STUDIES ON THE TOXINS PRODUCED BY AEROMONAS SPECIES ISOLATED FROM CLINICAL AND ENVIRONMENTAL SOURCES.

A thesis submitted to the Department of Biological Sciences,
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In partial fulfillment of the
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Master of Philosophy

in

BIOLOGY
(Microbiology)

By

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DECLARATION

The materials contained within this thesis is my original work and has not previously been submitted to this or any other university.

(NOOR-US-SABA)

This thesis by Noor-us-Saba is accepted in its present form by the Department of Biological Sciences, Quaid -I-Azam University, Islamabad, as satisfying the thesis requirements for the degree of Master of Philosophy in Biology (Microbiology).

Internal Examiner----------

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Chairman----------

Dated: 16.10.1996

In the Name of Allah ,

the Beneficent ,

the Merciful .

Dedicated to

My Family

CONTENTS

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LIST OF ABBREVIATIONS

Spp	Species
G -ve	Gram Negative
+ve	Positive
h	hour
°C	Degree Centigrade (Celsius)
ug	Microgram
mg	Milligram
g	Gram
ul	Microliter
ml	Milliliter
L	Liter
um	Micrometer
%	Percent (Percentage)
pH	log H+ Concentration
CFCF	Cell Free Culture Filtrate
CT	Cholera toxin
DNAase	Deoxyribose nuclease
HA	Haemagglutination Activity
rpm	Revolutions per minute
BSA	Bovine Serum Albumin
O.D	Optical Density
kd	Kilo Dalton
MEM	Minimum Essential Medium
PBS	Phosphate Buffer Saline
IW	Intestinal Weight
BW	Body Weight
IW/BW	Intestinal Weight/ Body Weight
W/W	Weight/ Weight
W/V	Weight/Volume
HU/ml	Hemolysin Unit/ Milliliter
Con	Concentration
Nos.	Numbers
C	Control

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ABSTRACT

Twelve strains of Aeromonas species (*Aeromonas hydrophila*- 5 strains, *Aeromonas caviae*- 6 strains and *Aeromonas sobria* -1 strain) from clinical and environmental sources were tested for (a), Production of different exotoxins (Hemolysin, Cytotoxin and Enterotoxins) (b), Enteropathogenic effects of the elaborated exotoxins to determine the biological activities of these exotoxins.

All the 12 Aeromonas strains were propagated in tryptic soya broth supplemented with yeast extract, 0.6% w/v, pH 7.4 and incubated with agitation at 300 oscillation / minutes for 24 hours at 37°C. On these specifications all the strain produced optimum amount of exotoxins in the form of CFCF (Cell Free Culture Filtrate). Each CFCF sample was tested for biochemical feature using protein estimation and Gel electrophoresis and the biological assay of exotoxin (hemolysin, cytotoxin and enterotoxin).

The protein concentration range in all CFCF solutions, irrespective of species and sources was 144.518 - 236.08 ug/ml. In CFCF of *A. hydrophila* it was 171.98 - 198.433 ug/ ml , in *A. caviae* 161.086- 236. 0768 ug/ml and in *A. sobria* it was 144.5108ug/ml.The high protein content of CFCF gave a relatively high hemolytic, cytopathic and enterotoxic effects. CFCF of all 12 strains were tested on Gel electrophoresis and protein bands were observed within molecular weight range of 15 kd (enterotoxin) and 55kd (hemolysin) along with many other unknown protein bands.

Hemolysin activity in vitro on 1% rabbit erythrocytes was observed in 100% (12 out of 12) of CFCF samples irrespective of species and sources of isolation, it was found in the range of 32-1025 HU/ml in *A. hydrophila*, 2-256HU/ml in *A. caviae* , and 16 HU/ml in *A. sobria*.

Cytotoxic activity on Hep-2 cells, MRC-5 cells and Vero cells was observed in all of the 12 CFCF sample (100%) of Aeromonas strains, with varying degree of cytotoxicity, and it was more commonly found in *A. hydrophila* than *A. caviae and A. sobria*

The MRC-5 cell line (human diploid cells) appears to have been first time subjected to cytotoxic studies using *Aeromonas* toxins. It was found that MRC-5 cells could also be used for CPE study of bacterial toxins . A significant association was observed between hemolysin and cytotoxin production by *Aeromonas*, the presence of these activities may play a significant role in the epidemiology of *Aeromonas* associated gastroenteritis. Cytotoxic activity did not correlate with the production of enterotoxin since only 66% (8 of 12) CFCF of *Aeromonas* species were found to be weakly enterotoxic , 60% (3of 5) *A. hydrophila*, 83% (5 of 6) *A. caviae* were enterotoxic. While only one CFCF sample of *A. sobria* was found to be non- enterotoxic. It could be inferred that *A. hydrophila* , as well as *A. caviae* and *A. sobria* are bonafide enteric pathogens as they possess the virulence factors (hemolysin , cytotoxin, and enterotoxin) that may operate in the gastrointestinal tract to provoke a diarrhoeal syndrome.

