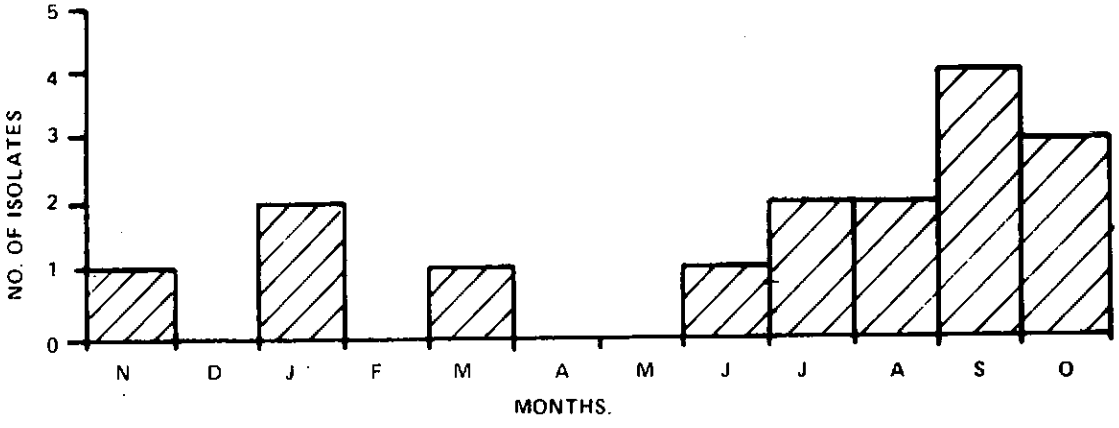


FIG. 4: MONTHLY ISOLATION OF *P. SHIGELLOIDES* FROM 16 PATIENTS WITH DIARRHOEA, SHOWING SEASONALITY IN THE DISTRIBUTION OF ISOLATES.

CHAPTER 3

BIOCHEMICAL CHARACTERISTICS AND PROPOSAL FOR A SIMPLE SCHEME FOR THE ISOLATION AND PRELIMINARY IDENTIFICATION OF AEROMONAS SP. AND P. SHIGELLOIDES FROM FAECAL SPECIMENS

INTRODUCTION

The recent upsurge in reports on the isolation of Aeromonas sp. and P. shigelloides from clinical specimens, particularly faecal specimens of patients with diarrhoea would probably result in attempts by various laboratories in different parts of the world to isolate and identify these organisms. In other words, many laboratories would now see the need for the routine culture of faecal specimens for these organisms in order to determine their clinical significance.

Aeromonas sp. and P. shigelloides usually grow well on commonly used enteric media such as MacConkey and desoxycholate citrate agars. As a result, their isolation from faecal specimens has not been much of a problem. However, on such media it is usually difficult to distinguish them from members of the larger family of Enterobacteriaceae. In addition, it has also been reported that if the pH of the medium is acidic as a result of lactose fermentation, a positive oxidase test may not always be obtained (Overman, D'Amato and Tomfohrde, 1979).

As an alternative to enteric media, several investigators had suggested the use of blood agar with 10 - 30ug per ml of ampicillin (ABA) for the isolation

of Aeromonas sp. and P. shigelloides from faecal specimens (Gracey, Burke and Robinson, 1982; Kay, Guerrero and Sack, 1985; Von Graevenitz, 1985; Mishra et al., 1987). A shortcoming of this medium however is that ampicillin will prevent the isolation of some sensitive strains (Hunt et al., 1987). It thus appears that, at the moment no single 'ideal' medium is available for these organisms. However, most authorities believe that the use of an enteric medium such as xylose desoxycholate citrate agar (XDCA) in conjunction with a selective medium such as ABA should be adequate for their optimal recovery from faecal specimens (Von Graevenitz, 1985; Kelly, Stroh and Jessop, 1988).

Similarly, several schemes have been suggested for the identification of Aeromonas sp. and P. shigelloides. These include those of Popoff and Veron (1976), Popoff (1984), Janda et al. (1984) and Schubert (1984). Furthermore, Von Graevenitz (1985) selected important tests in most of the earlier schemes and recommended about 50 biochemical characteristics for the identification of members of the two genera. This latest attempt by Von Graevenitz has no doubt reduced possible confusion that might have arisen from using different schemes to characterise Aeromonas sp. and P. shigelloides. However, the complexities, media

requirements and reagents for some of these tests are not within the reach of laboratories with limited resources, as often found in developing countries.

In such laboratories, the selection of a small number of simple and reliable biochemical tests for the preliminary identification of Aeromonas sp. and P. shigelloides is therefore an essential requirement for the routine examination of faecal specimens for these organisms. This study aims at (1) establishing the minimum number of biochemical tests required for the preliminary identification of Aeromonas sp., and P. shigelloides in a routine diagnostic laboratory; (2) proposing a simple scheme for the isolation and preliminary identification of Aeromonas sp. and P. shigelloides from faecal specimens.

MATERIALS AND METHODS

Bacterial Strains:

The 55 strains of Aeromonas sp. and 16 strains of P. shigelloides, used in this study, were isolated from faecal specimens submitted to the enteric laboratory of the Lagos University Teaching Hospital (LUTH), Idi-Araba, Lagos, between October 1986 and September, 1987. Of the 55 Aeromonas sp., 53 were from patients

with diarrhoea and 2 from controls. All *P. shigelloides* isolates were from patients with diarrhoea.

Isolates were identified as *Aeromonas sp.* or *P. shigelloides* by standard methods previously described (Popoff, 1984; Janda *et al.*, 1984; Schubert, 1984) as summarised by Von Graevenitz (1985). Isolates of *Aeromonas sp.* were identified to species level according to the scheme of Popoff (1984) as described by Von Graevenitz (1985). In all, there were 20 *A. hydrophila*, 28 *A. caviae* and 7 *A. sobria*.

Biochemical Characteristics:

All biochemical tests were performed by standard techniques, mostly as described by Cowan and Steel (1974). For the primary identification of isolates as *Aeromonas sp.* or *P. shigelloides*, isolates were tested for the production of oxidase, catalase, hydrogen sulphide, urease, haemolysis on blood agar (Oxoid), pigmentation on nutrient agar (Oxoid), gas, indole, deoxyribonuclease, amylase, B-lactamase, ornithine decarboxylase, and lysine decarboxylase. They were also tested for acid production from glucose, lactose, xylose, mannitol, inositol, maltose, sucrose, dulcitol, arabinose and adonitol. Other tests

include citrate utilisation, growth in 6.5% NaCl broth, nitrate reduction, motility, and the methyl-red, Voges - Proskauer (MRVP) test.

In addition to those characteristics observed on primary identification, tests for aesculin hydrolysis, growth in potassium cyanide, production of acid from salicin, gas from glucose, and production of acetyl methyl carbinol (VP test) were performed to differentiate isolates primarily identified as Aeromonas sp. into A. hydrophila, A. caviae and A. sobria. Production of B-lactamase was detected by the starch paper technique earlier described by Odugbemi, Hafiz and McEntegart (1977), as well as the chromogenic cephalosporin method described by O'Callaghan et al. (1972). Similarly, production of deoxyribonuclease (DNase) was determined on DNase Test Agar (Difco Laboratories, Detroit, Michigan, USA) to which 1.35 ml of 0.5% aqueous solution of methyl green was added per 100 ml of medium (Lior, 1984). All other tests were performed according to established standard procedures (Cowan and Steel, 1974).

Control Strains:

A positive control strain each of A. hydrophila and P. shigelloides, included in this study, were obtained

from Dr. D. M. Jones of the Public Health Laboratories, Withington Hospital, Manchester, England.

RESULTS

The biochemical profiles of 55 strains of *Aeromonas sp.* and 16 *P. shigelloides* examined during this study are shown in Table 5. All the strains produced oxidase, catalase and acid from glucose, Furthermore, over 80% of the strains were motile, produced acid from maltose, reduced nitrate to nitrite and were methyl red positive. Conversely, none of the strains produced hydrogen sulphide from KIA, urease enzymes, pigmentation on nutrient agar, growth in 6.5% NaCl broth, or acid from lactose, xylose, dulcitol and adonitol. Also, 27 (49.1%) of the *Aeromonas sp.* and 3 (18.8%) of the *P. shigelloides* strains produced B-lactamase.

Production of oxidase and inability to utilise xylose are two tests that are characteristics of the family Vibrionaceae.

Similarly, results obtained for pigmentation on nutrient agar and ability to grow in 6.5% NaCl broth were in total agreement with expected results. Both *Aeromonas sp.* and *P. shigelloides* are known not to produce pigmentation on nutrient agar and not able to grow in 6.5% NaCl broth.

The two characteristics separate them from most pseudomonads and the halophilic vibrios respectively.

Tests commonly used in the differentiation of *Aeromonas sp.* from *P. shigelloides* include production of ornithine decarboxylase, DNase, amylase, and acid production from mannitol and not inositol. All the strains of *Aeromonas sp.* produced acid from mannitol, while 42 (76.4%) and 40 (72.7%) produced DNase and amylase respectively. Also, none of the *Aeromonas* strains produced acid from inositol. On the other hand, all *P. shigelloides* strains tested produced acid from inositol while none produced DNase, amylase, or acid from mannitol. However, all the *P. shigelloides* strains and 12 (21.8%) of *Aeromonas sp.* were ornithine decarboxylase positive.

Table 6 shows the results of tests commonly used in the differentiation of *Aeromonas* strains into species. During this study, 20 *Aeromonas hydrophila*, 28 *A. caviae* and 7 *A. sobria* were examined. All *A. hydrophila* and

A. caviae strains produced acid from salicin, while none of the A. sobria strains was positive for the test. Also, all A. hydrophila and A. sobria strains produced gas from glucose, while none of the A. caviae strains was positive for the test. Results of these two tests were in agreement (100%) with criteria stated by Popoff (1984) for the speciation of Aeromonas isolates. Furthermore, 15 (75%) of A. hydrophila, 25 (89.3%) of A. caviae, and none of A. sobria hydrolysed aesculin. Also, 13 (65%) of A. hydrophila, 18 (64.3%) of A. caviae and 1 (14.3%) of A. sobria grew in KCN broth. For the VP test, 15 (75%) of A. hydrophila, 1 (3.6%) of A. caviae, and 1 (14.3%) of A. sobria were positive.

COMMENT

The results of this study are in agreement with that of others (Popoff, 1984; Janda et al., 1984; Von Graevenitz, 1985), in confirming the usefulness of the various biochemical tests in the identification of Aeromonas sp. and P. shigelloides in clinical as well as research laboratories. However, the results have also shown that some of the biochemical tests may be desirable but not compulsory, particularly in laboratories with limited resources.

In this study, tests for the production of oxidase, catalase, H_2S from KIA, pigmentation on nutrient agar, acid from glucose, xylose, dulcitol, adonitol, mannitol and inositol, as well as ability to grow in 6.5% NaCl broth are very specific and in agreement with expected results (Table 5). On the other hand, tests for the production of indole, haemolysis on blood agar, lysine decarboxylase, citrate utilisation, and acid production from maltose, sucrose and arabinose are less specific and may vary with different strains. Other workers have also observed variations in some of these tests among Aeromonas sp. and P. shigelloides isolates (Von Graevenitz, 1985; Gosling, 1986).

Furthermore, this study also confirms the reliability of mannitol and inositol fermentations in the differentiation of Aeromonas sp. from P. shigelloides. All Aeromonas sp. strains in this study fermented mannitol but not inositol. Conversely, all the P. shigelloides strains ferment inositol but not mannitol. It was observed that 12 (21.8%) of the Aeromonas strains were ornithine decarboxylase positive. For a very long time, Aeromonas strains were thought not to produce ornithine decarboxylase and the characteristic has been widely used for differentiating Aeromonas sp. from P. shigelloides. However, Hickman-Brenner et al. (1987) reported ornithine positive Aeromonas

strains to which they suggested the name Aeromonas veronii.

Similarly, among the tests used in the speciation of Aeromonas strains, production of acid from salicin and gas from glucose were very specific and in agreement with expected results (Table 6). Other tests such as hydrolysis of aesculin, growth in KCN and the Voges Proskauer (VP) test gave between 65 and 89% correlation. In addition, biochemical reactions of the two strains from control patients were not in any way significantly different from those of strains from patients with diarrhoea.

In summary, production of acid from salicin and gas from glucose may just be adequate for the speciation of Aeromonas isolates (Table 6). It is interesting to note that, using these six tests alone in this study, we would not have missed any of the isolates. Equally, the tests require 18 - 24 h incubation at 37°C and are therefore easy to perform.

Based on the results of this study, a simple scheme is proposed for the isolation and preliminary identification of Aeromonas sp. and P. shigelloides from faecal specimens (Fig. 5). The scheme is as follows: Inoculate a loopful of faecal specimen onto Ampicillin blood agar (ABA) and xylose desoxycholate citrate agar

(XDCA). Also inoculate about 2 grams of faecal specimens into alkaline peptone water (APW), pH 8.6. Incubate all inoculated media for 18 - 24 h at 37°C and subculture APW onto fresh ABA and XDCA. Test all non-xylose fermenting colonies on XDCA for oxidase production, and similarly flood ABA plates with oxidase reagent. Quickly subculture oxidase positive colonies to nutrient agar (NA) plates, and 6.5% NaCl broth. Non-pigmented colonies on NA that fail to grow in 6.5% NaCl broth should then be subcultured into mannitol and inositol broth for the preliminary identification as Aeromonas sp. or P. shigelloides. Speciation of Aeromonas sp. isolates is achieved by testing for the production of acid from salicin, and gas from glucose.

This scheme is simple since all the media and reagents are easily accessible to most laboratories. The scheme is also adequate for the following reasons. The pH 8.6 of APW provides a good enrichment for Aeromonas and Plesiomonas by inhibiting other common intestinal flora in the specimen. Inclusion of ampicillin in ABA make the medium very selective. Similarly, xylose utilisation and non-oxidase production excludes a large group - the Enterobacteriaceae at an early stage of the investigation, while growth in 6.5% NaCl broth and greenish pigmentation on

NA eliminate the halophilic vibrios and most pseudomonads (which are oxidase positive and non-xylose fermenting) respectively. Fermentation of mannitol and not inositol separate Aeromonas sp. from P. shigelloides, and vice versa. Also speciation of Aeromonas is completed by testing for the production of acid from salicin and gas from glucose.

In clinical laboratories, the rapid identification of bacteria that cause diarrhoea is essential for diagnostic, epidemiological, and public health reasons. Furthermore, when large numbers of faecal specimens are to be processed, there is need for rapid screening of negative specimens and confirmation of positive ones. This scheme is appropriate for this purpose and would save both time and materials. However, the scheme is not meant to replace existing schemes. Rather, it is to complement such schemes particularly in the screening of large samples. It is also believed that the media and reagents are simple enough to make the scheme accessible to laboratories with limited resources. Though, the scheme is based on faecal specimens, it can be adapted to other clinical specimens such as blood, wound swabs and other body exudates.