INTRODUCTION

The members of genus *Aeromonas* family *Aeromonadaceae* are recognized as an important intestinal and extraintestinal pathogens of human and a variety of other vertebrate and invertebrate animals. They occur widely in soil, water and food etc. They may be motile or non-motile, the non-motile strains are recognized as frequent pathogens of cold-blooded marine and fresh water animals, while the motile *Aeromonas* are increasingly being reported to cause various infections in human being such as wound infections, meningitis, endocarditis, osteomyelitis (Nakano, *et al.,* 1990), diarrhoea, and septicemia (Majeed and Macrae, 1994)

The diarrhoea caused by *Aeromonas* may be mild or severe as dysentery-like (Champsaur, *et al.,* 1982; Gracey, *et al.,* 1984). In some well controlled studies *Aeromonas* species were isolated more frequently from diarrhoeal stools than from normal ones (Burke, *et al.,* 1983; Agger, *et al.,* 1985). The role of *Aeromonads* in enteric infection in immunologically compromised adults and children have been the subject of a number of studies.

Members of genus *Aeromonad* are Gram-negative, short and straight rod shaped with rounded ends and sometime may look like coccoid, size ranges from 0.31 micron by 1.0-3.5 micron. They may occur singly or in pairs or short chains and are non sporing, facultatively anaerobic and motilo by means of single polar flagella, however, one species is non-motile (*A. salmonicida*) (Lee, 1990).

The biochemical reactions are most characteristic at 30°C. They are positive for amylase, catalase, DNAase, protease, gelatinase, indol production, glucose fermentation, and cannot grow in 6.5% NaCl, and do not require NaCl for growth (Carnahan, *et al.,* 1989). They are also positive for hemolysis and gas production (Palumbo, *et al.,* 1989). They are resistant to vibrostatic compound e.g., 150 µg of 2,4-diamino 6,7-di-isopropylpetridin phosphate and ampicillin 10µg (Palumbo, *et al.,* 1989) and carbenicillin (Carnahan, *et al.,* 1991).

The colonies of *Aeromonas* on nutrient agar after 24 hours are 1-3mm diameter, circular smooth, convex, dull-white and translucent becoming bigger on further incubation. On blood agar most strains produce a wide zone of beta-hemolysis. Optimum temperature for growth is 22-28°C (Lee, 1990).

The genus *Aeromonas* is currently placed in the family Vibrionaceae (Havelaar, *et al.*, 1990). Two major subdivisions or groups of genus *Aeromonads* were recognized on the basis of their phenotypic differences and pathogenicity .

a - Psychrophilic - non motile do not grow at 35-37°C and are obligate fish pathogen. (*Aeromonas salmonicida*)

b - Mesophilic - motile grow at 35-37 °C occur in the environment and are opportunistic human pathogen, *Aeromonas hydrophila*, *Aeromonas caviae,* and *Aeromonas sobria* were included in this group by Popoff (1984).

Mishra, *et al.*, (1987) studied different media for the isolation of *Aeromonas* spp. to access their significance in the etiology of human diarrhoea. It was found that sheep blood agar with 30 mg of ampicillin per liter (ASBA30) yielded a significant higher percentage of positive specimens as compared to the other media which were generally used for the isolation of other enteric organisms.

Some strain of *Aeromonas* have been found to grow in bacteriological media at refrigeration temperatures. This may be significant from a public health point of view, as bacteria free supernatant containing *Aeromonas*

exotoxins were able to induce fluid accumulation (watery diarrhoea) in rodents and rabbits, and hence may be able to do so in human (Kirov, *et al.*, 1993).

Nishikawa (1988), had investigated the occurrence of motile *Aeromonas* species in stool, food and environmental specimens to assess their pathogenic significance and to determine sources and routes of infection and found that the *Aeromonas* counts in food specimens which included minced beef, pork and chicken, sea food and various vegetables and their products were unexpectedly high suggesting that infection might be food borne rather than water borne. About 70% of the isolates from meat products were *Aeromonas hydrophila* and *Aeromonas sobria* while *Aeromonas caviae* was the most common in sea fish, vegetables and their products. Most *Aeromonas hydrophila* and *Aeromonas sobria* produced haemolysin, but haemagglutinin was found more frequently in *Aeromonas sobria*

Unlike other gram-negative bacteria, pathogenic strains of motile *Aeromonas* produces a wide range of exotoxins. Several exotoxins have been described that may be associated with the virulence of *Aeromonas* spp. These includes *hemolysin*, *cytotoxin*, and *enterotoxins*. These toxins were found to be cytopathic in cell cultures, beta-hemolytic, and

enterotoxic in mice assay (Singh and Sanyal, 1992). While the organisms can grow at a temperature range of 22°-37°C the toxins are best produced at 37°C (Kirov, *et al.*, 1993).