Table 5: Biochemical Characteristics of 55 Aeromonas sp. and 16 P. shigelloides Strains Isolated from Patients Seen at LUTH, Lagos, Nigeria

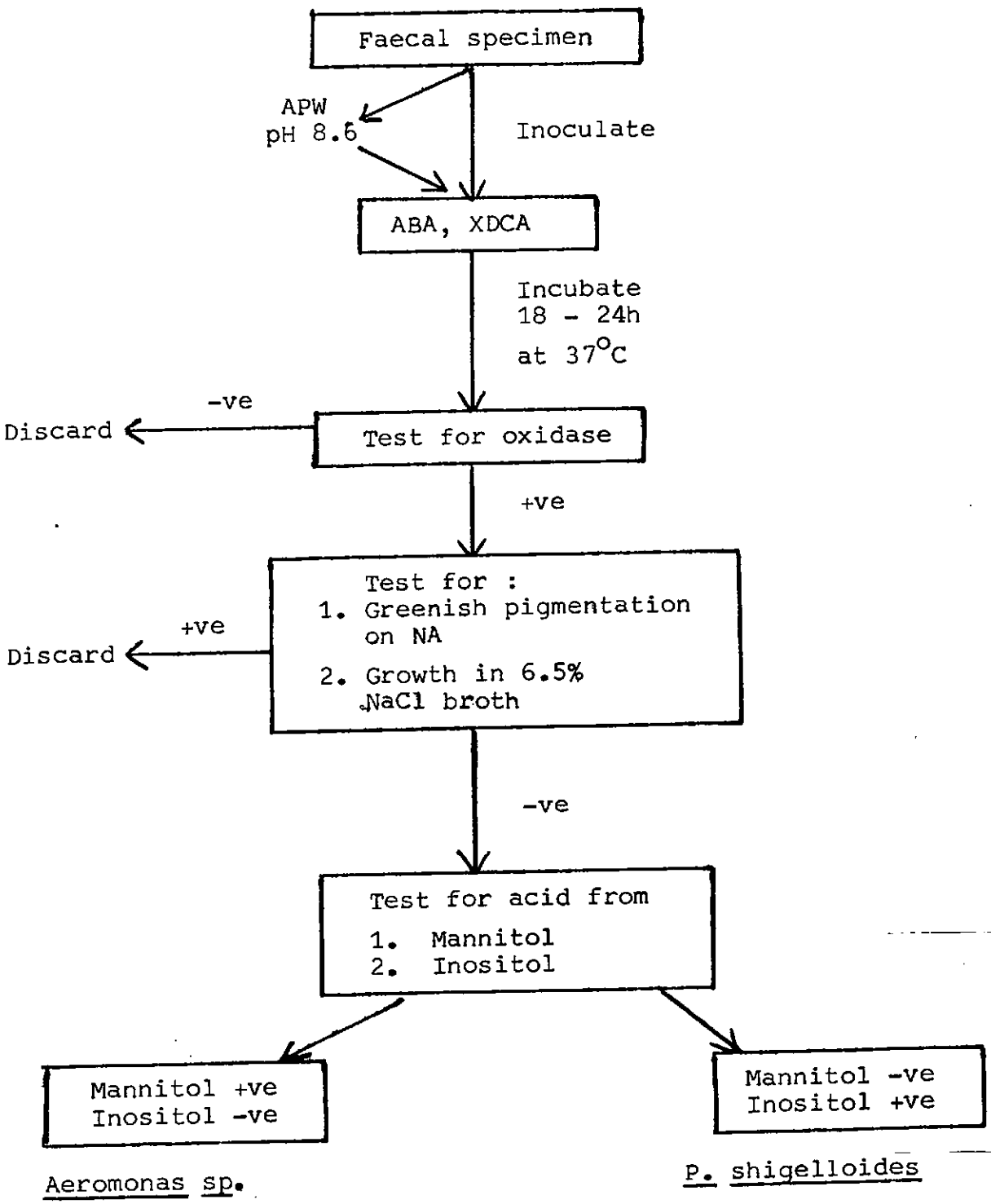
Tests	Number (%) of isolates giving positive reactions	
	Aeromonas sp. (n = 55)	P. shigelloides (n = 16)
Production of:		
Oxidase	55(100)	16(100)
Catalase	55(100)	16(100)
Urease	0	0
H_2S from KIA	0	0
Haemolysis on BA	39(70.9)	2(12.5)
Pigmentation on NA	0	0
Gas	37(67.3)	4(25)
Indole	41(74.6)	14(87.5)
DNase	42(76.4)	0
Amylase	40(72.7)	0
B-lactamase	29(52.7)	4(25)
Ornithine decarboxylase	12(21.8)	16(100)
Lysine decarboxylase	41(74.6)	13(81.3)
Acid Production from:		
Glucose	55(100)	16(100)
Lactose	0	0
Xylose	0	0
Mannitol	55(100)	0
Inositol	0	16(100)
Maltose	48(87.3)	14(87.5)
Sucrose	35(63.6)	5(31.3)
Dulcitol	0	0
Arabinose	22(40)	6(37.5)
Adonitol	0	0
Citrate utilisation	6(10.9)	0
Growth in 6.5% NaCl Broth	0	0
Nitrate reduction	49(89.1)	16(100)
Motility	48(87.3)	14(87.5)
Methyl red	45(81.8)	16(100)
Voges-Proskauer	17(30.9)	(0)

Table 6: Biochemical Characteristics Used in the Differentiation of Aeromonas sp. Isolated from Patients Seen at LUTH, Lagos, Nigeria

Tests	Number (%) of isolates giving positive reactions		
	A. hydrophila (n = 20)	*A. caviae* (n = 28)	*A. sobria* (n = 7)
Aesculin hydrolysis	15(75)	25(89.3)	0
Growth in KCN	13(65)	18(64.3)	1(14.3)
Acid from salicin	20(100)	28(100)	0
Gas from glucose	20(100)	0	7(100)
Voges Proskauer reaction	15(75)	1(3.6)	1(14.3)

[a] Based on the scheme of Popoff (1984)

Figure 5: A Simple Scheme for the Isolation and Preliminary Identification of Aeromonas sp. and P. shigelloides from Faecal Specimens



CHAPTER 4

SURVIVAL OF AEROMONAS SP. AND P. SHIGELLOIDES IN COMMONLY USED TRANSPORT MEDIA

INTRODUCTION

Transport media, as the name suggests, are primarily used for the transportation of clinical specimens from the point of collection to the laboratory where such specimens are processed. They are therefore essential for the survival of pathogens in transit. In additon, a good transport medium also maintains the status quo in the bacterial population in clinical specimens during transportation, so that a good representation of indigenous bacterial flora in an infection is obtained when the specimens is eventually cultured.

Transport media are widely used in diagnostic as well as research laboratories worldwide. Infact, some highly fastidious organisms die soon after leaving the host body unless they are kept in appropriate transport media. Furthermore, in most community-based epidemiological studies, transport media are required for transporting specimens from the rural communities to laboratories usually situated in the cities for processing.

Over the past two decades, *Aeromonas sp.* and *P. shigelloides* have been increasingly implicated in human infections, notably diarrhoea (Davis, Kane and Garagusi, 1978; Ljungh and Wadstrom, 1985; Holmberg

et al., 1986; Nolte et al., 1988). This increased awareness of their potential pathogenic roles, would probably results in attempts by various laboratories to isolate them from clinical specimens. Hence there is need to identify appropriate transport media that would ensure the survival of these organisms during transportation.

A review of literature revealed that alkaline peptone water (APW) pH 8.6, and modified Amies transport medium have been used for the transportation of specimens containing Aeromonas sp. or P. shigelloides (Millership and Chattopadhyay, 1984; Kay, Guerrero and Sack, 1985). However, Von Graevenitz (1985) reported that the survival of Aeromonas or Plesiomonas in various transport media have not been specifically investigated.

Therefore, the present study primarily set out to investigate the survival of Aeromonas and Plesiomonas in transport media commonly used in the enteric laboratory. During the course of the study, alkaline peptone water (APW) was modified by adding 0.01% w/v ferrous sulphate. This modified alkaline peptone water (M-APW) was evaluated along with the other transport media.

MATERIALS AND METHODS

Bacterial Strains:

Five strains of *Aeromonas sp.* and 2 strains of *P. shigelloides* were evaluated in this study. The five *Aeromonas sp.* consisted 2 strains of *A. caviae*, a strain each of *A. hydrophila* and *A. sobria* and a positive control strain. On the other hand, the 2 strains of *P. shigelloides* consist of one local isolate and a positive control strain. The 2 positive control strains were obtained from Dr. D.M. Jones of the Public Health Laboratory, Withington Hospital, Manchester, England.

Transport Media:

The following transport media were evaluated:

1. Amies transport medium (Difco Laboratories, Detroit, Michigan, USA).
2. Stuart transport medium (Difco Laboratories, Detroit, Michigan, USA).
3. Cary-Blair transport medium (Oxoid Ltd., Basingstoke, England).
4. Phosphate buffered saline (Oxoid Ltd., Basingstoke, England).
5. Alkaline peptone water (Oxoid Ltd., Basingstoke, England).
6. Modified alkaline peptone water (pH 8.6).

The conventional media were prepared according to manufacturers' instructions, dispensed into bijou bottles (5 ml each) and sterilized at 121°C for 15 mins. The modified alkaline peptone water was prepared by adding 0.01% w/v of ferrous sulphate to the conventional peptone water. The pH was then adjusted to 8.6 after which it was dispensed and sterilized.

Inoculation of Transport Media:

Each of the test strains was plated on Mueller - Hinton agar (Oxoid) and incubated at 37°C for 18 - 24 h to obtain pure cultures. A sterile cotton wool swab was then used to scrape all the growth on a given plate, and this was inserted into an appropriate transport medium. For each of the test strains, five bijou bottles of each transport medium were inoculated as described earlier. The inoculated transport media were kept at room temperature (22° - 28°C) for further observation. Though, a cold environment (4°C) is ideal during transportation of clinical specimens, room temperature was chosen in this case as being within the reach of every laboratory.

Measurement of Survival Rates:

To measure the survival rates of *Aeromonas sp.* and *P. shigelloides* inoculated into the various transport media, subcultures were made fortnightly onto xylose

desoxycholate citrate agar (XDCA) and blood agar supplemented with 15 ug/ml of ampicillin (ABA - 15). Recovered isolates were identified as Aeromonas sp. or P. shigelloides using standard procedures (Popoff, 1984; Schubert, 1984). Results obtained over a six month period were then analysed.

During each screening, the mean survival rate ($\bar{X}_s$) for a test strain was calculated as the number of bijou bottles from which the strain was recovered, over the number (5) of bijou bottles initially inoculated. Similarly, the cumulative mean survival rate ($\bar{X}_c$) for the seven test strains inoculated into a particular transport medium was obtained by adding up the individual mean survival rates ($\Sigma\bar{X}_s$), and dividing by the number of strains.

That is,

$$\bar{X}_c = \frac{\Sigma\bar{X}_s}{7}$$

RESULTS

A plot of the cumulative mean survival rates ($\bar{X}_c$) for each of the six transport media evaluated in this study is shown in Figure 6. For the first one month, all test strains were recovered from all the transport media. In which case, they would all be adequate for the storage of Aeromonas sp. and P. shigelloides for a short

period. However, during the next one month, all strains were recovered only from alkaline peptone water (APW) and the modified alkaline peptone water (M-APW). There were various degrees of survival rates (< 1) of the strains in phosphate buffered saline, Amies, Stuart and Cary-Blair.

Furthermore, two months after inoculating the transport media, all test strains were recovered from the modified APW only. The survival rate of the strains in APW was still good (> 0.9), whereas the survival rates in phosphate buffered saline (PBS), Amies, Stuart and Cary-Blair was between 0.6 and 0.8.

Four months after inoculation, all strains were still recovered from the modified APW, while the recovery rate for APW was 0.7. The rate was between 0.3 and 0.5 for PBS, Amies, Stuart and Cary-Blair. It was noteworthy that six months after inoculation, recovery rate was still greater than 0.9 in the modified APW. By this time, it was only 0.5 in APW, and between 0.1 and 0.3 in PBS, Amies, Stuart and Cary-Blair.

In which case, while the Aeromonas sp. and P. shigelloides can still be recovered fully at least six months after inoculation into modified APW, only about 50% can be recovered if APW alone is used. Similarly, between 10 and 30% would be recovered if PBS, Amies, Stuart, or Cary-Blair had been used.

COMMENT

The results of this study have shown that Aeromonas and Plesiomonas strains would survive in the various transport media evaluated, for at least one month. In additon, the positive effect of ferrous sulphate (FeSO4) on the survival of these organisms in alkaline peptone water was also demonstrated. Specifically, the modified APW (0.01% FeSO4 in APW) provided an excellent medium that ensured the survival of the organisms for at least six months after inoculation (Figure 6). As such, the medium appeared suitable for the storage of Aeromonas and Plesiomonas isolates.

Among the conventional transport media evaluated in this study, suitability in terms of ensuring the survival of Aeromonas and Plesiomonas is in the following order: APW, followed by phosphate buffered saline (PBS), Amies, Stuart, and then Cary-Blair. Furthermore, the results of this study also indicate that all the transport media evaluated can be used for the transportation of specimens containing Aeromonas or Plesiomonas. However, it is most probable that an added advantage of the APW and the modified APW is their alkalinity (pH 8.6). This would probably reduce the survival of normal intestinal flora usually contaminating such specimens.

Several other workers have reported on the effect of metal salts on microorganisms (Browning, 1931;

Waring and Werkman, 1942; Odugbemi, McEntegart and Hafiz, 1978). In a review on iron metabolism in infection and immunity, Bullen, Rogers and Griffiths (1974) observed that the injection of iron compounds can enhance the virulence of certain bacteria, and in some cases can abolish passive immunity. They further stated that the characteristic feature of the effect of iron is the stimulation of rapid bacterial multiplication in circumstances where it previously did not occur. In this study, it is most probable that the ferrous sulphate also acted as a reducing agent, thereby preventing the formation of toxic end-products of oxidation during the storage.

In view of the positive effect of iron on micro-organisms, there is the question of how much iron should be added to culture media to avoid iron contamination. Bowen (1966) quoted references citing levels of 3.5, 0.25, and 0.13 mg/g dry weight as the iron content in plankton, bacteria, and fungi respectively. Furthermore, Waring and Werkman (1942) suggested that the common enteric bacteria needed 0.02 - 0.03 mg/liter iron for maximum growth. However, in our environment, preliminary reports from a study on the effect of ferrous sulphate, manganese chloride, and magnesium chloride on the growth rate of some enteric bacteria indicate that ferrous

sulphate at a concentration of 0.01% w/v gave the best result (unpublished data). Hence, this concentration was chosen in this study.

In conclusion, this study has shown that Aeromonas and Plesiomonas remained viable in the various transport media evaluated, for at least one month after inoculation, thereby suggesting that any of the media may be useful in transporting specimens suspected to contain these organisms. Furthermore, the modified alkaline peptone water would be the most suitable medium for storing Aeromonas and Plesiomonas isolates, though the conventional alkaline peptone water (APW) appeared adequate for storage not exceeding 6 weeks. To the best of my knowledge, this is the first study on the survival of Aeromonas and Plesiomonas in various transport media.

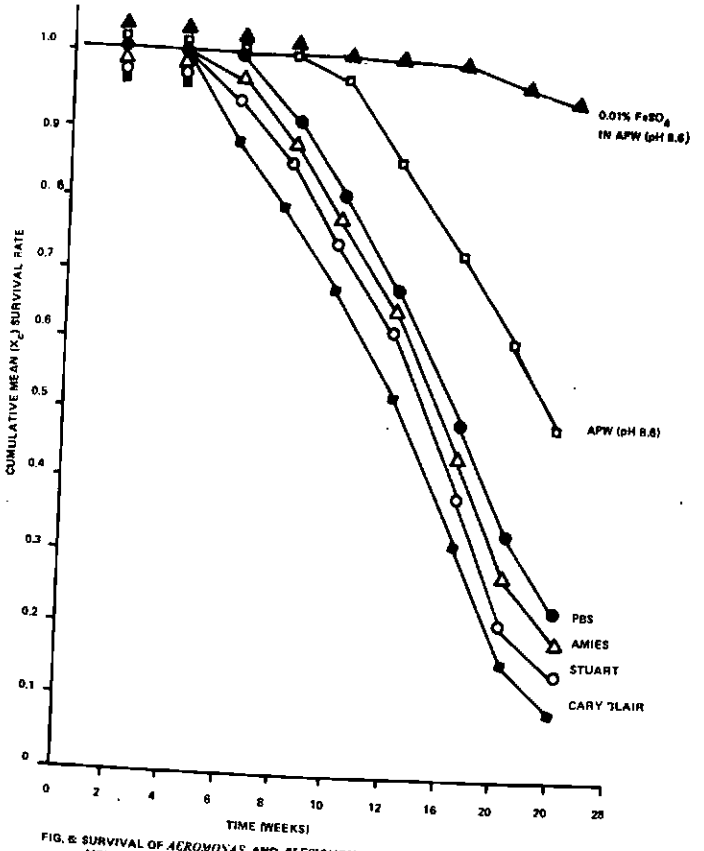


FIG. 6. SURVIVAL OF *AEROMONAS* AND *PLESIOMONAS* ISOLATES IN COMMONLY USED TRANSPORT MEDIA KEPT AT ROOM TEMPERATURE (22–25°C)

CHAPTER 5

PRODUCTION OF HAEMOLYSINS, HEAT-STABLE (ST) ENTEROTOXIN AND INVASIVENESS BY AEROMONAS SP. AND P. SHIGELLOIDES ISOLATED IN LUTH, LAGOS NIGERIA

INTRODUCTION

The enteropathogenic roles of *Aeromonas sp.* and *P. shigelloides* have not been proven conclusively, and possible mechanisms by which they may cause diarrhoea are still being investigated worldwide. In clinical bacteriology, a common method of determining whether a bacterium is capable of causing diarrhoea is to identify virulence characteristics which are recognised as important in the pathophysiology of diarrhoea and to relate the presence of these characteristics to strains isolated from persons with and without diarrhoea.

Hanson *et al.* (1977) as well as Rigney, Zilinsky and Rouf (1978) have reported that haemorrhage is a conspicuous feature of *Aeromonas* induced infections, which are often referred to as 'red sore disease' and 'red leg disease' in some ectothermic animals. They further reported that soft tissue *Aeromonas* infections in humans may be indistinguishable from infections caused by *Streptococcus pyogenes*. With these clinical manifestations and the fact that majority of strains are haemolytic on blood agar, they suggested that haemolysin may be an important virulence factor in the pathogenesis of *Aeromonas* - associated infections.

In the same vein, Boulanger *et al.* (1977) were the first to suggest the presence of a heat-stable (ST) enterotoxin in *Aeromonas* strains. Subsequently, James *et al.*

(1982) confirmed the earlier observation of Boulanger et al. (1977) and reported that in rats immunised with cholera toxin, the fluid secretion induced by heated (100°C) samples of Aeromonas culture filtrates was inhibited. Although, enterotoxigenicity has not been clearly demonstrated in P. shigelloides, Sanyal et al. (1980) reported distension of rabbit intestinal loops with live cells as well as culture filtrates of 13 strains of P. shigelloides. The suckling mouse test was also positive with these strains.

On the other hand, few experiments to demonstrate invasiveness in strains of A. hydrophila and P. shigelloides using the guinea-pig eye model (Sereny test) have been negative (Ljungh and Krønevi, 1982; Sanyal, Saraswathi and Sharma, 1980). However, some patients with Aeromonas or Plesiomonas intestinal infections have been reported to present with clinical features characteristic of invasive organisms (Rahman and Willoughby, 1980; Taylor et al., 1986; Ingram, Morrison and Levitz, 1987). More recently, Nolte et al. (1988) reported a case of proctitis and fatal septicaemia caused by P. shigelloides in an immunocompetent 42 - year old man. Furthermore, on the basis of histopathological studies, Pitarangsi et al. (1982) stated that Aeromonas can be invasive, and Watson et al. (1985) clearly demonstrated invasion of

HEp-2 cells by faecal isolates of *A. hydrophila*.

However, possible roles of these virulence properties and others such as the heat-labile (LT) enterotoxin (Sanyal, Singh and Sen, 1975; Wadstrom, Ljungh and Wretlind, 1976; Sanyal, Saraswathi and Sharma, 1980), cytotoxins (Donta and Haddow, 1978), proteases (Dahle, 1971) and surface adhesins (Atkinson and Trust, 1980) in the pathogenesis of *Aeromonas* or *Plesiomonas* - associated diarrhoea remain unclear. In some studies, *Aeromonas* strains isolated from patients with diarrhoea were enterotoxigenic, while strains from healthy people rarely showed enterotoxigenic characteristics (Burke *et al.*, 1983; Gracey, Burke and Robinson, 1982). In other surveys, workers have found no difference in enterotoxin production whether strains were isolated from patients, controls, environmental or animal sources (Annapurna and Sanyal, 1977; Pitarangsi *et al.*, 1982; Turnbull *et al.*, 1984).

In Nigeria, very little is known regarding the virulence properties of *Aeromonas sp.* or *P. shigelloides* in our environment. However, Agbonlahor *et al.* (1982) reported a case of *Aeromonas* - associated food poisoning in a 64-year old male at the University of Jos Teaching Hospital. Furthermore, Agbonlahor (1983) also noted that five of six *Aeromonas* strains isolated from patients with diarrhoea at the Lagos University Teaching Hospital

produced both cytotoxin and haemolysin. In addition, 3 of the strains also produced heat-labile (LT) enterotoxin. To the best of my knowledge, there is no single report yet on the virulence properties of *P. shigelloides* in Nigeria.

The aim of this study, therefore was to investigate the production of haemolysin, heat-stable (ST) enterotoxin, and invasiveness by strains of *Aeromonas sp.* and *P. shigelloides* isolated from patients with and without diarrhoea in LUTH, Lagos, Nigeria.

MATERIALS AND METHODS

Bacterial Strains:

A total of 55 *Aeromonas sp.* and 16 *P. shigelloides* strains were examined for the production of haemolysins, heat-stable (ST) enterotoxin, and invasiveness. The *Aeromonas* strains consisted of 20 *A. hydrophila*, 7 *A. sobria* and 26 *A. caviae*, all from patients with diarrhoea; as well as 2 *A. caviae* from patients without diarrhoea. All the 16 *P. shigelloides* strains examined were from patients with diarrhoea.

Haemolysins Production:

Production of haemolysins was evaluated on trypticase soya agar (Difco) containing 5% sheep red blood cells.

Aeromonas and Plesiomonas strains were radially streaked on well-dried plates. The inoculated plates were then incubated for 72 h at 37°C, and examined daily for complete (β) or partial (α) haemolysis.

Heat-Stable (ST) Enterotoxin Production:

Production of heat-stable (ST) enterotoxin was detected in two stages:

Toxin Extraction:- Each strain of the Aeromonas sp. or P. shigelloides was grown in trypticase soy broth (Difco) with 0.6% yeast extract (Difco) at 37°C on a roller drum (22 rpm) for 18 h. The cultures were centrifuged at 3,000 rpm for 30 minutes and each supernatant was passed through a 0.45 um membrane filter (millipore) to remove remaining bacterial cells. The sterile filtrates were heated at 100°C for 30 minutes to inactivate haemolysin and heat-labile enterotoxins that may be present. All filtrates were then kept frozen (- 20°C) until required for use.

Suckling Mouse Assay:- The method of Dean et al. (1972), modified by Giannella (1976) was used for the suckling mouse assay. Suckling mice (2 - 3 day - old) were arranged into groups of 3 each, after separation from their mothers. To each mouse in a group, 0.1 ml

of stained (a drop of sterile 2% Evans blue dye per ml) filtrate was injected intraperitoneally into the milk - filled stomach (Plate A). The mice in the control groups each received 0.1 ml of phosphate buffered saline (PBS). The animals were killed after 4 h and the ratio of gut to body weight was determined. Tests that gave ratios of intestinal weights to remaining body weights equal to or greater than 0.09 were considered positive (Plate B). Ratios between 0.08 and 0.084 were taken as doubtful and were repeated.

Sereny Test:

The test was performed according to the procedures described by Sereny (1955). The guinea-pigs used in the test were obtained from the animal house of the College of Medicine, University of Lagos, Lagos, Nigeria. Each test strain was grown on brain heart infusion agar (Oxoid) for 18 h, and cultures obtained were harvested into 2 ml sterile saline. Using a sterile Pasteur pipette, a drop of the thick saline suspension was inoculated into a guinea-pig's eye, and the other uninoculated eye served as control. Inoculated eyes were observed daily for 72 h for the development of keratoconjuctivitis, indicated by redness, swelling and pus discharge (Plate C).

Control Strains:

Control strains included in this study were *E. coli* 0128: H12 (E5263/76) producing ST only, and *E. coli* 0124. H (E12860/78) invasive in the sereny test. They were earlier received from Dr. B. Rowe (Director, Public Health Laboratory, Enteric Division, Colindale, London).

RESULTS

Results of tests to determine production of haemolysins, heat-stable (ST) enterotoxin, and invasiveness by strains of *Aeromonas* and *Plesiomonas* examined in this study are summarised in Table 7. Out of 55 *Aeromonas sp.* (20 *A. hydrophila*, 28 *A. caviae* and 7 *A. sobria*) examined, 39 (70.9%) produced α or B-haemolysis on sheep blood agar. On the other hand, only 2 (12.5%) of *P. shigelloides* strains produced α haemolysis. Furthermore, haemolysin production according to species showed that 16 (80%) of 20 *A. hydrophila*, 17 (60.7%) of 28 *A. caviae*, and 6 (85.7%) of 7 *A. sobria* were haemolytic. It is noteworthy that none of the two *A. caviae* isolated from control patients produced haemolysin. However, the difference in haemolysin production between isolates from patients with diarrhoea and controls was not statistically significant ($\chi^2 = 3.33$, $p > 0.05$).

Heat-stable (ST) enterotoxin production was observed in 11 (20%) of 55 Aeromonas strains and 4 (25%) of 16 P. shigelloides examined (Table 7). Of the 11 strains of Aeromonas that were positive for ST, 4 were A. hydrophila and 7 were A. caviae. However, none of the 7 A. sobria isolates examined in this study produced ST. Though, all ST positive strains were isolated from patients with diarrhoea, the difference between diarrhoea and none diarrhoea isolates was also not statistically significant ($\chi^2 = 0.72$, $p > 0.05$).

In this study, 6 (10.9%) of 55 Aeromonas strains and 4 (25%) of 16 P. shigelloides strains examined demonstrated invasive properties in the guinea-pig eye model (Table 7). The positive Aeromonas strains consisted of 2 A. hydrophila, 3 A. caviae and 1 A. sobria.

The present report is probably the first of a positive Sereny test for Aeromonas or Plesiomonas isolates. However, the difference in invasiveness between isolates from patients with diarrhoea and controls was not statistically significant ($X^2 = 0.06$, $p > 0.05$).

Comparative production of haemolysin, ST and invasiveness by 20 A. hydrophila strains examined, as well as the age and sex of patients from whom these strains were isolated, are shown in Table 8. Sixteen of the strains produced haemolysin, 4 produced ST and only 2 were invasive. Production of these virulence factors were almost with equal frequency from males and females. Also, the ages of the patients range from 2 weeks to 95 years. In which case, production of these factors in A. hydrophila appeared not to be age or sex - related. Similarly, 3 of the test strains produced haemolysin and ST thereby suggesting the possibility of an interplay of different virulence factors in the pathogenesis of diarrhoea due to A. hydrophila.

Similarly, of 28 A. caviae strains examined, 17 produced haemolysin, 7 produced ST, and only 3 were invasive (Table 9). As in A. hydrophila, age and sex did not appear to be associated with the production of

these factors. It is equally noteworthy that 7 strains that produced haemolysin were also positive for ST. Also, there is a strong possibility of an interplay of various virulence factors in the outcome of *A. caviae* - associated diarrhoea.

For the 7 *A. sobria* examined in this study, 6 produced haemolysin and only one strain was invasive (Table 10). None of the strains examined was positive for ST, thus suggesting that ST production may be rare in this species. Also, there appeared to be no correlation between haemolysin, enterotoxin or invasiveness within the *A. sobria* isolates examined.

On the other hand, 2 of 16 *P. shigelloides* isolates examined produced partial haemolysis on sheep blood agar, 4 produced ST and 4 strains were also invasive. Furthermore, 2 of the strains that produced ST were also invasive, suggesting some correlation between these two factors.

COMMENT

The present study has shown that a good number of *Aeromonas* and *Plesiomonas* strains isolated from patients with diarrhoea, in our environment, produced haemolysins, heat-stable (ST) enterotoxin, or were invasive. Like in this study, results of several studies in other parts of the world have shown production of virulence factors by *Aeromonas* or *Plesiomonas* strains (Sanyal, Singh and Sen, 1975; Wadstrom *et al.*, 1976; Sanyal, Saraswathi and Sharma, 1980; Asao *et al.*, 1984; Ljungh and Wadstrom, 1985; Chopra *et al.*, 1986; Figura *et al.*, 1986). More recently in Wurzburg, Germany, Fischer *et al.* (1988) described a case of pseudoappendicitis caused by a *P. shigelloides* strain that elaborated a heat-stable (ST) toxin.

However, there is a growing confusion on the actual roles of these factors in the pathogenesis of diarrhoea due to these two organisms. While some investigators believe they contribute significantly to infection (Gracey, Burke and Robinson, 1982; Burke *et al.*, 1983;

Fischer et al., 1988), others believe they play no contributory role in infection (Annapurna and Sanyal, 1977; Pitarangsi et al., 1982; Turnbull et al., 1984; Morgan et al., 1985). In this study, all isolates that produced enterotoxin, haemolysin or invasiveness were from patients with diarrhoea. Thus, our results support the view that virulence factors are more often demonstrated by clinical isolates. The results also suggest that these factors possibly play significant roles in the pathogenesis of diarrhoea due to Aeromonas or Plesiomonas in our environment.

At the moment, there is a controversy on whether an association exists between enterotoxin, haemolysin and cytotoxin among Aeromonas isolates. For instance, while Chopra et al. (1986) suggested that a strong correlation exists between production of enterotoxin, haemolysin and cytotoxin in Aeromonas isolates, Figura et al. (1986) demonstrated that there was no close relationship between haemolysins, cytotoxin or enterotoxin production in their isolates. Though cytotoxin was not included in the present study, results obtained did not show any strong correlation between haemolysin and enterotoxin. It is however most probable that results of enterotoxin assay in some of the earlier studies might have been influenced by haemolysins and

other heat-labile cytotonic extracellular products. As a matter of fact, Asao et al. (1984) reported the production by A.hydrophila of a haemolysin which had enterotoxic and cytotoxic effects. The purified haemolysins caused fluid accumulation in infant mice intestines and rabbit ileal loops and was cytotoxic to Vero cells. Subjecting toxin filtrates in the present study to heat treatment (100°C for 30 mins) made the test more specific for ST enterotoxin.

Similarly, attempts have been made to correlate enterotoxin production with species and it has been suggested by some authors that A. hydrophila and A. sobria are pathogenic, while A. caviae are environmental strains that are non-pathogenic (Daily et al., 1981; Watson et al., 1985; Barer, Millership and Tabaqchali, 1986). In this study, production of ST enterotoxin was not species - related. Presently, neither Aeromonas species nor P. shigelloides has been conclusively proven as an enteropathogen and classification of strains into pathogenic and non-pathogenic seems premature and erroneous.

In this study, 11% of our Aeromonas strains and 25% of P. shigelloides demonstrated invasive properties (Table 7). To the best of my knowledge, this is the first report of a positive Sereny test for Aeromonas or Plesiomonas, though there were earlier reports

suggesting that both organisms may be invasive (Mandal Whale and Morson, 1982; Taylor et al., 1986; Holmberg et al., 1986). In fact, some patients with Aeromonas intestinal infection have been reported to present with dysentery (Rahman and Willoughby, 1980). Similarly, Nolte et al. (1988) reported proctitis and fatal septicaemia caused by P. shigelloides in a 42-year-old, apparently healthy host.

As indicated by Holmberg and Farmer (1984), many experts believe that Aeromonas sp. and P. shigelloides cause diarrhoea in humans, though possible pathogenic mechanisms remain largely unknown. However, virulence factors have been shown to be important in the pathogenesis of other established enteropathogens such as V. cholerae, Salmonella or Shigella. For instance, fluid pouring into ileal loops is considered to be due to the liberation of enterotoxic substances by the bacteria during multiplication in the intestine. Similarly, Scheffer et al. (1988) observed that the presence of haemolysin may contribute to bacterial infections, since the release of inflammatory mediators may account for the increase in vascular permeability, oodema formation, and granulocyte accumulation. It has also been shown in in vivo studies that the mouse

lethality increased when *E. coli* expressed haemolysin production (Hacker *et al.*, 1983). It is therefore expected that these will contribute to virulence in *Aeromonas* and *Plesiomonas* isolates producing them.

In conclusion, this study clearly established the production of haemolysins, heat-stable (ST) enterotoxin, and invasiveness by strains of *Aeromonas sp.* and *P. shigelloides* from patients with diarrhoea than from controls in Lagos, Nigeria. Furthermore, the results suggest that these factors may contribute to the pathogenicity of strains possessing them, and that pathogenicity of these organisms would probably involve more than one mechanism.

Table 7: Production of haemolysins, heat-stable (ST) enterotoxin, and invasiveness by Aeromonas sp. and P. shigelloides Strains isolated from patients in LUTH Lagos, Nigeria

	A. hydrophila	A. caviae	A. sobria	Total	P. shigelloides
No. of Strains Tested	20	28	7	55	16
No (%) of Strains Positive for Haemolysin Production	16(80)	17(60.7)	6(85.7)	39(70.9)	2(12.5)
No (%) of Strains Positive for ST Enterotoxin Production	4(20)	7(25)	0	11(20)	4(25)
No (%) of Strains Positive for Invasiveness	2(10)	3(10.7)	1(14.3)	6(10.9)	4(25)

Table 8: Sex and Age of Patients whose Faecal Specimens Yielded A. hydrophila Isolates Producing Haemolysins, Heat-Stable (ST) Enterotoxin, or Invasiveness

		Production of :		
Sex of Patients	Age of Patients	Haemolysin	ST Enterotoxin	Invasiveness
M	63 yrs	+	+	-
F	75 yrs	+	-	-
M	3 yrs	+	+	+
F	78 yrs	+	-	-
F	30 yrs	-	+	-
M	95 yrs	+	-	+
M	17 yrs	+	-	-
F	4 yrs	+	-	-
F	25 yrs	+	-	-
F	69 yrs	+	-	-
F	3 months	+	-	-
M	9 yrs	+	-	-
M	2 weeks	+	-	-
F	15 yrs	+	-	-
M	92 yrs	+	-	-
F	36 yrs	+	-	-
F	1 yr	+	+	-

\+ = Positive

\- = Negative

Table 9: Sex and Age of Patients whose Faecal Specimens Yielded A. caviae Isolates Producing Haemolysins, Heat-Stable (ST) Enterotoxin, or Invasiveness

		Production of :		
Sex of Patients	Age of Patients	Haemolysin	ST Enterotoxin	Invasiveness
M	43 yrs	+	+	-
F	1 yr	-	-	+
M	5 yrs	+	+	-
M	10 yrs	+	+	-
F	3 yrs	+	-	-
F	72 yrs	-	+	+
F	1 yr	+	-	-
F	9 months	+	-	-
M	23 yrs	+	+	-
F	2 days	+	-	-
F	5 yrs	+	-	-
F	6 months	+	-	-
F	29 yrs	+	+	-
F	60 yrs	+	+	-
M	82 yrs	+	-	-
M	8 yrs	+	-	-
M	30 yrs	+	-	-
M	76 yrs	+	-	-
F	8 yrs	+	-	+

+ = Positive

- = Negative

Table 10: Sex and Age of Patients whose Faecal Specimens Yielded A. sobria Isolates Producing Haemolysins or Invasiveness

Sex of Patients	Age of Patients	Production of : Haemolysin	ST Enterotoxin	Invasiveness
F	29 yrs	+	-	-
F	65 yrs	+	-	-
F	6 yrs	+	-	-
F	2 yrs	+	-	-
F	2 yrs	+	-	+
F	9 yrs	+	-	-

\+ = Positive

\- = Negative

Table 11: Sex and Age of Patients whose Faecal Specimens Yielded P. shigelloides Isolates Producing Haemolysins, Heat-Stable (ST) Enterotoxin, or Invasiveness

Sex of Patients	Age of Patients	Production of : Haemolysin	ST Enterotoxin	Invasiveness
F	16 yrs	-	+	-
M	74 yrs	-	-	+
F	81 yrs	-	-	+
M	27 yrs	+	-	-
M	37 yrs	-	+	+
F	7 months	+	-	-
M	41 yrs	-	+	-
F	15 yrs	-	+	+