Relatively little is known about the relationship between enterotoxin, hemolysin and cytotoxin produced by *Aeromonas* isolates, however, several reports suggests a parallel activity of these three factors in *Aeromonas* species. Although B-hemolysin production might seem to be a likely pathogenic factor of *Aeromonas* species (Asao, *et al.*, 1986), the failure of hemolytic strains to cause diarrhoea in human volunteers suggests that hemolysin *per se* is not the sole determinant of virulence (Nishikawa, *et al.*, 1994).

The haemagglutinating assay (HA) is the commonly used procedure to determine the ability of organism to adhere to eukaryotic cells. Burke, *et al.*, (1984) reported that the enterotoxigenic diarrheal isolates of *Aeromonas hydrophila* showed HA activity but no such activity was observed with non toxigenic diarrheal infections or the environmental isolates.

Turnbull, *et al.*, (1984) studied the enterotoxin production in relation to taxonomic grouping and source of isolation of *Aeromonas* spp. A strong

correlation was noted between ability to produce enterotoxin and some of the biochemical characters used for scanning. A mouse assay for the enterotoxigenic activity has been described.

However, some immunological differences between the hemolysins produced by the two strains was noted. Janda, *et al.*, (1985) developed a mouse lethality assay as an appropriate model for the study of invasive disease clinically produced by *Aeromonas* spp.

Kindschuh, *et al.*, (1987) studied the clinical and biochemical significance of toxin produced by *Aeromonas* and their cross reactivity with *Shigella* toxin and evaluated the role of cytotoxin and enterotoxin production by *Aeromonas* strains in the pathogenesis of acute gastroenteritis. The relationship between certain biochemical traits of *Aeromonas* spp. and toxin production has also been reported. No significant correlation of exotoxin production and gastroenteritis, and the toxins of *Aeromonas* spp. has been established. No cross reaction with the antiserum raised against the *Shigella* toxins are evident.

The enterotoxin production by *Aeromonas hydrophila* for biological activity by the rabbit ileal loop and suckling mouse assays, as well as by elongation of CHO cells was investigated by Chopra, *et al.*, (1986). Antigenic evaluation of the culture filtrates from various isolates of

A hydrophila was performed by enzyme linked immunosorbent assay with anti-cholera toxin and anti-Aeromonas enterotoxin, and found that heat stability data demonstrated the presence of a heat-labile cholera toxin cross-reactive factor and a heat stable non-cholera toxin cross-reactive enterotoxin The biological activities of both enterotoxins were heat labile at 56°C for 20 minutes.

Kirov, *et al*, (1986) examined the virulence characteristics of *Aeromonas* spp , i e., the production of hemolysin and enterotoxin and the ability of the *Aeromonas* spp. to grow at elevated temperatures. For enterotoxin activity mice assay was used and for hemolysin assay rabbit erythrocytes were used. A procedure for these tests were devised and the toxins were extensively tested in the animal model. It was found that enterotoxigenic human and environmental isolates tended to have high hemolysin titers and the non-toxigenic isolates had no hemolysin titers.

Asao, *et al*, (1986) purified and characterized a hemolysin produced by *Aeromonas hydrophila* strain no CA-11 isolated from environmental sources and a strain AH-1 isolated from a diarrheal case. This hemolysin caused fluid accumulation in infant mouse intestine and rabbit intestinal loops and killed Vero cells.

Notermans, *et al.*, (1986) studied the three toxins production by two strains of *Aeromonas* spp. *A. sobria* and *A. hydrophila*. Cytotoxic activity was studied on the Vero cells. The strains used were isolated by Asao, *et al* , (1986) and were used for toxin production by Notermans, *et al.*,(1986) independently and some differences of toxin activity was noted.

Potomski, *et al.*, (1987) purified the cytotoxic enterotoxin of *Aeromonas sobria* by the use of monoclonal antibodies. The purified enterotoxin was found to be a single protein and caused fluid accumulation in rat ileal loops and in infant mice, was cytotoxic to cultured cells, and hemolytic to human erythrocytes, was also lethal to mice after intramuscular injection.