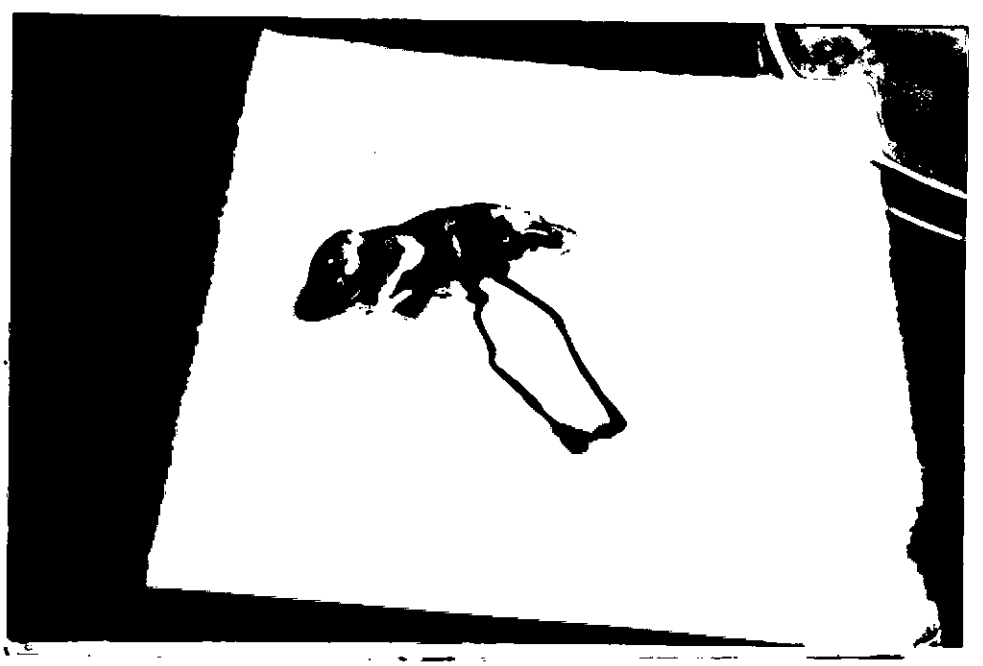
\+ = Positive

\- = Negative

PLATE A

A SUCKLING MOUSE BEING INOCULATED THROUGH THE MILK - FILLED STOMACH WITH A CULTURE FILTRATE OF AEROMONAS HYDROPHILA FOR THE DETERMINATION OF HEAT-STABLE (ST) ENTEROTOXIN PRODUCTION

PLATE B



AN EXPERIMENTALLY INOCULATED SUCKLING MOUSE WITH THE INTESTINE TAKEN OUT FOR WEIGHING. THE RATIO OF THE INTESTINE TO THE REMAINING BODY WEIGHT WAS GREATER THAN 0.09, HENCE, IT WAS POSITIVE FOR HEAT-STABLE (ST) ENTEROTOXIN PRODUCTION

PLATE C

A GUINEA-PIG WHOSE RIGHT EYE WAS INOCULATED WITH AN INVASIVE STRAIN OF AEROMONAS HYDROPHILA. 72 HRS AFTER INOCULATION, THERE WAS KERATOCONJUCTIVITIS INDICATED BY REDNESS, RATCHES AND PUS DISCHARGE FROM THE INFECTED EYE.

CHAPTER 6

LACK OF CORRELATION BETWEEN DEMONSTRATED VIRULENCE FACTORS OF AEROMONAS SP. AND P. SHIGELLOIDES, AND ENTEROPATHOGENICITY IN MICE

INTRODUCTION

The controversial nature of *Aeromonas* and *Plesiomonas* as agents of acute diarrhoeal diseases has long been expressed by several workers (Pitarangsi *et al.*, 1982; Von Graevenitz, 1985; Figura *et al.*, 1986). Although these organisms have been frequently associated with enteric diseases, conclusive evidence of their enteropathogenicity is still not available. Furthermore, the role of the various virulence factors described in *Aeromonas* and *Plesiomonas* in the pathogenesis of diarrhoea associated with these organisms is poorly understood.

In medical science, experimental animals are widely used in the study of the pathogenesis of infectious diseases. This is because the clinical manifestations of such infections in animals have been found to mimic those of the respective disease in man. Hence, the ability of an organism to initiate infection in healthy animals is, in most cases, an indication of its potential virulence in humans.

For *Aeromonas* and *Plesiomonas*, most of the previous attempts to induce experimental diarrhoea in animals have failed. For instance, Ljungh and Krenevi (1982) reported the failure of *A. hydrophila* to induce experimental diarrhoea in rabbits, guinea-pigs, hamsters and rats

even after elimination of the resident intestinal flora by prior antibiotic treatment as well as neutralisation of gastric pH and slowing of intestinal motility. In the same vein, Pitarangsi et al. (1982) reported that an isolate of P. shigelloides from an individual with severe diarrhoea did not cause diarrhoea in five rhesus monkeys.

Therefore, there is a concerted effort worldwide for a satisfactory animal model for demonstrating Aeromonas and Plesiomonas - associated diarrhoea. In Nigeria, I am not aware of any report on experimental infection in animals using Aeromonas or Plesiomonas. Hence, this study is an attempt to experimentally induce diarrhoea, via the oral route, in healthy adult mice using apparently virulent local isolates of Aeromonas and Plesiomonas.

MATERIALS AND METHODS

Bacterial Strains:

Fifteen apparently virulent strains of Aeromonas sp. and P. shigelloides were evaluated for their ability to induce diarrhoea in healthy adult mice (Table 12). This consisted of 4 A. hydrophila strains, 5 A. caviae, 3 A. sobria and 3 P. shigelloides. The 4 A. hydrophila

strains were made up of a strain that produced haemolysin, heat-stable (ST) enterotoxin, and was invasive; another strain that produced haemolysin and was invasive; and 2 strains that produced both haemolysin and ST. Similarly, the 5 A. caviae strains consisted of a strain that produced ST and was invasive, and 4 others that produced both haemolysin and ST. The 3 A. sobria strains all produced haemolysins only. For the 3 P. shigelloides strains, 2 produced ST and were invasive while the third strain produced haemolysin.

Mice:

Inbred mice aged between 2 - 3 months and weighing 20 - 30g were used. The mice were obtained from the Swiss albino colony at the Animal House of the College of Medicine, University of Lagos, Idi-Araba, Lagos. Before challenging the mice with test strains, their faecal samples were cultured and found to be negative for known bacterial enteropathogens, Aeromonas or Plesiomonas.

Preparation of Inoculum:

Test bacterial strains were cultured on Mueller-Hinton agar (MHA), and incubated aerobically at 37°C for 24 h. At least 5 colonies of each strain on MHA

were picked and subcultured into 10 ml of trypticase soy broth (Difco) with 0.6% yeast extract (Difco). The inoculated broths were incubated at 37°C for 8 h and then centrifuged at 3,000 r.p.m. for 30 mins. The deposits obtained were washed twice in phosphate buffered saline (PBS), pH 7.3. The washed cells were resuspended in normal saline and then adjusted to match a McFarland 0.5 barium sulphate standard containing approximately 1×10^8 c.f.u. per ml. The surface agar plate count method of Miles and Misra (1938) was used to confirm the concentration of each suspension.

Oral Challenge of Mice:

After 24 h of starvation (food and water withdrawal), a group of 2 mice were allowed to drink from each of the standardised saline suspensions containing approximately 1×10^8 c.f.u. per ml of a given test strain. The mice were fed with the bacterial suspension for 24 h before being replaced with fresh water. Thereafter, mice were examined daily for 2 weeks for symptoms of diarrhoea. Collected faeces were cultured to recover inoculated strains which were then identified by standard techniques (Popoff, 1984; Schubert, 1984). Control mice included

in this study were given normal saline only instead of bacterial suspension.

Histological Studies:

Mice fed with invasive strains of Aeromonas and Plesiomonas were sacrificed and samples of their lung, kidney and heart were examined histologically for evidence of invasion. The organs were fixed in 10% buffered formalin for 24 h, dehydrated in graded alcohols, embedded in parafin and sectioned at 5 - 7 un. The sections were stained by Taylor's modification of Gram's method (Bartholomew, 1980).

RESULTS

Apparently virulent strains of Aeromonas sp. and P. shigelloides evaluated in this study failed to cause diarrhoea in any of the experimentally infected mice. However, the organisms were recovered from their faeces for between five to seven days after the mice were challenged. This is an indication that the organisms were able to survive the gastric acid barrier of the stomach.

Furthermore, examination of histological sections of the lung, kidney and heart muscles of mice fed with apparently invasive strains of Aeromonas or Plesiomonas

did not reveal bacterial invasion of any of the organs (Plates D, E & F).

COMMENT

The results of this study have shown the inability of apparently virulent strains of *Aeromonas* and *Plesiomonas* to cause diarrhoea in healthy adult mice via the oral route. It is noteworthy however, that the organisms were able to survive the gastric acid barrier of the stomach, since they were excreted in the faeces of the animals. Their inability to initiate diarrhoea in mice may be due to a number of factors such as their inability to attach to intestinal epithelial cells, and consequent failure to proliferate in the intestine; inability to produce the virulence factors *in vivo*; or that mice are not usually susceptible to *Aeromonas* and *Plesiomonas*.

A review of literature also indicate that similar results have been obtained by various workers in other parts of the world. In Thailand, Pitarangsi *et al.* (1982) reported that rhesus monkeys fed 10^9 cytotoxic *A. hydrophila* strains, after 24 h of starvation and oral administration of sodium bicarbonate, failed to develop diarrhoea over the ensuing 5 days. In the same study, an isolate of *P. shigelloides* from an individual

with severe diarrhoea did not cause diarrhoea in five rhesus monkeys. More recently, Morgan et al. (1985) reported lack of correlation between known virulence properties of A. hydrophila and enteropathogenicity for humans. Among 57 volunteers used in their study, 55 had no gastrointestinal symptoms, and only 2 had mild symptoms of less than 24 hours after being orally fed with suspected virulent strains of A. hydrophila.

Furthermore, histological sections of the lung, kidney and heart of mice fed with apparently invasive strains of Aeromonas and Plesiomonas in the present study, did not reveal bacterial invasion of any of the organs. Essentially, the organs appeared normal with no cellular infiltration (Plates D, E & F). However, in an earlier study in the United States of America, Brenden and Huizinga (1986) reported an intrasmuscular infection of mice with a virulent fish strain of A. hydrophila, and that histological examination revealed severe necrosis at the injection site, oedema, haemorrhage or neutrophil infiltration of the lung; and focal parenchyma necrosis in the liver. The route of infection probably played a significant role in this particular case.

A number of reasons can be adduced to explain the apparent lack of correlation between enteropathogenicity

and known virulence factors as observed in this study. It may be that the various virulence factors have to combine in certain ratios, or that host factors may be a strong determinant of the outcome of an attack.

More recently, Namdari and Bottone (1988) described what they termed "suicide phenomenon" in certain Aeromonas species, and suggested that the phenomenon probably accounts for the numerous discrepant observations regarding species - specific (A. caviae) and strain - specific (A. hydrophila and A. sobria) human entero-pathogenicity and animal virulence properties of Aeromonas species. According to these authors, when certain Aeromonas strains are inoculated into broth containing 0.5% glucose, there is accumulation of acetic and other fatty acids, reduced pH, and consequent cessation of growth and death of the organisms within 24 hours. However, the phenomenon appeared not to operate in the present study, since the organisms were reisolated 5 to 7 days post inoculation.

In conclusion, it is probably surprising that despite extensive studies on the enteropathogenicity of Aeromonas and Plesiomonas, their mechanisms of infection have remained largely unknown and probably more confusing. Contrary to expectation, the results of this pioneering

work in our environment showed lack of correlation between demonstrated virulence factors and enteropathogenicity in mice. However, more studies need to be done, using strains isolated from different geographical locations within the country, as well as different animal species, for a better understanding of the enteropathogenic nature of Nigerian isolates.

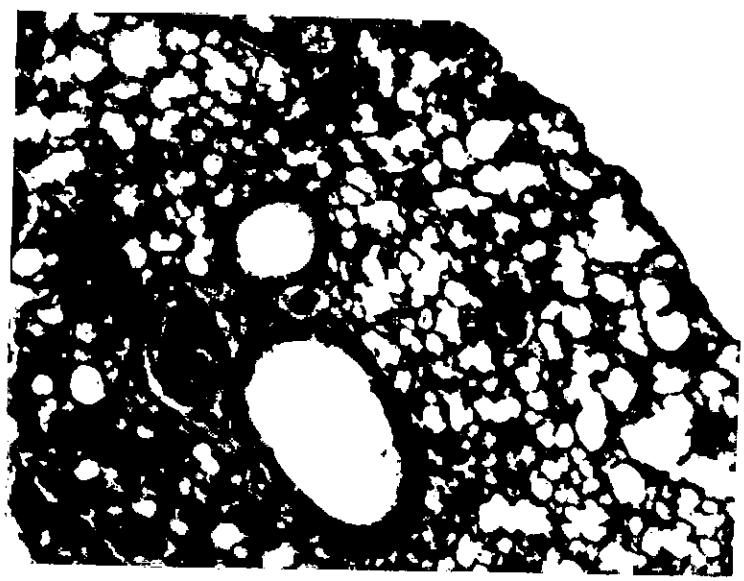
Table 12: Virulence Characteristics of Aeromonas sp. and P. shigelloides Strains used in the experimental infection of mice

Strain No.	Species	Production of : Haemolysin	ST Enterotoxin	Invasiveness
1005	A. hydrophila	+	+	+
1600	A. hydrophila	+	-	+
116	A. hydrophila	+	+	-
2350	A. hydrophila	+	+	-
1322	A. caviae	-	+	+
65	A. caviae	+	+	-
1400	A. caviae	+	+	-
1610	A. caviae	+	+	-
1865	A. caviae	+	+	-
589	A. sobria	+	-	-
1501	A. sobria	+	-	-
1524	A. sobria	+	-	-
1344	P. shigelloides	-	+	+
2339	P. shigelloides	-	+	+
1242	P. shigelloides	+	-	-

\+ = Positive

\- = Negative

PLATE D



HISTOLOGICAL SECTION OF THE LUNG (X 100) OF A MOUSE ~~FED WITH AN INVASIVE~~ AEROMONAS HYDROPHILA STRAIN. THE CELLS ARE ESSENTIALLY NORMAL : ALVEOLAR WALLS INTACT, NO CELLULAR INFILTRATES, AND NORMAL COLUMNAR LINING GLOMERULI

PLATE E

HISTOLOGICAL SECTION OF THE KIDNEY (X 100) OF A MOUSE FED WITH AN INVASIVE AEROMONAS HYDROPHILA STRAIN. THE GLOMERULI APPEARED NORMAL WITH NO CELLULAR INFILTRATION AND HYPERPLASIA, AND THERE IS NORMAL TUBULAR ARCHITECTURE.

PLATE F

HISTOLOGICAL SECTION OF THE HEART (X 400) OF A MOUSE FED WITH AN INVASIVE AEROMONAS HYDROPHILA STRAIN. THE MYOCARDIAL CELLS ARE NORMAL, NO CELLULAR INFILTRATES AND NO CELL WALL HYPERPLASIA OF VASAVASORUM.

CHAPTER 7

IN VITRO SUSCEPTIBILITY PATTERN OF AEROMONAS SP. AND P. SHIGELLOIDES TO SELECTED ANTIMICROBIAL AGENTS

INTRODUCTION

The increasing frequency of reports worldwide indicates that both *Aeromonas sp.* and *P. shigelloides* can be pathogenic for humans, producing focal or systemic infections of varied severity particularly in immuno-compromised hosts (Von Graevenitz and Mensch, 1968; Davis, Kane and Garagusi, 1978; Agbonlahor *et al.*, 1982; Agbonlahor, 1983; Ljungh and Wadstrom, 1985; Von Graevenitz, 1985; Ingram, Morrison and Levitz, 1987; Nolte *et al.*, 1988).

More specifically, *Aeromonas* has been reported to cause a variety of human infections such as cellulitis (McCraken and Barkely, 1972), hepatobiliary infections (DeFronzo, Murray and Maddrey, 1973), conjuctivitis (Smith, 1980), food poisoning (Agbonlahor *et al.*, 1982), peritonitis (Saito and Schick, 1973), osteomyelitis (Weinstock *et al.*, 1982), meningitis (Qadri *et al.*, 1976), and septicaemia (Ketover, Young and Armstrong, 1973). Similarly, *P. shigelloides* has been implicated in cellulitis and wound infections (Von Graevenitz and Mensch, 1968; McCraken and Barkely, 1972), endophthalmitis (Cohen *et al.*, 1983), cholecystitis (Claesson *et al.*, 1984), septicaemia and blood stream infections (Ellner and McCarthy, 1973; Appelbaum *et al.*, 1978; Curti, Lin and Szebo, 1985) and pseudoappendicitis (Fischer *et al.*,

1988).

However, the most commonly Aeromonas and Plesiomonas associated clinical condition is diarrhoea which has been reported in immunocompromised as well as in immunocompetent hosts (Mohieldin et al., 1966; Von Graevenitz and Mensch, 1968; Chatterje and Neogy, 1972; Bhat, Shanthakumari and Rajan, 1974; Sanyal, Singh and Sen, 1975; Holmberg and Farmer, 1984; Reinhardt and George, 1985a; Ljungh and Wadstrom, 1985).

The use of antimicrobial agents in the treatment of infectious diseases has produced great advances in human and veterinary medicine. However, the use of antimicrobial agents is generally not encouraged in the treatment of acute diarrhoeal diseases. Apart from the adverse effect of such treatment on normal intestinal flora, most diarrhoeal episodes are usually mild and self - limiting, requiring only supportive treatment with oral rehydration solutions (ORS). Antimicrobial usage is however justified in cases of prolonged diarrhoea or diarrhoea due to invasive enteropathogens such as Salmonella typhi, Yersinia enterocolitica and enteroinvasive E. coli. Recent reports in literature have indicated that Aeromonas sp. and P. shigelloides can be invasive and are therefore capable of causing severe

extraintestinal infections following diarrhoea attack (Lawson, Burke and Chang, 1985; Watson et al., 1985; Holmberg et al., 1986; Ingram, Morrison and Levitz, 1987; Nolte et al., 1988).