Cross reactivity of *Aeromonas* enterotoxin with Cholera toxin (CT) was studied by Potomski and Burke, *et al.*, (1987). The enterotoxin which was cross reactive with Cholera toxin caused fluid accumulation and rounding of Y-1 adrenal cells in rat ileal loops and in infant mice. All these activities were neutralized by antiserum to CT. There was no hemolytic or cytotoxic activities associated with this CT-cross reactive cytotoxic enterotoxin.

The attachment of enteric pathogens to mucosal surface has been recognized as an important step in the pathogenesis of gastrointestinal

infections in man and animals. Motile *Aeromonas* species have properties that have been reported to be associated with virulence such as adherence and invasiveness. Corrello, *et al.*, (1988) studied the ability of *Aeromonas* species to adhere to Hep-2 cells. An association between diarrhoa and a high level adhesion was observed in the majority of the environmental isolates.

Namdari and Bottonone (1990) studied the cytotoxin and enterotoxin production among the clinical and environmental isolates of *Aeromonas caviae* and further tested the toxins effect on the Hep-2 cell line. On the basis of their data they presumed that since *A. caviae* can survive at elevated pH, as found in gastrointestinal tract of formula-fed infants, and because of the adherence and cytotoxin production capabilities of the species, it should be regarded as an enteric pathogen in pediatric patients and most probably among adults as well.

Gosling, *et al.*, (1993) conducted a thorough study to establish the optimum conditions for *Aeromonas* enterotoxin production, and hence detection of enterotoxigenic strains, and to separate and purify enterotoxin and hemolysin to clarify their roles as virulence factors in man.

On the basis of review of literature following three important toxins were found to be reported to be produced by the *Aeromonas* spp.:

Cytotoxin: Which are toxic to CHO cells (Janda, *et al.*, 1985), HELA cells (Kindschuh, *et al.*, 1987), EY-1 adrenal cells (Potomski, *et al.*, 1987). The toxic effects have also been observed on Hep2 cells (Namdari and Bottone, 1990) and Vero cells (Majeed and Macrae, 1994) Cytotoxins generally gave rise to cell damage or death and may produce dysentery like illness, Gracey, *et al.*, (1984) reported that 20% of gastrointestinal infections due to *Aeromonas* spp. are of the dysenteric type. Furthermore, it has been reported repeatedly that the majority of clinical isolates of *Aeromonas* spp. produces cytotoxins (Cumberbatch, *et al.*, 1979; Johnson, *et al.*, 1981; Janda, *et al.*, 1983; Gosting, *et al.*, 1986). Carrello, *et al.*, (1988) studied the ability of *Aeromonas* species to adhere to Hep2 cells An association between diarrhoea and a higher level of adhesion was observed in the majority of the fecal isolates and in none of the environmental isolates.

Heat labile like and Heat stable like enterotoxin (Chopra, *et al.*, 1986, Kirov, *et al.*, 1986; Notermans, *et al.*, 1986).

alpha and Beta hemolysins . Hemagglutinins (Burke, *et al.*, 1984) and the ability to adhere to and invade epithelial cells (Kirov, *et al.*, 1986).

The purpose of the present study was to determine the toxigenic activity of the three toxins (hemolysin, cytotoxin, and enterotoxin) produced by the 12 isolates of three *Aeromonas* spp. (*A. hydrphila, A. sobria, A. caviae*) isolated from clinical and environmental sources from Rawalpindi and Islamabad.

MATERIALS AND METHODS

1. Source and Selection Of Strains of *Aeromonas* spp.:

Twelve pathogenic strains of *Aeromonas* were selected for the present study, out of these 12 strains, eight were from stool samples of diarrhoeal patients, one strain was from stool sample of normal child, and three strains were from water sources including stored water, spring water, and polluted water.

These 12 strains included, *Aeromonas hydrophila* (5 strains) *Aeromonas caviae* (6 strains), and *Aeromonas sobria* (1 strain). (Details of strains as follows)

Strain Nos.	*Species*	*Sources*
1.	Aeromonas hydrphila	Diarrhoeal stool
2.	Aeromonas hydrphila	Diarrhoeal stool
3.	Aeromonas sobria	Diarrhoeal stool
4.	Aeromonas caviae	Diarrhoeal stool
5.	Aeromonas caviae	Diarrhoeal stool
6.	Aeromonas caviae	Diarrhoeal stool
7.	Aeromonas caviae	Normal stool
8.	Aeromonas caviae	Diarrhoeal stool
9.	Aeromonas hydrophila	Diarrhoeal stool
10.	Aeromonas hydrophila	Stored water
11.	Aeromonas hydrophila	Spring water
12.	Aeromonas caviae	Polluted water

Those strains were chosen for the present study because of their pathogenic properties demonstrated with whole life cells in the rabbit ileal loop test.

In the present study the ability of these 12 strains to produced all the three *Aeromonas* exotoxins (i.e. enterotoxins, hemolysins, and cytotoxins) is to be investigated .

2. ***Identification Of Strain***

Identification:

Those 12 strains were identified on the basis of standard morphological, cultural, and biochemical characteristics.

Cultural characteristics.

Primary culturing for the identification of Aeromonas was done on nutrient agar plates, media plates were streaked (which were prepared as follows), and were incubated at 30°C for 24 hours.

Nutrient Agar (Difco) medium was prepared using manufacturer s instructions by suspending 23 gm of dehydrated medium in 1 liter distilled water and heated to dissolve completely. Sterilized in autoclave at 121°C for15 minutes and 15 lb. pressure. The medium was cooled to

50°C and about 20 ml medium was dispensed into pre-sterilized Petri dishes. The dull-white colonies were suspected to be Aeromonas.

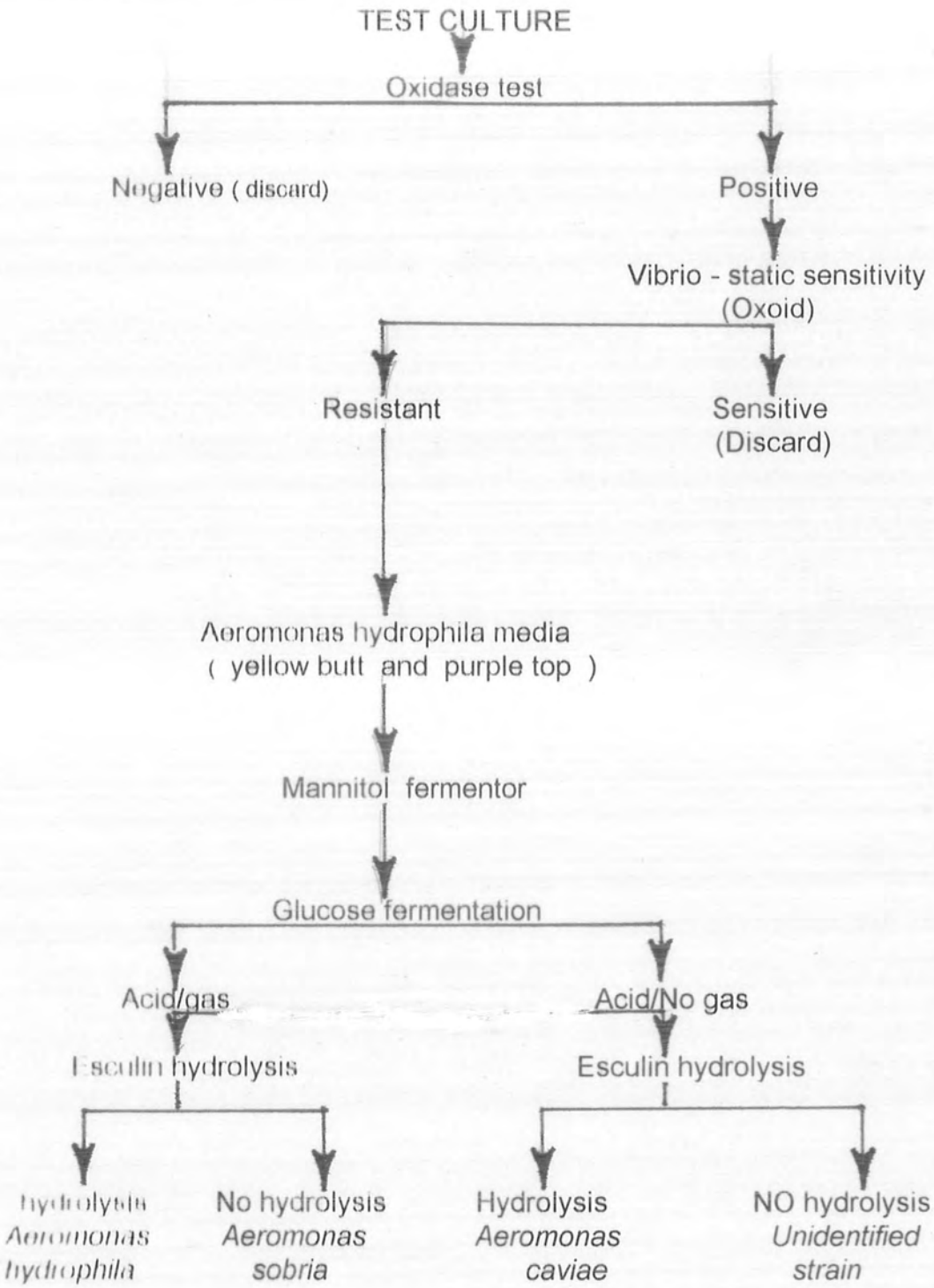
Morphological characteristics.