As a matter of fact, reports from other parts of the world indicate that both Aeromonas sp. and P. shigelloides have been widely tested for their susceptibilities to various antimicrobial agents (Overman, 1980; Fass and Barnishan, 1981; Fainstein, Weaver and Bodey, 1982; Von Graevenitz, 1985; Reinhardt and George, 1985b; Motyl, Mckinley and Janda, 1985). In Nigeria, however, I am aware of only a report by Agbonlahor (1983) on the susceptibility of six A. hydrophila isolates from patients with diarrhoea in Lagos to a few antibiotics. To the best of my knowledge, there is no documented report yet on P. shigelloides.

Furthermore, we cannot continue to rely on results of other laboratories abroad since the antimicrobial resistance pattern of common pathogenic bacteria is known to vary from one geographical location to another. For instance, O'Brien et al. (1978) observed a 6 - to 14 - fold differences in resistance to antibiotics between a general hospital in Boston, USA and a similar one in Paris, France. The present study was therefore carried out to determine the susceptibility of isolates of

Aeromonas sp. and P. shigelloides in our environment to selected antimicrobial agents.

MATERIALS AND METHODS

Bacterial Strains:

The 55 strains of Aeromonas sp. and 16 strains of P. shigelloides, used in this study, were isolated from faecal specimens of patients with and without diarrhoea submitted to the enteric laboratory of the Lagos University Teaching Hospital (LUTH), Idi-Araba, Lagos, between October 1986 and September 1987. The Aeromonas strains consisted of 20 A. hydrophila, 7 A. sobria and 26 A. caviae, all from patients with diarrhoea; as well as 2 A. caviae strains from patients without diarrhoea. All the 16 P. shigelloides strains were from patients with diarrhoea.

Susceptibility Testing:

Antimicrobial susceptibility was determined on Mueller-Hinton agar (OXOID) using the disk-agar diffusion technique (disk method) of Bauer et al. (1966). Oxoid multodisks codes 1788E and U4, as well as single disks of chloramphenicol, erythromycin and ceftriaxone were used in this study. In addition single disks of ofloxacin kindly provided by Nigerian Hoechst Ltd,

Ikeja, Lagos were included. The list and concentrations of the 15 antimicrobial agents used in this study are shown in Table 13.

Standardization of Inoculum:

Four to five colonies of each isolate (from an overnight culture plate) were inoculated into 2 ml of sterile nutrient broth in bijou bottles. This was incubated for 6 hrs at 37°C, and the turbidity was then adjusted to match a McFarland 0.5 barium sulphate standard, to contain approximately 1×10^8 cells/ml of the inoculum. A sterile cotton wool swab was dipped into the standardised bacterial suspension. Excess fluid was drained off by rotating the swab with firm pressure against the inside of the bijou bottle above the fluid level. The swab was then used to streak the dried surface of a Mueller-Hinton agar plate, ensuring an even distribution of inoculum.

Appropriate disks of the different antimicrobial agents were aseptically placed on the inoculated plates, and pressed firmly to ensure complete contact with the agar. The plates were incubated for 18 h at 37°C, and then observed for zones of growth inhibition around each disk.

Interpretation of the Zone Sizes:

The diameter of the growth inhibition zone, surrounding each antimicrobial agent was measured in millimetre (mm) using a ruler, and the results were interpreted as susceptible, intermediate, or resistant according to the World Health Organization (WHO) zone size interpretation standards for antimicrobial agents (Table 14).

Standard Reference Strains:

Internationally recognised standard reference strains for antimicrobial susceptibility testing, E. coli ATCC 25922, Staphylococcus aureus ATCC 25923 and Pseudomonas aeruginosa ATCC 27853 were included in the test as controls. They were obtained from Dr. Clyde Thornsberry of the Centers for Disease Control (CDC), Atlanta, Georgia, USA.

RESULTS

Table 15 shows the in vitro susceptibilities of Aeromonas sp. and P. shigelloides strains to the 15 antimicrobial agents evaluated in this study. All isolates were susceptible to ofloxacin (10 ug), a quinolone

recently introduced into the market by Nigerian Hoechst Limited, Ikeja, Lagos. Similarly, all P. shigelloides, A. hydrophila, A. sobria and 22 (78.6%) of A. caviae strains tested were susceptible to nalidixic acid, another quinolone.

Among the aminoglycosides, gentamicin (10 ug) was the most active with all isolates of P. shigelloides, A. hydrophila, A. caviae, and 6 (85.7%) of A. sobria susceptible. Also, over 50% of Aeromonas sp. and P. shigelloides examined in this study were susceptible to streptomycin. Also in this study, 36 (65.5%) of Aeromonas sp. and 10 (62.5%) of P. shigelloides isolates examined were susceptible to cephaloridine (25 ug).

Furthermore, between 60% and 80% of Aeromonas sp. and P. shigelloides isolates were susceptible to chloramphenicol (10 ug), erythromycin (30 ug), and co-trimoxazole (25 ug). In addition, 47 (85.5%) of Aeromonas and 13 (81.3%) of P. shigelloides isolates examined were susceptible to tetracycline (50 ug).

Other antimicrobial agents with good activities against Aeromonas sp. and P. shigelloides as observed in this study include ceftriaxone (30 ug), nitrofurantoin (200 ug) and colistin (10 ug). All P. shigelloides isolates and 54 (98.2%) Aeromonas sp. were susceptible to ceftriaxone. Similarly, there was a 100% and 94.6% activity of nitrofurantoin against P. shigelloides and Aeromonas strains respectively. For colistin, 51 (92.7%) of Aeromonas strains and 15 (93.8%) of P. shigelloides were susceptible. However, there was a reduced activity of sulphonamides (300 ug) against the isolates with about 50% of Aeromonas and 60% of P. shigelloides being susceptible.

Results of this study also indicate a good activity of carbenicillin (100 ug) against the isolates. All P. shigelloides and 51 (92.7%) of Aeromonas strains were susceptible to this drug. On the other hand, majority of the strains (>70%) were not susceptible to ampicillin (25 ug).

COMMENT

The results of this study have shown that strains of Aeromonas sp. and P. shigelloides from our environment

are susceptible to most of the 15 antimicrobial agents evaluated. Specifically, all the strains were susceptible to ofloxacin, and there was a good activity of gentamicin, carbenicillin and ceftriaxone against these organisms (Table 15). Similarly, over 70% of the strains were susceptible to colistin, nalidixic acid, nitrofurantoin, erythromycin, chloramphenicol, tetracycline and co-trimoxazole. In addition, about 60% of strains tested were susceptible to cephaloridine and streptomycin, while about 50% were susceptible to compound sulphonamide. On the other hand, less than 30% of the strains were susceptible to ampicillin.

Based on the results of this *in vitro* susceptibility testing, recommended drugs of choice in cases of serious *Aeromonas* or *Plesiomonas* - associated infections requiring antimicrobial therapy would be ofloxacin, gentamicin, ceftriaxone and carbenicillin. Other drugs such as chloramphenicol, tetracycline and co-trimoxazole could also be useful. It should be noted however that *in vitro* antimicrobial susceptibility testing is only a guide, and that conditions *in vivo* may be quite different from those obtained *in vitro*. Therefore, the ultimate decision to use a particular antimicrobial agent depends on a number of other factors including its associated side effects, toxicity, pharmakokinetic properties, diffusion

in different body sites, attainable serum levels, the pathogenic significance of the microorganism, and the immune status of the patient.

In a related study in this environment by Agbonlahor (1983), all six A. hydrophila isolates from patients with diarrhoea were uniformly sensitive to chloramphenicol, tetracycline, streptomycin, gentamicin, sulphafurazole and compound sulphonamide. The strains were equally resistant to ampicillin and cephalothin. Though, the results of the current study are similar to that of Agbonlahor (1983), a uniform sensitivity to streptomycin was not observed in the isolates.

Studies in other parts of the world by Fainstein, Weaver and Bodey (1982) showed that chloramphenicol, tetracycline, co-trimoxazole and the aminoglycosides were very active against A. hydrophila isolates. Similarly, Motyl et al. (1985) reported that both Aeromonas and P. shigelloides are usually sensitive to tetracycline, chloramphenicol, gentamicin and the quinolones. Furthermore, in a recent case of pseudoappendicitis caused by P. shigelloides, the patient was treated with cefazolin for 4 days, and within 2 days of therapy, the fever subsided (Fischer et al., 1988). Results of these studies are not significantly different from the current findings.

In this study, the choice of antimicrobial agents was based on a number of factors. Chloramphenicol and tetracycline were selected for their broad spectrum effectiveness, and common use in our environment. Ampicillin and gentamicin were included for their usefulness in treating gastroenteritis and therapeutically diffucult Gram-negative rod infections as earlier reported by Joseph, Debell and Brown (1978). Ofloxacin, a quinolone was recently introduced into the market by the Nigerian Hoechst Limited, and it was thought worthwhile to evaluate its effectiveness against these organisms.

In Nigeria, like most developing countries, there is wide-spread and indiscriminate use of antimicrobial agents (Odugbemi et al., 1986). Hence, there is need for constant susceptibility testing for surveillance and detection of resistance and susceptibility of clinically important bacteria. At present, there appeared to be no problem with antimicrobial treatment of Aeromonas and Plesiomonas infections in our environment, since many of the drugs showed effective in vitro activity.

Table 13: Symbols and concentration of antimicrobial agents used in the susceptibility testing

Name	Symbol	Concentration
Ampicillin	AMP	25 ug
Colistin sulphate	CT	10 ug
Nalidixic acid	NA	30 ug
Nitrofurantoin	F	200 ug
Compound sulphonamide	S3	300 ug
Streptomycin	S	25 ug
Tetracycline	TE	50 ug
Co-trimoxazole	SXT	25 ug
Gentamicin	CN	10 ug
Cephaloridine	CR	25 ug
Carbenicillin	CAR	100 ug
Ofloxacin (Tarivid)	OFX	10 ug
Ceftriaxone	CRO	30 ug
Chloramphenicol	C	10 ug
Erythromycin	E	30 ug

Table 14: Recommended zone size interpretation standards for antimicrobial agents used in this study

Antimicrobial agent	Disk Potency	Inhibition zone diameter to nearest mm		
		Resistant	Intermediate	Sensitive
Ampicillin	10 ug	14	15 - 16	17
Colistin sulphate	10 ug	8	9 - 10	11
Nalidixic acid	30 ug	13	14 - 18	19
Nitrofurantoin	300 ug	14	15 - 16	17
Sulphanamides	300 ug	12	13 - 16	15
Streptomycin	10 ug	11	12 - 14	15
Tetracycline	30 ug	14	15 - 18	19
Gentamicin	10 ug	12	13 - 14	15
Cephalothin	30 ug	14	15 - 17	18
Chloramphenicol	30 ug	12	13 - 17	18
Erythromycin	15 ug	13	14 - 17	18
Carbenicillin	100 ug	13	14 - 16	17
Co-trimoxazole	25 ug	10	11 - 15	16

Source: Bailey and Scott's Diagnostic Microbiology by Finegold, S.M., and Martin, W. J. (6 eds.) 1982. Mosby Co. London p. 546.

Table 15: Susceptibility Patterns of A. hydrophila, A. caviae, A. sobria and P. shigelloides to Different Antimicrobial Agents Evaluated

Antimicrobial Agent	A. hydrophila (n = 20)	A. caviae (n = 28)	A. sobria (n = 7)	Total	P. shigelloides (n = 16)
OFX	20(100)	28(100)	7(100)	55(100)	16(100)
CN	20(100)	28(100)	6(85.7)	54(98.2)	16(100)
CRO	20(100)	27(96.4)	7(100)	54(98.2)	16(100)
CAR	18(90)	27(96.4)	6(85.7)	51(92.7)	16(100)
CT	18(90)	28(100)	5(71.4)	51(92.7)	15(93.8)
NA	20(100)	22(78.6)	7(100)	48(89.1)	16(100)
F	20(100)	26(92.9)	6(85.7)	52(94.6)	16(100)
E	17(85)	25(89.3)	3(42.9)	45(81.8)	15(93.8)
C	16(80)	24(85.7)	4(57.1)	44(80)	14(87.5)
TE	17(85)	26(92.9)	4(57.1)	47(85.5)	13(81.3)
SXT	15(75)	18(64.3)	4(57.1)	37(67.3)	13(81.3)
CR	13(65)	19(67.9)	4(57.1)	36(65.5)	10(62.5)
S	13(65)	16(57.1)	4(57.1)	33(60)	10(62.5)
S3	12(60)	13(46.4)	3(42.9)	28(50.9)	10(62.5)
AMP	6(30)	8(28.6)	2(28.6)	16(29.1)	4(25)

CHAPTER 8

PLASMID SCREENING AMONGST AEROMONAS SP. AND P. SHIGELLOIDES ISOLATES FROM PATIENTS IN LUTH, LAGOS, NIGERIA

INTRODUCTION

Plasmids are extra chromosomal deoxyribonucleic acids (DNA) capable of stable autonomous replication. Their recognition was in the early 1950's soon after the discovery of conjugation in E. coli by Joshua Lederberg (1952). Since that discovery, plasmids have been identified in almost every bacteria group where they have been investigated. While plasmids are not essential for the survival of bacteria, they may code for a variety of genetic determinants which permit their hosts to survive better in adverse environment, or to compete better with other microorganisms occupying the same ecological niche.

Plasmids are initially recognised by the phenotypic properties or biological functions they confer on their bacterial hosts. These include resistance to antimicrobial agents (Datta, 1962; Anderson and Lewis, 1965; Rotimi, Emina and Eke, 1983; Olukoya et al., 1988a; 1988b), some metal ions (Novic, 1967; Smith, 1967), colicins (Fredericq, 1958) and ultraviolet radiation (Howarth, 1965). Other properties that have been documented to be plasmid-mediated include virulence (Zink et al., 1978; Gotuzzo et al., 1987), enterotoxin production (Smith and Halls, 1968), ability to hydrolyse

certain compounds (Ishiguro et al., 1979), haemolysin production (Smith and Halls, 1968) and adherence to mammalian cells (Nandadasa et al., 1981).

Another area where plasmids have been widely used is in the epidemiological studies of bacterial infections. For instance, among penicillinase producing Neisseria gonorrhoeae (PPNG), two distinct types of penicillin - resistance plasmids have been recognised. One with molecular weight of 4.4 megadaltons is associated with isolates of Far Eastern origin, while plasmid with 3.2 megadaltons were linked with West Africa (Robert and Falkow, 1977; Perine et al., 1977; Dillon and Pauze, 1981). However, Anderson, Odugbemi and Johnson (1982) reported the detection of a 4.4 megadaltons plasmid in two epidemiologically unrelated strains of PPNG from Nigeria. Furthermore, Odugbemi et al. (1983a) in an epidemiological study of N. gonorrhoeae isolates from Africa concluded that all different patterns except the combination of 24.5 megadaltons and 3.2 megadaltons plasmid reported from all other geographical areas are present in Africa. More recently, Olukoya et al. (1988a; 1988b) have used plasmid analysis to study the epidemiology of some pathogenic bacteria in Nigeria.

At present, not much is known on the prevalence of plasmids in Aeromonas or Plesiomonas. However, Shotts et al. (1972) reported plasmid-mediated antibiotic resistance in A. hydrophila isolated from fish, while Olsen and Wright (1971) reported that A. salmonicida can be the recipient of resistance plasmids from Enterobacteriaceae and the pseudomonads. On the other hand, Holmberg et al. (1986) reported that many Plesiomonas strains in the United States possessed plasmids with molecular weights of 150 megadaltons. More recently, Nolte et al. (1988) reported that an isolate from a patient with proctitis and fatal septicaemia possessed a plasmid > 100 megadaltons.

In Nigeria, there is no report yet on the presence or absence of plasmids in Aeromonas or Plesiomonas. Therefore, the present study was carried out to establish the prevalence of plasmids in isolates of Aeromonas and Plesiomonas from patients seen at LUTH, Lagos, Nigeria.

MATERIALS AND METHODS

Bacterial Strains:

Fifty five Aeromonas sp. and 16 P. shigelloides were screened for plasmids in this study. The Aeromonas strains consisted of 20 A. hydrophila, 7 A. sobria,

and 26 A. caviae, all from patients with diarrhoea; as well as 2 A. caviae from patients without diarrhoea. All the 16 P. shigelloides were from patients with diarrhoea.

Isolation of Plasmid DNA:

The method used for the isolation of plasmid DNA was the rapid alkaline lysis method of Birnboim and Doly (1979). The method is based on the principle of the alkaline denaturation of chromosomal DNA while covalently closed circular DNA remains double - stranded. The method involves the gentle lysis of the bacterial cells by lysozyme and centrifugation to remove the bulk of the chromosomal DNA. The plasmid DNA thus obtained is stained with a dye, electrophoressed and then visualized using an ultraviolet (uv) light.