The morphological characteristics of suspected colonies were studied by Gram staining , the colonies with morphology of G-ve , short , straight rods with rounded ends were Aeromonas.

Biochemical characteristic.

Those colonies were biochemical identified by using the following specification of Popoff (1984).

BIOCHEMICAL IDENTIFICATION SCHEME OF POPOFF



Each isolate was tested for oxidase, catalase, glucose and Mannitol fermentation reactions. Esculin hydrolysis further confirm the identity of isolates on species level.

Oxidase test:

A 1% solution of oxidase reagent was freshly prepared by dissolving 0.05 g of tetramethyl paraphenylene diamine dihydrochloride (Sigma) in 5ml sterilized distilled water. A sterile filter paper was impregnated with 2-3 drops of the oxidase solution and smeared with a heavy suspension of the bacterial growth. Development of purple coloration within 10-20 seconds was a positive test.

Catalase test:

The visible 24 hours growth on nutrient agar was mixed aseptically with a drop of 3% hydrogen peroxide (Merck) over the surface of a clean microscopic slide, using a sterile inoculating loop. The formation of bubble indicates catalase positive reaction, where as no bubble formation was a catalase negative reaction.

Sugar fermentation test.

Peptone water culture medium was used for different sugar fermentation tests. Composition of the medium was:

Peptone(BBL)	10.0g
Sodium chloride	5.0g
Distilled water	1000ml
pH	7.4

Peptone and sodium chloride were dissolved in distilled water and autoclaved at 121°C for 15 minutes Following sugars were used: Esculin, Glucose, Mannitol. 10% solution of each sugar was prepared and filter sterilized.

Phenol red indicator.

Phenol red	0.2 g
Alcohol 95%	500ml
Distilled water	500ml

In each screw capped test tube, 10 ml of peptone water, 1ml of sugar solution and 0.5ml phenol red indicator were mixed. A visible bacterial growth was inoculated into the test tubes and inoculated at 37°C

for 24 to72 hours. Acidification of sugar was confirmed by a yellow coloration in the medium. No color change was a negative reaction.

Esculin hydrolysis.

Esculin Iron Agar (EIA) was used for this test. Composition of the media was as follows;

Esculin Iron Agar

Esculin	1.0 g
Ferric ammonium citrate	0.5 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.1

The ingredients were mixed and boiled to dissolve. After cooling pH was adjusted to 7.1, poured to tubes (3ml / tube) and autoclaved at 121°C-124°C for 15 minutes. The isolates were stab cultured in the tubes using a sterile needle and incubated at 30°C for 24 hours. Appearance of black color around the stab culture line was taken as positive for esculin hydrolysis.

B hemolysis test.

B-hemolysis test was performed on 5%sheep blood agar.

Preparation Of 5% Sheep Blood Agar Plates

For the preparation of blood agar plates, following procedure was used:

Nutrient agar (Difco) 23 g and sodium chloride 7 g were dissolved in one liter of distilled water , and autoclaved at 121°C for 15 minutes. The medium was cooled to 50°C and 50 ml of defibrinated sheep blood was added to the medium aseptically. About 15-20 ml of this final medium was dispensed into the pre-sterilized Petri plates. Plates were checked for sterility by incubation at 37°C for 24 hours before use.

Preparation of Defibrinated Sheep Blood :

Sheep blood was collected aseptically from the jugular vein in a sterile flask containing glass beads. As the blood was poured the flask was swirled in a clockwise direction to avoid clotting.

Prepared blood agar media plates were streaked with pure culture of the isolates and incubated at 30°C for 24 hours, after 24 hours, and checked for B-hemolysis. The clearing

of the media around the colonies was taken as positive test for B- hemolysis.

Maintenance of isolated strains:

Identified strains of *Aeromonas* spp. were stored on nutrient agar plates/slants at 4°C. The cultures were maintained by transferring on fresh nutrient agar plates/slants at 15 days intervals, incubated for 24 hours at 30°C. When colonies appeared the cultures were again transferred to +4°C for storage for another 15 days.

3. ***Preparation of Bacterial Toxin Cell Free Culture Filtrate (CFCF)*** *(Represented by the flow Diagram of CFCF production)*

The Cell Free Culture Filtrate (CFCF) of the isolates positive for fluid accumulation in the rabbit ileal loop was prepared using the method described by Gosling, *et al.,* (1993), and Majeed and Macrae (1994). Toxins were produced and recovered from each of the twelve isolated strains.

The *in vitro* production of CFCF was carried out in 7 steps, which are as under:

Step-1:Preparation Of Bacterial Seed Culture.

For toxin production the seed cultures were prepared from the stored stock cultures. Nutrient agar media plates were streaked for isolation of separate colonies and were incubated at 30°C for 24 hours.1-2 Colonies from nutrient agar plates were picked and sub-cultured on 5% sheep blood agar plates for the enhancement of pathogenicity. Plates were incubated at 30°C for 24 hours.

Step-2:Preparation Of Production Medium .

For the production of toxin, Tryptic Soya Broth (Difco) supplemented with 0.6% w/v yeast extract was used. The medium was formulated according to the manufacturers instructions as under.

30 grams of dehydrated Tryptic Soya Broth medium was dissolved in 1 liter of distilled water, and medium was supplemented with yeast extract 0.6 % w/v, pH was adjusted to 7.4. Then this medium was boiled to dissolve the ingredients, and distributed 10 ml in each of 100 ml Erlenmeyer flask, autoclaved at 121°C for 15 minutes. The sterilized medium was incubated at 37°C to check the sterility before use.

Step-3 Inoculation Of Production Medium

The pre-incubated production medium flasks were labeled for : Date of Inoculation, Strain Number of *Aeromonas*, and the *Aeromonas* culture were inoculated into the respective medium flask under aseptic conditions. The seed cultures had been prepared on 5% sheep blood agar plates. Only 24 hours old colonies were used for inoculation of the toxin production medium. The seeded culture flasks were incubated at 37°C by the following procedure.

Step-4 <u>Cultivation.</u>

After inoculation each flask was kept in shaker incubator for 20-24 hours at 37°C with agitation at 300 oscillations:

Growth Period	20-24h
Growth Temperature	37°C
Rate Of Shaking	300 Oscillation

Step-5 <u>Harvesting And Centrifugation.</u>

Cell free preparations were prepared by centrifuging the 24 hours old cultures medium. For this purpose each of the production Erlenmeyer flask was taken out from the shaker incubator and all the contents from each flask were transferred aseptically in biohazard safety cabinet to the respective centrifuging tube (marked with Strain number and Date). The cells were separated

by centrifugation at 10,000 g, + 4°C for 30 minutes. The tubes were processed as follows.

Step-6 Filtration.

Separation of cells and toxin containing supernatant fluid was performed in multiple steps. After centrifugation the supernatants were separated from sediments by transferring from centrifuging tubes to the sterile test tubes under aseptic conditions. The supernatants were collected and filtered through a 0.2 micron syringe filters (pore size 0.2 micron, Sartorious minisart, gas sterilized) using separate 10 ml disposable pre- sterilized syringes for each filtration. The filtrates were collected in pre-sterilized Nalgene Storage Ampoules.

Step-7 Storage.

The ampoules of each of the *Aeromonas* strain Cell Free Culture Filtrate (CFCF) were stored at -20°C. Samples were tested for biological assays of exotoxins (*Cytotoxin* activity , *enterotoxin* activity , *hemolysin* activity) and biochemical tests such as Protein estimation and SDS-PAGE Gel electrophoresis. The below produced flow diagram is the summary of the steps carried out for the preparation of Cell Free Culture Filtrate (CFCF).

CELL FREE CULTURE FILTRATE PRODUCTION FLOW DIAGRAM

STEP -1 PREPARATION OF BACTERIAL CULTURE	Culture used Aeromonas strains isolated from clinical and environmental samples
	Culture used Aeromonas strains isolated from clinical and. environmental samples
	Colonies of Aeromonas picked from stored nutrient agar plates by sterile wire loop.
	Streaked on 5% sheep blood agar plates. Incubated at 30 °C sheep blood agar plates.
	2-3 separate colonies were picked by sterile wire loop.
STEP -2 PREPARATION OF PRODUCTION MEDIUM	Tryptic Soya broth, supplemented with 0.6% yeast extract sterilized under steam at 121 °C for 15 minutes .
STEP - 3 INOCULATION OF PRODUCTION MEDIUM	The medium (Tryptic Soya broth) were inoculated with Aeromonas colonies picked from 5% blood agar plates.
STEP - 4 CULTIVATION	The flasks were incubated at 37°Cfor 24 hours at 300 rpm in environmental shaking incubator.
STEP - 5 HARVESTING AND CENTRIFUGATION	After 24 hours the medium was transferred to Centrifuge tubes. Centrifuged at 10000 rpm at 4°C for 30 minutes. The supernatants were collected in separate tube and the sediments were discarded.
STEP - 6 FILTRATION	The supernatant (Cell free culture filtrate-(CFCF) were filtered through syringe filters (0.2 um)
STEP -7 STORAGE	Stored at -20 °C .