Briefly, the procedure involves growing bacteria overnight at 37°C on Mueller-Hinton agar plates. Colonies obtained for each test strain were scraped into an Eppendorf tube containing 0.5 ml of 50 mM glucose, 10 mM EDTA and 25 mM Tris (pH 8.0); and lysozyme. The mixture was vortexed and stored for 5 mins at room temperature (22 - 25°C). To each tube was added 200 ul of a freshly prepared solution of 0.2N NaOH, 1% sodium dodecyl sulphate (SDS). This was mixed by inverting

tube 2 or 3 times, and mixture was stored on ice for 5 mins. A 150 ul of an ice-cold solution of potassium acetate (pH 4.8) was added and tube was vortexed gently in an inverted position for 10 seconds. The mixture was stored for 5 mins on ice and centrifuged for 5 mins at 4°C. The supernatant was transferred to a clean tube and an equal volume of chloroform added, vortexed and centrifuged for 2 mins.

The supernatant was transferred into a fresh tube and 600 ul of 95% ethanol added to precipitate the DNA. The mixture was vortexed and centrifuged for 5 mins at room temperature. The supernatant was then removed and the tube inverted on a paper towel for the fluid to drain off. One ml solution of 70% ethanol was added, vortexed and recentrifuged for 3 mins. All supernatants were again removed and tubes inverted on a paper towel to allow excess fluid to drain off and pellet to dry. The pellet was then resuspended in 50 ul of TE buffer (pH 8.0).

Agarose gel electrophoresis:

Electrophoresis of DNA was carried out on 0.8% horizontal agarose slab gels in Tris-borate buffer (89 mM Tris-borate, 89 mM boric acid, 25 mM EDTA pH 8.0).

A dye solution, consisting of bromophenol blue (0.25%) and sucrose (40% W/V) in water was added to DNA samples before elctrophoresis. Loaded gels were electrophoresed at 100 mA for 3 hours (Plate G). The gels were then stained with ethidium bromide (0.5 ug/ml) solution in water for 45 mins, and the DNA was visualized by transmitted short wave ultraviolet light. The distance migrated from the origin by plasmid DNA was measured in milimetres and the photograph of the plasmid bands on gel was then taken.

Determination of Plasmid Size:

When plasmid DNA is subjected to electrophoresis in gel, the migration of the different DNA species is related inversely to their molecular weights. Hence a straight line relationship is obtained when the distance migrated from the origin by the plasmid DNA is plotted against the $\log_{10}$ of the molecular weights of the plasmid DNA. Molecular weights of unknown plasmids are extrapolated from the straight line graph, using standard plasmids of known weights.

Control Strains:

A reference strain of E. coli V517 carrying 8 plasmids of molecular weight standards pVA 517A - H

(Macrina et al., 1978) was included in the study. It was provided by Dr. J. Crossa of Oregon Health Sciences University, Portland, Oregon, USA.

RESULTS

Table 16 shows the isolates harbouring one or more plasmids with molecular weights ranging from 2.4 to 16.8 x 10^6 daltons. Of 20 A. hydrophila strains examined, 3 (15%) harboured plasmid. Similarly, 9 (32.1%) of 28 A. caviae, and 1 (6.3%) of 16 P. shigelloides harboured plasmids. No plasmid was discovered in 7 A. sobria examined, while on the whole, a total of 12 (21.8%) of 55 Aeromonas strains examined harboured one or more plasmids.

Also in this study, an A. hydrophila strain harboured the plasmids 2.4×10^6 and 4.8×10^6 daltons while an A. caviae similarly harboured two plasmids 3.0×10^6 and 5.3×10^6 daltons. Furthermore, the only P. shigelloides isolate that was positive for plasmids harboured two distinct plasmids of 9.7×10^6 and 16.8×10^6 daltons.

It is equally noteworthy that all the strains harbouring plasmids were isolated from patients with diarrhoea. Though the number of control strains included in this study is small, this preliminary finding seems to suggest that plasmids may be important in the pathogenesis of strains harbouring them.

COMMENT

The results of this study showed that isolates of *Aeromonas sp.* and *P. shigelloides* from patients with diarrhoea in our environment harboured plasmids. Though the picture was not of the best quality (Plate H), it was possible to measure the distance migrated by plasmids in the gel. The molecular weight of these plasmids range from 2.4×10^6 to 16.8×10^6 daltons. More specifically, 15% of 20 *A. hydrophila* isolates, 32.1% of 28 *A. caviae* and 6.3% of *P. shigelloides* examined in this study harboured one or more plasmids. However, no plasmid was detected in 7 isolates of *A. sobria* examined (Tables 16 & 17).

Worlwide, plasmid studies have not been widely carried out in *Aeromonas* or *Plesiomonas* strains. However, McNicol *et al.* (1980) reported that 57% of the environmental *Aeromonas* isolates recovered in Bangladesh were resistant to multiple antibiotics including tetracycline and that the resistance appeared to be plasmid-mediated. Also, Holmberg *et al.* (1986) reported that many

Plesiomonas strains in the United States possessed plasmids with molecular weights 150 x 10^6 daltons; while Nolte et al. (1988) reported that a P. shigelloides isolate from the blood of a patient with proctitis and fatal septicaemia possessed a plasmid 100 x 10^6 daltons. Therefore, it is possible that some of the plasmids in the test strains encode for specific properties of such strains.

Previous studies on other bacterial pathogens in our environment as well as in other parts of the world, have equally suggested that plasmids code for some virulence factors thereby enhancing the establishment of infection by such pathogens. These include resistance to antibiotics in E. coli (Adetosoye, 1980; Rotimi et al., 1983; Olukoya et al., 1988a; 1988b), enterotoxin production in E. coli (Gyles, So and Falkow, 1974), B-lactamase production in N. gonorrhoeae and Staphylococcus aureus (Odugbemi et al., 1983a; 1983b; Novic, 1967), invasiveness in Y. enterocolitica (Zink et al., 1978) and adherence in E. coli (Nandadasa, 1981).

An attempt to correlate plasmid size with species shows that the plasmid 2.4×10^6 daltons occurred with equal frequencies among A. hydrophila and A. caviae isolates (Table 17). Also, the plasmid 4.0×10^6 daltons occurred in only A. hydrophila while plasmids 3.0×10^6 and 5.6×10^6 daltons occurred in only A. caviae. Similarly, plasmids 6.4×10^6 and 6.7×10^6 daltons occurred in A. caviae and A. hydrophila respectively. Furthermore, the plasmid 8.4×10^6 daltons was found only in A. caviae, while plasmids 9.7×10^6 and 16.8×10^6 daltons were found only in P. shigelloides isolates. While these findings may suggest some species - relatedness among some plasmids, more elaborate studies would have to be done before a definite conclusion can be drawn.

Finally, this pioneering effort has established the presence of plasmids among Aeromonas and Plesiomonas isolates from our environment. Furthermore, some of the phenotypic properties observed in such isolates are possibly plasmid-mediated. It is most probable therefore that more elaborate studies of plasmids in these organisms would enhance a better understanding of their pathogenic mechanisms and epidemiology of their infections.

Table 16: Isolates of Aeromonas and Plesiomonas from LUTH harbouring one or more plasmids

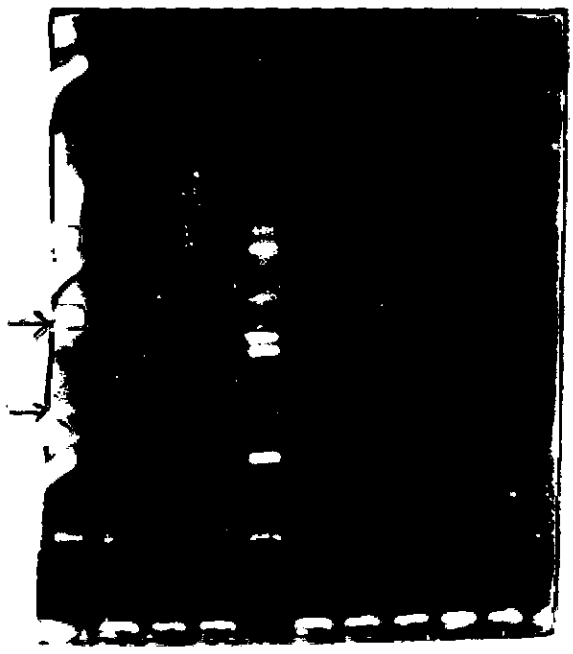
	A. hydrophila	A. caviae	A. sobria	Total	P. shigelloides
No. of isolates Examined	20	28	7	55	16
No. of positives	3	9	0	12	1
Percentage	15	32.1	0	21.8	6.3

Table 17: Plasmid positive Aeromonas and Plesiomonas isolates and their molecular weights

Isolate Number	Species	Plasmid Size (Megadaltons)
832	A. caviae	2.4
1653	A. hydrophila	2.4
1708	A. hydrophila	2.4
1430	A. caviae	2.4
234	A. caviae	3.0
		5.3
156	A. caviae	6.4
2269	A. hydrophila	6.7
65	A. caviae	8.4
596	A. caviae	8.4
577	P. shigelloides	9.7
		16.8

PLATE G

AGAROSE GEL ELECTROPHORESIS APPARATUS WITH TWO TANKS ATTACHED. THE DISTANCES MIGRATED BY PLASMIDS IN THE GEL ARE INVERSELY PROPORTIONAL TO THEIR RESPECTIVE SIZES.



Agarose gel electrophoresis of plasmids

L → R: The 1st column is an isolate of A. hydrophila harbouring two plasmids of different sizes (arrowed). The 5th and 10th columns each contains a standard control strain, E. coli V517, showing at least six different plasmids in this gel.

CHAPTER 9

GENERAL DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

GENERAL DISCUSSIONS

Acute diarrhoeal disease, characterised usually by the frequent passing of watery stools and dehydration, is a major cause of morbidity and mortality throughout the world (Rohde and Northrup, 1976; Giannella, 1981; Dosunmu-Ogunbi *et al.*, 1982; Odugbemi *et al.*, 1987). In the developing countries where the problem is endemic, Snyder and Merson (1982) estimated that diarrhoeal diseases cause nearly 5 million deaths per year in children under 5 years old.

While the mortality and impact of acute diarrhoeal disease is less in the developed countries, it remains a major cause of morbidity, hospitalization, and loss of economic resources. In the United States for instance, it has been reported that acute diarrhoeal disease is second only to the common cold in causing time lost from work (Dingle, Badger and Jordan, 1964). In Nigeria, precise statistical data are lacking but Bojuwoye (1987) remarked that acute diarrhoeas would probably rank second to malaria from the point of view of production time lost. Such is the magnitude of the problem of acute diarrhoeal diseases worldwide.

As highlighted by Carpenter (1981), several microbial agents are now known to cause acute gastrointestinal diseases in man. These include the traditional enteric pathogens such as *V. cholerae*, *Salmonella sp.*, *Shigella sp.*,

enteropathogenic *E. coli* (EPEC), *Clostridium dificile*, *Entamoeba histolytica* and *Giardia lamblia*. Other agents recently recognised as enteropathogens include additional *E. coli* groups that are classified as enterotoxigenic (ETEC), enteroinvasive (EIEC), and enterohaemorrhagic (EHEC); *Campylobacter sp.*, *Yersinia sp.*, the rotavirus and the Norwalk virus (Agbonlahor and Odugbemi, 1982; Riley *et al.*, 1983; Skirrow, 1977; Coker and Dosunmu-Ogunbi, 1984; Alabi *et al.*, 1986; Bishop *et al.*, 1973).

However, despite the development of newer diagnostic techniques and tremendous improvement on the part of the clinical microbiologists, an aetiological agent can still be identified in only about 80% of cases of acute diarrhoeal diseases (Sack, 1975; Rohde and Northrup, 1976). Therefore, the search for additional agents, that may be hitherto unknown, has resulted in the identification of a third group of microorganisms that are being proposed as agents of acute diarrhoea. This include *Cryptosporidium* (Bogaerts *et al.*, 1984), *Listeria monocytogenes* (Schlech *et al.*, 1983), the *Morganella - Proteus - Providencia* group (Muller, 1986), *Aeromonas* and *Plesiomonas* (Sanyal, Singh and Sen, 1975; Agbonlahor, 1983; Ljungh and Wadstrom, 1985; Von Graevenitz, 1985).

In the recent past, there has been an upsurge in the number of reports on the isolation of *Aeromonas sp.* and

P. shigelloides from faecal specimens of patients with diarrhoea as well as controls in different parts of the world. However, while majority of these reports ascribed aetiological roles to these organisms, a few but important epidemiological studies could not (Cumberbatch et al., 1979; Gracey, Burke and Robinson, 1982; Agbonlahor, 1983; Arai et al., 1980; Reinhardt and George, 1985a; Pitarangsi et al., 1982; Figura et al., 1986). There is therefore a controversy on the clinical significance of Aeromonas or Plesiomonas in relation to acute diarrhoeal diseases.

Between October 1986 and September 1987, faecal specimens of 2,350 patients with diarrhoea and 500 patients without diarrhoea (controls) were cultured for the isolation of established bacterial enteropathogens as well as Aeromonas sp. and P. shigelloides. During the study, Aeromonas sp. was isolated from 53 (2.26%), P. shigelloides from 16 (0.68%), Shigella sp. from 4 (0.17%), Salmonella sp. from 3 (0.13%) and Y. enterocolitica from 2 (0.09%) of 2,350 specimens from patients with diarrhoea. Furthermore, enteropathogenic E. coli (EPEC) was isolated from 39 (2.85%) of 1,368 faecal specimens examined while V. cholerae was not found in any of the specimens (Table 1). On the other hand, Aeromonas sp. was isolated from 2 (0.4%) of the 500 faecal specimens from the control group.

The results of this prospective study have shown that both Aeromonas sp. and P. shigelloides were more frequently isolated from patients with diarrhoea than from controls in our environment (Table 2). In addition, the difference in the frequency of isolation of Aeromonas sp. from patients with diarrhoea and the control group was statistically significant ($p < 0.01$).

Furthermore, it is interesting that when compared with other established enteric pathogens isolated in this study, Aeromonas sp. and P. shigelloides came second and third respectively after EPEC in terms of isolation rates (Table 1). They were also more frequently isolated than Shigella sp., Salmonella sp., Y. enterocolitica and V. cholerae. Equally noteworthy is the fact that no other enteric pathogen was isolated from stools that yielded Aeromonas sp. or P. shigelloides in this study. Therefore, while the mere isolation of an organism from a diseased condition does not always confers clinical significance on such an isolate, the findings of this study are strongly suggestive of aetiologic roles for Aeromonas and Plesiomonas in our environment.

Further evidence of possible clinical significance of Aeromonas sp., and P. shigelloides isolated in this study was provided by the epidemiological features observed in

diarrhoea associated with these organisms. The clinical features observed were similar to those of classical diarrhoea due to other established enteropathogens, and these include abdominal pains, anorexia, weight loss and vomiting (Table 3). Similarly, Aeromonas and Plesiomonas - associated diarrhoea, as observed in this study, was not sex-related; and it affected all age-groups but relatively more frequent in children under 5 years old (Figs. 1 & 2). This finding is in agreement with an earlier one in this environment by Agbonlahor (1983). In that study, two out of six Aeromonas isolates from acute diarrhoea cases were from children. Similar features were also observed in a recent study in the United States by Agger, McCormick and Gurwith (1985).

Another striking feature of this study is the seasonality in the distribution of isolates (Figs. 3 & 4). For Aeromonas sp., majority of the isolates were obtained during the wet months of July, August and October which constitute the bulk of the rainy season in Nigeria. A similar picture was also obtained for P. shigelloides with peaks in September and October. This pattern of distribution is indicative of a water-related infection as earlier observed for Aeromonas and Plesiomonas (Ljungh and Wadstrom, 1985; Agger, McCormick and Gurwith, 1985).

Furthermore, biochemical characterisation of 55 Aeromonas sp. and 16 P. shigelloides strains isolated during the course of this study had shown that 11 simple tests including production of oxidase, catalase, H_2S from KIA, pigmentation on nutrient agar, acid from glucose, xylose, dulcitol, adonitol, mannitol and inositol, as well as ability to grow in 6.5% NaCl broth are very specific. The tests may just be enough for the preliminary identification of suspected Aeromonas sp. and P. shigelloides (Table 5). Similarly, acid production from salicin and gas from glucose appeared adequate for the speciation of Aeromonas species into A. hydrophila, A. sobriae and A. caviae.

Based on the results of this study, a simple scheme is proposed for the isolation and preliminary identification of Aeromonas sp. and P. shigelloides from faecal specimens (Fig. 5). However, the scheme is meant to complement and not to replace existing internationally recognised schemes for these organisms. It would also be useful for the screening of large samples of specimens since only suspicious isolates would require further confirmation. It is equally expected that the scheme should be within the reach of

laboratories with limited resources, that may be unable to bear the additional cost of media and reagents needed for a comprehensive identification scheme for Aeromonas sp. (Popoff, 1984) and P. shigelloides (Schubert, 1984).

In many research as well as diagnostic laboratories, transport media are widely used to ensure the survival of pathogens in clinical specimens during transportation. The need to identify appropriate transport medium for Aeromonas sp. or P. shigelloides is now more imperative than before, owing to the increased awareness of their pathogenic potentials. Von Graevenitz (1985) reported that the survival of these organisms in various transport media have not been specifically investigated. To date, I am not aware of any such investigation. I therefore examined the survival of strains of Aeromonas sp. and P. shigelloides in commonly used transport media over a six month period.

The results obtained indicate that all the media can be used for the transportation of specimens containing Aeromonas or Plesiomonas. However, alkaline peptone water (APW) pH 8.6 was the best suitable followed by phosphate buffered saline (PBS), Amies, Stuart, and then Cary-Blair (Fig. 6). Equally significant is the positive effect of ferrous sulphate on the survival of Aeromonas and Plesiomonas in APW. The organisms were recovered from

modified APW containing 0.01% w/v ferrous sulphate for at least six months after inoculation (Fig. 6).

Other workers have equally reported on the positive effect of some metal ions on microorganisms of medical importance (Borwning, 1931; Odugbemi, McEntegart and Hafiz, 1978). In particular, Bullen, Rogers and Griffiths (1974) had observed that the injection of iron compounds can enhance the virulence of certain bacteria, and in some cases can abolish passive immunity. In the present study, ferrous sulphate probably acted as a reducing agent thus minimizing the toxic effect of end products of oxidation during storage.

During the past two decades, several studies in different parts of the world have demonstrated the production of potential virulence factors in strains of Aeromonas sp. and P. shigelloides. These include haemolysins, enterotoxin, cytotoxins, proteases and surface adhesins (Wretlind, Mollby and Wadstrom, 1971; Donta and Haddow, 1978; Sanyal, Singh and Sen, 1975; Dahle, 1971; Atkinson and Trust, 1980). In the same vein, a good number of strains of Aeromonas sp. and P. shigelloides examined in the present study produced haemolysins, heat-stable (ST) enterotoxin, or were invasive. Of the 55 Aeromonas sp. examined, about 70% produced α or B-haemolysin, 20% produced ST while about

11% were invasive. Similarly, about 25% of 16 *P. shigelloides* studied produced ST, and an equal number demonstrated invasive ability in the guinea-pig eye model (Sereny, 1955). In addition, about 13% produced haemolysin (Table 7).

Although the production of virulence factors has been shown to be important in the pathogenesis of established enteropathogens, the role of such factors in *Aeromonas* and *Plesiomonas* - associated diarrhoea is yet to be fully understood. This is because of the lack of correlation between strains producing virulence factors *in vitro* and experimental infections in animals and human volunteers (Pitarangsi *et al.*, 1982; Ljungh and Krenovi, 1982; Morgan *et al.*, 1985). There are suggestions now that the various virulence factors of *Aeromonas* and *Plesiomonas*, particularly enterotoxins, would require further purification before their mode of action can be fully elaborated (Wadstrom, 1978; Sanyal, Saraswatchi and Sharma, 1980; Chakraboty *et al.*, 1984; Chopra *et al.*, 1986).

Over the years, experimental animals have become ready tools in the study of the pathogenesis of infectious diseases, including diarrhoea. More often than not, the ability of an organism to initiate clinical manifestations

in healthy animals has been taking as a demonstration of its potential virulence in humans. For Aeromonas and Plesiomonas, the search for suitable animal models for experimental studies is continuous. Previous attempts to induce diarrhoea in animal species such as rabbits, guinea-pigs, hamsters, rats and rhesus monkeys have not been successful (Ljungh and Krenevi, 1982; Pitarangsi et al., 1982).

In the present study, attempt to experimentally induce diarrhoea in healthy adult mice, using apparently virulent strains of Aeromonas sp. and P. shigelloides was unsuccessful. Each of the animals was starved for 24 h and then orally fed with approximately 1×10^8 c.f.u. per ml of washed cells in phosphate buffered saline (PBS). Two weeks after the mice were orally fed, none had diarrhoea though Aeromonas and Plesiomonas were recovered from their faeces. Furthermore, histological sections of the kidney, lung and heart muscles of mice fed with astrains that demonstrated invasive ability in the guinea-pig eye model (Sereny, 1955); revealed no signs of bacterial invasion of the organs. The cells appeared normal, no cellular infiltration or hyperplasia.

However, the fact that the organisms were excreted in the feaces of the animals showed that they survived

the gastric acid barrier in the stomach. Their inability to initiate diarrhoea in these animals may be due to a number of factors, including inability to attach and establish themselves in the intestinal lumen, competition with intestinal flora of the animals; or host specificity that may make mice unsuitable as has been observed in enterotoxigenic E. coli strains (Cantey, 1985). It may also be that the various virulence factors have to combine in certain ratio to produce diarrhoea as recently suggested by Ljungh and Wadstrom (1985).

In recognition of their seemingly clinical significance, both Aeromonas sp. and P. shigelloides have been widely tested for their susceptibilities to various antimicrobial agents (Overman, 1980; Fass and Barnishan, 1981; Fainstein, Weaver and Bodey, 1982; Reinhardt and George, 1985b; Motyl, Mckinley and Janda, 1985; Chang and Bolton, 1987, Bakken et al., 1988). In Nigeria, Agbonlahor (1983) also reported on the susceptibility of six A. hydrophila strains isolated from patients with diarrhoea at the Lagos University Teaching Hospital (LUTH). However, frequent antimicrobial susceptibility testing is required for surveillance and for the detection of the constantly changing pattern of resistance among bacterial pathogens.

The results of the present study have shown that Aeromonas sp. and P. shigelloides from our environment

are susceptible to most of the antimicrobial agents evaluated (Table 15). Ofloxacin (Tarivid), a new quinolone, was most active *in vitro* against the strains. There was also a good activity (>80%) of gentamicin, carbenicillin and ceftriaxone against the organisms. Furthermore, over 70% of the strains were susceptible to colistin, nalidixic acid, nitrofurantoin, erythromycin, chloramphenicol, tetracycline and co-trimoxazole. Between 50 and 60% of the strains were susceptible to cephaloridine, streptomycin and compound sulphonamide; while less than 30% were susceptible to ampicillin.

It thus appear that the treatment of *Aeromonas* and *Plesiomonas* - related infections requiring antimicrobial therapy, may not be of much problem for now since a good choice can be made among the many antimicrobials to which the organisms are susceptible. However, in a situation like ours where indiscriminate use of antimicrobial agents is common (Odugbemi *et al.*, 1986), the need for constant susceptibility testing cannot be overemphasized. This line of argument is further buttressed by the notable difference in the results of this study and that of Agbonlahor (1983) regarding antimicrobial susceptibilities of isolates. In that study, all the six *A. hydrophila* isolates were uniformly sensitive

to chloramphenicol, tetracycline, streptomycin, gentamicin, and compound sulphonamide; while in the present study, some level of resistance to the same drugs was observed.

Bacterial plasmids are extra chromosomal deoxyribonucleic acid (DNA) that are capable of autonomous replication. Primary interest in their study has been as a result of the biological functions they confer on their hosts. These include resistance to various compounds such as antimicrobial agents, colicins, some metal ions, and ultraviolet radiation (Datta, 1962; Anderson, 1965; Rotimi, Emina and Eke, 1983; Olukoya et al., 1988a; 1988b: Novic, 1967; Fredericq, 1958; Howarth, 1965). In additon, plasmid analysis has over the years become a very useful epidemiological marker in the studies of bacterial infections (Robert and Falkow, 1977; Anderson, Odugbemi and Johnson, 1982; Odugbemi et al., 1983a; 1983b).

In the present study, results obtained from the screening of a total of 55 Aeromonas sp. and 16 P. shigelloides for plasmids have shown that some of the strains harboured plasmids with molecular weights ranging from 2.4×10^6 to 16.8×10^6 daltons (Table 17). Precisely, 3 (15%) of 20 A. hydrophila, 9 (32.1%) of 28 A. caviae and 1 (6.3%) of 16 P. shigelloides harboured

one or more plasmids. However, no plasmid was discovered in 7 A. sobria examined in this study (Table 16). The probability is quite high that some of the properties exhibited by bacteria harbouring these plasmids are plasmid-mediated.

On a global scale, not much of plasmid studies have been done on Aeromonas and Plesiomonas strains. In Nigeria, this is probably the first. However, McNicol et al. (1980) reported that 57% of Aeromonas isolates recovered in Bangladesh were resistant to multiple antibiotics including tetracycline and the the resistance appeared to be plasmid-mediated. Similarly, Holmberg et al. (1986) and Nolte et al. (1988) have separately reported Plesiomonas strains harbouring plasmids with molecular weights greater than 150×10^6 daltons and 100×10^6 daltons respectively. The smaller-sized plasmids observed in local strains are probably peculiar to our environment.

CONCLUSIONS AND RECOMMENDATIONS

This study sets out to establish the prevalence and characteristics of Aeromonas sp. and P. shigelloides isolated in Lagos, Nigeria. During the course of the work, Aeromonas sp. were significantly more frequently

recovered from patients with diarrhoea than from controls ($p < 0.01$). Likewise, *P. shigelloides* was more frequently isolated from the diarrhoea group than the control group, though the difference between the two groups was not statistically significant ($p > 0.05$). Furthermore, clinical features observed in patients with *Aeromonas* or *Plesiomonas* - associated diarrhoea were not in any way significantly different from those of diarrhoea due to other established enteropathogens. Similarly, isolation rates of *Aeromonas sp.* and *P. shigelloides* compared favourably with those of these known enteropathogens. Thus, it can be rightly said that both *Aeromonas* and *Plesiomonas* are more often associated with diarrhoea; and as a matter of fact, the findings are suggestive of aetiological roles for these organisms in acute diarrhoeal diseases in our environment.

Biochemical characterisation of the isolates further confirms the usefulness of internationally recognised schemes of Popoff (1984) and Schubert (1984) for differentiating these organisms. Also, results of the various biochemical tests did not reveal any property that may be said to be peculiar to strains isolated in our locality. In addition, none of the biochemical tests correlated with virulence properties of isolates,

as has been observed in some earlier studies. It is my strong conviction then that with the level of information available on Aeromonas and Plesiomonas, it may be erroneous to use phenotypic characters to predict the "virulence" nature of isolates.

Based on the results obtained from the extensive biochemical characterisation of the isolates, the scheme proposed for the isolation and preliminary identification of Aeromonas sp. and P. shigelloides seems adequate. The scheme is simple and should thus be within the reach of small laboratories. For bigger ones, the scheme should be adequate for the screening of large samples during emergencies.

Virulence factors demonstrated in strains tested include haemolysins, heat-stable (ST) enterotoxin and invasiveness. These are recognised factors that are known to contribute significantly to the pathogenicity of other established enteropathogens. Whether they play any significant role in Aeromonas or Plesiomonas - associated diarrhoea in this study remain unclear, as virulent properties were not correlated in experimental animal studies in mice. As stated earlier, antimicrobial treatment of acute diarrhoea is usually not recommended since less dangerous, cheaper and effective treatment

are available in form of the oral rehydration solutions (ORS). However, results of antimicrobial susceptibility testing in this study indicate that for cases of Aeromonas or Plesiomonas - associated diarrhoea requiring therapy; ofloxacin, gentamicin, tetracycline, and chloramphenicol could be useful.

Furthermore, results of this work also indicate that the commonly used conventional transport media seem adequate for Aeromonas and Plesiomonas strains. However, alkaline peptone water (APW) and the modified APW appeared suitable for long-term storage of these organisms. Likewise, the application of plasmid analysis to clinical isolates of Aeromonas and Plesiomonas on a wider scale could enhance a better understanding of their epidemiology and possible pathogenic mechanisms.

Based on the results of this study, the following recommendations, if implemented, should further enhance our understanding of Aeromonas and Plesiomonas in relation to acute diarrhoeal diseases:

1. More epidemiological studies should be carried out on Aeromonas and Plesiomonas - associated diarrhoea in other parts of the country, to provide information on a national scale.

2. Aeromonas and Plesiomonas should be routinely cultured for, particularly in the diagnostic laboratories, to improve the present level of awareness and reporting which is grossly inadequate.
3. The search for additional virulence factors in these organisms should be intensified to enhance better understanding of possible pathogenic mechanisms.
4. Efforts should be intensified to identify suitable animal models for the study of Aeromonas and Plesiomonas - associated diarrhoea.
5. Since diarrhoea is more endemic in developing countries, including Nigeria, these countries should be more involved in the search for newer agents, better preventive and control measures, as well as effective management of this scourge of our time.

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APPENDICES

APPENDIX I

MEDIA AND REAGENTS USED

The following media were prepared according to the manufacturers' instructions :

MacConkey Agar (Oxoid CM7)

Peptone	20.0g
Lactose	10.0g
Bile Salts	5.0g
Sodium chloride	5.0g
Neutral red	0.075g
Agar	12.0g
Distilled water	1.0 litre

pH 7.4

Desoxycholate Citrate Agar (Oxoid CM35)

'Lab-Lemco' Powder	5.0g
Peptone	5.0g
Lactose	10.0g
Sodium citrate	5.0g
Sodium thiosulphate	5.0g
Ferric citrate	1.0g
Sodium desoxycholate	2.5g
Neutral red	0.025g
Agar	15.0g
Distilled water	1.0 litre

pH 7.0

Xylose Desoxycholate Citrate Agar (XDCA)

This is desoxycholate citrate agar to which 1% w/v of D-Xylose (Difco) is added. The pH is adjusted to 7.0.

Blood Agar Base No. 2 (Oxoid CM271)

Proteose Peptone	15.0g
Liver Digest	2.5g
Yeast Extract	5.0g
Sodium Chloride	5.0g
Agar	12.0g
Distilled water	1.0 litre

pH 7.4

To prepare blood agar, suspend 40g in 1 litre of distilled water, bring to boil to dissolve completely. Mix and sterilize by autoclaving at 121°C for 15 minutes. Cool to 40 - 45°C and add 5 - 10% sterile blood. Dispense about 15mls to each petri dish.

Ampicillin (15 ug/ml) Blood Agar

Prepare blood agar base appropriately and sterilize at 121°C for 15 minutes. Cool to 40 - 45°C, add 5 - 10% sterile blood and add 15,000 ug per litre of medium.

Brain Heart Infusion Agar (Oxoid CM375)

Calf Brain Infusion Solids	12.5g
Beef Heart Infusion Solids	5.0g
Proteose Peptone	10.0g
Sodium Chloride	5.0g
Dextrose	2.0g
Disodium phosphate	2.5g
Agar	10.0g
Distilled water	1.0 litre

pH 7.4

Nutrient Broth (Oxoid CM1)

'Lamb-Lemco' Powder	1g
Yeast Extract Powder	2g
Peptone	5g
Sodium Chloride	5g
Distilled water	1.0 litre

pH 7.4

Nutrient Agar (Oxoid CM3)

'Lab-Lemco' Powder	1.0g
Yeast Extract	2.0g
Peptone	5.0g
Sodium chloride	5.0g
Agar	15.0g
Distilled water	1.0 litre

pH 7.4

Phosphate Buffered Saline (Oxoid BR14a)

Sodium Chloride	8.0g
Potassium chloride	0.2g
Disodium hydrogen phosphate	1.15g
Potassium dihydrogen phosphate	0.2g
Distilled water	1.0 litre

pH 7.3

Amies w/o Charcoal (Difco 0832)

Sodium chloride	3.0g
Potassium chloride	0.2g
Calcium chloride	0.1g
Magnessium chloride	0.1g
Monopotassium phosphate	0.2g
Disodium phosphate	1.15g
Sodium thioglycolate	1.0g
Charcoal	10.0g
Agar	4.0g
Distilled water	1.0 litre

pH 7.4

Stuart Medium (Difco 0621)

Sodium thioglycollate	0.9g
Sodium glycerophosphate	10.0g
Calcium chloride USP	0.1g
Methylene blue	0.002g
Agar	3.0g
Distilled water	1.0 litre

pH 7.4

Peptone Water (Oxoid CM9)

Peptone	10.0g
Sodium chloride	5.0g
Distilled water	1.0 litre

pH 7.2

Alkaline Peptone Water

Prepare as per peptone water and adjust to pH 8.6 with IN sodium hydroxide. Dispense and sterilize.

Modified Alkaline Peptone Water

Peptone	10.0g
Sodium chloride	5.0g
Ferrous sulphate	1.0g
Distilled water	1.0 litre

Adjust pH to 8.6 with IN NaOH, dispense and sterilize.

Cary-Blair Medium (Oxoid CM519)

Disodium hydrogen phosphate	1.1g
Sodium thioglycollate	1.5g
Sodium chloride	5.0g
Calcium chloride	0.09g
Agar	5.6g
Distilled water	1.0 litre

pH 8.4

Trypticase Soy Broth (Difco 0370)

Tryptone	17.0g
Soytone (Soy Bean Peptone)	3.0g
Dextrose	2.5g
Sodium chloride	5.0g
Dipotassium phosphate	2.5g
Distilled water	1.0 litre

pH 7.3

Trypticase Soy Agar (Difco 0369)

Tryptone	15.0g
Soytone	5.0g
Sodium chloride	5.0g
Agar	15.0g
Distilled water	1.0 litre

pH 7.3

DNase Test Agar (Difco 0632)

Tryptose	20.0g
Deoxyribonucleic acid	2.0g
Sodium chloride	5.0g
Agar	15.0g
Distilled water	1.0 litre

pH 7.3

Mueller Hinton Agar (Oxoid CM337)

Beef	300.0g
Casein hydrolysate	17.5g
Starch	1.5g
Agar	10.0g
Distilled water	1.0 litre

pH 7.4

Kligler Iron Agar (Oxoid CM33)

'Lab-Lemco' Powder	3.0g
Yeast Extract	3.0g
Peptone	20.0g
Sodium chloride	5.0g
Lactose	10.0g
Dextrose	1.0g
Ferric citrate	0.3g
Sodium thiosulphate	0.3g
Phenol red	0.05g
Agar	12.0g

pH 7.4

Simmons Citrate Agar (Oxoid CM155)

Magnesium sulphate	0.2g
Ammonium dihydrogen phosphate	0.2g
Sodium ammonium phosphate	0.8g
Sodium citrate, tribasic	2.0g
Sodium chloride	5.0g
Bromo-thymol blue	0.08g
Agar	15.0g
Distilled water	1.0 litre

pH 7.0

Aesculin Medium

Brain heart infusion broth	25.0g
Aesculin	1.0g
Agar	1.0g
Distilled water	1.0 litre

pH 7.4

Decarboxylase Media (Moller, 1955)

Peptone	5.0g
Beef Extract	5.0g
Pyridoxal	5.0mg
Glucose	0.5g
Bromothymol blue (0.2%)	5.0ml
Cresol red (0.2%)	2.5ml
Distilled water	1.0 litre

pH 6.0

Dispense in 4 ml amounts and sterilize by autoclaving at 115°C for 20 minutes. For use, the following additions were made:

1. L-arginine hydrochloride 1%
2. L-lysine hydrochloride 1%
3. L-ornithine hydrochloride 1%
4. No additions (control)

Re - adjust the pH to 6.0 and distribute into 1 ml volume into tubes containing liquid paraffin. Sterilize at 115°C for 10 minutes.

Motility Indole Urea (MIU) Medium

Basal medium:

Peptone (rich in tryptophan)	30.0g
Potassium dihydrogen phosphate	1.0g
Sodium chloride	5.0g
Agar	4.0g
Phenol red (alcoholic solution)	2.0ml
Distilled water	1.0 litre

Preparation:

Add the ingredients to the water, boil to dissolve, and filter through paper. Dispense in 90-ml quantities in screw-cap bottles. Sterilize by autoclaving at 121°C for 15 minutes.

Prepare a 20% urea solution in water. Sterilize by filtration. Add 10 ml of the 20% urea solution to 90 ml of basal medium that has been melted and cooled to 50°C. Distribute in sterile tubes, 5 ml in each and allow to solidify in an upright position.

N.B. Alcoholic solution of phenol is prepared by dissolving 0.25g of phenol red in 50 ml ethyl alcohol and adding 50 ml of distilled water.

MRVP Medium (Oxoid CM43)

Peptone	5g
Dextrose	5g
Phosphate buffer	5g
Distilled water	1.0 litre

pH 7.5

Saline Tablets (Oxoid BR53)

Dissolve 1 tablet in 500 ml of distilled water to prepare 0.85% saline (i.e. 'normal' saline). Sterilize by autoclaving at 121°C for 15 minutes.

Iodine Solution

Iodine (powdered crystals)	10.0g
Potassium iodide (KI)	20.0g
Distilled water	1.0 litre

Dissolve the KI in the water, and add the iodine crystals slowly and shake thoroughly. Not all the crystals will dissolve, therefore filter or decant before use (WHO Document CDD/83.3 p. 80).

Lysozyme Solution

50 mM glucose

10 mM Na_2 EDTA

25 mM Tris

2 mg/ml of lysozyme

pH 8.0

SDS/NaOH

1% SDS

0.4 N NaOH

TE Buffer

0.05 M Tris

0.039 M Na_2 EDTA

APPENDIX II

MAJOR TESTS USED IN THE SCREENING OF AEROMONAS SPECIES AND PLESIOMONAS SHIGELLOIDES

Utilisation of glucose and lactose/Production of H_2S and Gas

Each test strain was inoculated into one KIA tube by stabbing with a straight wire. Inoculated tube was incubated at 37°C for 18 - 24 hours.

Yellow colouration of the butt alone indicated glucose utilisation.

Complete yellow colouration of the medium indicated glucose and lactose utilization.

Blackening of medium indicated H_2S production.

Bubbles, cracks or displacement of medium indicated gas production.

Motility, Indole and Urease Activity

Each test strain was inoculated, with a straight wire, into one MIU tube and incubated at 37°C for 18 - 24 hours.

Diffuse growth of test organism from the straight line of inoculation into the medium indicated that the organism is motile.

Red colouration of medium indicated positive urease activity.

Red ring formation on surface of medium upon addition of Kovac's (indole) reagent indicated production of indole.

Cytochrome Oxidase Activity (Kovacs, 1956)

Two or three drops of 1% solution of N, N, N', N' - tetramethyl-p-phenylenediamine dihydrochloride (oxidase reagent) were added onto a filter paper, and the test organism was smeared across the surface of the impregnated paper. Oxidase positive organisms gave a purple colour within 10 seconds.

Decarboxylase Activities

Each test organism was inoculated into 4 tubes : lysine, ornithine, arginine and the control. These were incubated at 37°C and read daily for 4 days. Decarboxylation of the amino acids was indicated by a change in colour to violet. The control remained yellow.

Methyl Red/Voges Proskauer (MRVP) Test

Two sets of MRVP medium bottles were inoculated with each test strain, and incubated at 37°C for 48 hours :

Methyl red Test - Two or 3 drops of methyl red solution was added to one MRVP medium bottle, and positive strains gave a red colouration on shaking.

Voges Proskauer Test - The test detects production of acetyl methyl carbinol from carbohydrates. After incubation, 1 ml of 40% potassium hydroxide and 3 ml of 5% α - naphthol solution were added. Positive strains gave a pink colour in 2 - 5 minutes, upon aeration.

Nitrate Reduction

Solution A : 8.0g of sulphalinic acid in 1 litre of 5N acetic acid.

Solution B : 0.5g of ∝ - naphthylamine in 1 litre of 5N acetic acid.

Equal volumes of solutions A and B were mixed immediately before use to give the test reagent.

0.1 ml of test reagent was added to each test culture. Reduction of nitrate to nitrite was indicated by a red colour developing within a few minutes.

B-Lactamase Production

1. Chromogenic cephalosporin method (O'callaghan et al., 1972) :

Chromogenic cephalosporin (Glaxo) an orange dye was added to bacterial colonies on the culture media. Development of red colouration within 2 minutes indicated a B-lactamase producing organism.

2. Starch paper technique (Odugbemi, Hafiz and McEntegart, 1977):

Strips of starch paper were soaked for 10 minutes in the benzyl penicillin solution (100,000 ug/ml in PBS) and then spread smoothly in petri dishes. Colonies of each

test organism was spread over an area (2 - 3mm) of the soaked paper, and then incubated at 37°C for 30 minutes. The papers were then flooded with iodine solution which was drained off immediately. Area surrounding a B-lactamase producing organisms is decolourised (white) while other areas surrounding non B-lactamase producing organisms (controls) remain blue - black.

Amylase Production

Each test strain was inoculated onto a nutrient agar plate supplemented with 1% (w/v) starch. The plate was incubated at 37°C for 18 - 24 hours, and then flooded with iodine solution. A clear zone surrounding a test organism indicated that the organism is an amylase producer.

DNase Production

Each test strain was inoculated onto a DNase test agar plate supplemented with 1% methyl green solution. The plates were incubated at 37°C for 24 hours. A clear zone surrounds a DNase producing organism while non DNase producers remain green.

APPENDIX III

SOURCES OF MEDIA, REAGENTS, CHEMICALS AND SENSITIVITY DISKS

Sources of Media:

Oxoid Ltd.,
Basingstoke,
Hants, England.

Difco Laboratories,
Detroit, Michigan, U.S.A.

BBL (Baltimore Biological Laboratories),
Div. Becton, Dickinson and Co.,
Cockeysville, MD 21030, U.S.A.

Sources of Chemicals/reagents:

Aesculin	BDH Chemicals Ltd., Poole, England.
Carbohydrates	BDH
Ethidium bromide	BDH
Ethylenediaminetetra - Acetic acid (EDTA)	BDH
Sodium chloride	BDH
Sodium hydroxide	BDH
Ferrous sulphate	BDH

Lysozyme	Sigma Chemical Co., P. O. Box 14508, St. Louis, MO 63178, U.S.A.
Agarose	Sigma
P-Dimethylaminobenzaldehyde	Sigma
Tris (hydroxymethyl) methylamine	Sigma
Sodium dodecyl sulphate (SDS)	Sigma
Antisera	Wellcome Reagents Ltd., Wellcome Research Laboratories, Beckenham, England.
Chromogenic Cephalosporin dye	Glaxo, Glaxo Laboratories Ltd., Greenford, Middlesex.
Sensitivity Disks:	
Code 1788E	Oxoid
Code U4	Oxoid
Ofloxacin (Tarivid)	The Nigerian Hoechst, Plot 144, Oba Akran Avenue, P. O. Box 261, Ikeja.

APPENDIX IV

STATISTICAL ANALYSIS

The Chi-square (χ^2) test was used to determine if there is an association between diarrhoea and the isolation of *Aeromonas sp.* or *P. shigelloides* from the faecal specimens of patients.

A. *Aeromonas sp.*

Table 18. Contigency table of observed (O) frequencies of *Aeromonas sp.* from faecal specimens of patients with and without diarrhoea.

	No. of Specimens Positive for *Aeromonas sp.*	No. of Specimens Negative for *Aeromonas sp.*	Total
Patients with Diarrhoea	53	2297	2350
Patients without Diarrhoea	2	498	500
Totals	55	2795	2850

$$\text{Expected frequency (E)} = \frac{\text{Column total x Row total}}{\text{Grand total}}$$

Using the above formula, we obtained table 19 which gave expected frequencies.

Table 19. Expected frequencies (E) of *Aeromonas sp.* from faecal specimens of patients with and without diarrhoea

	No. of expected Positives for *Aeromonas sp.*	No. of expected Negatives for *Aeromonas sp.*	Totals
Patients with Diarrhoea	45.35	2304.65	2350
Patients without Diarrhoea	9.65	490.35	500
Totals	55	2795	2850

Therefore,

Null hypothesis, Ho: There is no association between diarrhoea and isolation of *Aeromonas sp.*

Alternate hypothesis, H_A: There is an association between diarrhoea and isolation of *Aeromonas sp.*

But,

$$X^2 = \sum \frac{(F_O - F_E)^2}{F_E}$$

$$= \sum \frac{(53-45.35)^2}{45.35} + \frac{(2-9.65)^2}{9.65} + \frac{(2297-2304.65)^2}{2304.65} + \frac{(498-490.35)^2}{490.35}$$

$$= \sum \frac{(7.65)^2}{45.35} + \frac{(-7.65)^2}{9.65} + \frac{(-7.65)^2}{2304.65} + \frac{(7.65)^2}{490.35}$$

$$= \sum \frac{58.52}{45.35} + \frac{58.52}{9.65} + \frac{58.52}{2304.65} + \frac{58.52}{490.35}$$

$$= \sum 1.29 + 6.06 + 0.03 + 0.12$$

$$= 7.5$$

Also,

Degree of freedom $= (n_r - 1)\ (n_c - 1)$

$= (2 - 1)\ (2 - 1)$

$= 1$

However,

$\chi^2_{0.01}$ value from the χ^2 distribution

Table = 6.635

Decision Rule: Reject H_o, if calculated value of χ^2 is greater than the value of χ^2 from distribution table.

Decision: H_o is rejected since calculated value of χ^2, 7.5, is greater than the value of χ^2 from table, 6.635 at 99.99% confidence level.

Interpretation: Since H_o is rejected, H_A is accepted. Hence, there is a statistically significant association between diarrhoea and isolation of Aeromonas species. In other words, Aeromonas species are significantly more frequently isolated from patients with diarrhoea than from patients without diarrhoea (controls).

B. P. shigelloides

A similar chi-square testing for P. shigelloides gave the value of calculated χ^2 as 3.43, while the value of χ^2 from the distribution table was 3.841 at $\chi^2_{0.05}$. Hence we accepted H_o that there is no statistically significant association between diarrhoea and the isolation of P. shigelloides, since the calculated value of χ^2 is less than the value of χ^2 from the distribution table.

APPENDIX V

PUBLICATION FROM THIS WORK AND RECENT WORLD CONFERENCES ON AEROMONAS AND PLESIOMONAS

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PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF *AEROMONAS* SPECIES AND *PLESIOMONAS SHIGELLOIDES* ISOLATED IN LAGOS, NIGERIA

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Aeromonas **spp. and** ***Plesiomonas shigelloides*** **are increasingly being reported world-wide amongst the newer agents of acute diarrhoeal diseases. A prospective 12-month (Sept. 1986–Oct. 1987) study was undertaken at the Diarrhoeal Research Laboratories of Lagos University Teaching Hospital (LUTH) to determine the prevalence of these agents and their sensitivity to 15 commonly used antimicrobial agents. All isolates were identified by standard procedures; sensitivity testing was by the disk diffusion technique with Oxoid disks.** *Aeromonas* **spp. and** *P. shigelloides* **were isolated from 53 (2·3%) and 16 (0·7%) respectively of 2350 stools of patients with diarrhoea, while 6 (1·2%) and 1 (0·2%) were isolated from 500 stools of controls. Speciation of the 53** *Aeromonas* **spp. showed 26 (49·1%) were** *A. caviae*, **20 (37·7%)** *A. hydrophila*, **and 7 (13·3%)** *A. sobria*. **All isolates were sensitive to ofloxacin, a quinolone recently introduced into Nigeria. There was good in-vitro activity (>90%) for gentamicin, carbenicillin, colistin; and varying degrees of resistance (10–30%) to ampicillin, chloramphenicol, tetracycline, streptomycin, sulphonamide, nalidixic acid, ceftriaxone, co-trimoxazole and erythromycin. This study showed that** *Aeromonas* **spp. and** *P. shigelloides* **should be considered and investigated in diarrhoeal diseases in Nigeria, and in cases of systemic infections due to these organisms in which antimicrobial therapy is indicated, ofloxacin, gentamicin and carbenicillin could be useful.**

Microbe 86

1st INTERNATIONAL WORKSHOP ON AEROMONAS & PLESIOMONAS

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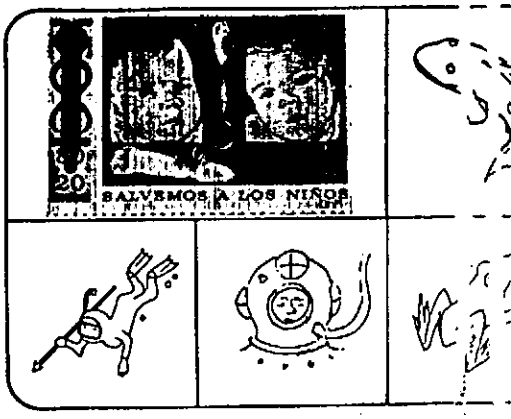

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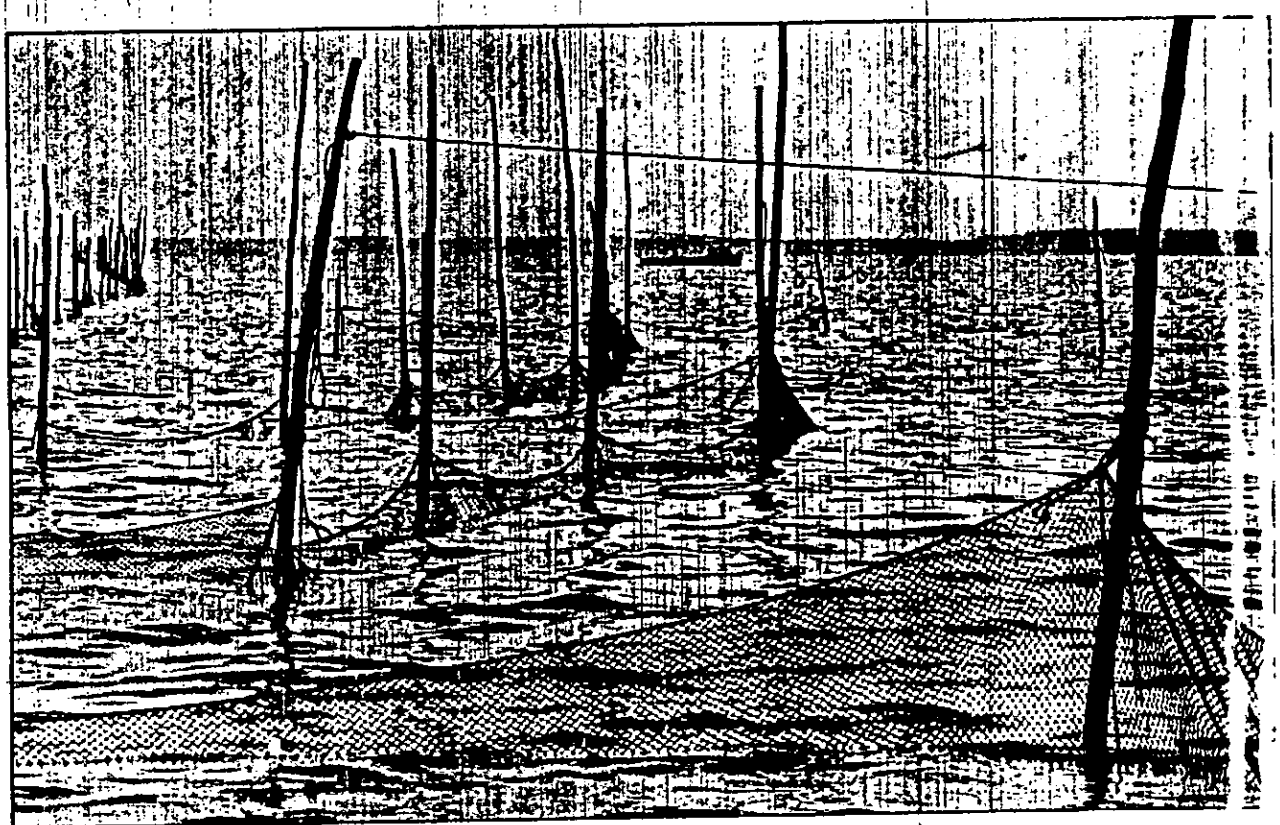

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