4. *Biochemical Analytical Procedures*

Protein Estimation (By Lowry' s Method)

The protein present in the CFCF were estimated by the method of Lowry, *et al.,* (1951). In this method proteins were treated with copper in alkaline medium and then reacted with folin - phenol reagent. The final color was a result of birut reaction of proteins with copper ions in the alkaline medium and reduction of phosphomolybdic-phosphotungstic reagent by tyrosine and tryptophan present in the sample proteins. The readings of optical density were taken with a spectrophotometer Spectronic -20. The reagents used were as follows :

Reagent A

Sodium Carbonate	2 g
Sodium Hydroxide (0.1 N)	0.4 g
Sodium Potassium tartarate	1 g
Distilled Water	100 ml

Reagent B

Copper sulphate	0.5 g
Distilled water	100 ml

Reagent C

Solution -A	50 ml
Solution -B	1 ml

Reagent D

Folin phenol with distilled water	1:1

Standard. (Bovine Serum Albumin, BSA) :

Bovine Serum Albumin	0.5g
Distilled Water	50 ml

Procedure for protein estimation:

Preparation of CFCF samples.

Cell Free Culture Filtrate of each strain was diluted as 100 ul in 400 ul of distilled water to obtained 1:5 dilution. 0.1 ml (100 ul) of each diluted sample was taken in duplicate in their respective test tubes for protein estimation, the number of samples, respective tube number and sample volume are shown in Table - 1.

Preparation of Bovine serum albumin standard

A stock solution of Bovine serum albumin was prepared by dissolving 0.05g of Bovine serum albumin, in 50ml distilled water to obtain 1mg/ml concentration of Bovine serum albumin. Different volumes of bovine serum albumin stock solution were taken in duplex of test tubes to obtain different concentrations of BSA for the preparation of BSA standard curve as shown in Table-2.

Procedure For Protein Estimation.

Samples and the Standard were processed at the same time by the following procedure. The volume of each tube of sample and standard except blank (Tube Numbers 21 & 22,) was made to 1 ml by adding required amount of distilled water. 1 ml Solution-C was added, shaken well and kept at room temperature for 10 minutes. Then 0.1 ml of 1:1 diluted folin phenol reagent was added to each tube, mixed well, and kept for 30 minutes at room temperature The optical density (O.D) was measured at 650 nm on spectrophotometer Spectronic 20. A graph of standard curve of BSA was made by taking the concentration of BSA

against their optical density .The protein concentration of each CFCF was determined by using this standard curve.

Table - 1. Preparation Of CFCF Sample For Protein Estimation, Sample Numbers ,Test Tube Numbers And Sample Volumes.

Sample Nos.	Test Tubes Nos.	Volumes
1.	1 - 2	100ul
2.	3 - 4	100ul
3.	5 - 6	100ul
4.	7 - 8	100ul
5.	9 - 10	100ul
6.	11 - 12	100ul
7.	13 - 14	100ul
8.	15 - 16	100ul
9.	17 - 18	100ul
10.	19 - 20	100ul
11.	21 - 22	100ul
12.	23 - 24	100ul

Table - 2. Bovine Serum Albumin Concentrations For BSA Standard Curve

S. Nos.	Tubes Nos.	BSA Concentrations
1.	1 - 2	60ul
2.	3 - 4	80ul
3.	5 - 6	100ul
4.	7 - 8	120ul
5.	9 - 10	140ul
6.	11-12	160ul
7.	13-14	180ul
8.	15-16	200ul
9.	17-18	220ul
10.	19-20	240ul
11.	21-22	Blank

Gel Electrophoresis (SDS - PAGE)

Protein fraction of *Aeromonas* toxins was analyzed by a modified method of the SDS - PAGE technique of Laemmli (1970) electrophoresis was performed in vertical slab gel apparatus (Bio-Rad mini protein - 2).

Resolving and Stacking Gels were prepared according to the recipe given as follows.

Composition Of Resolving And Stacking Gels For SDS-PAGE

Resolving Gel (12.5 %).

H_2O	3.98 ml
3 M Tris - HCl (pH - 8.8)	1.25 ml
10 % Ammonium persulphate	0.1 ml
30% acrylamide mixture*	4.17 ml
TEMED	0.005 ml
Total volume	9.505 ml

* Acrylamide with bis-acrylamide in a ratio of 30:0.8.

Preparation Of Resolving Gel Buffer Stock. 3M Tris HCl pH (8.8).

36.5 g Tris and 48 ml 1 N HCl were mixed and brought to 100 ml final volume with distilled water. This buffer was than filtered through Watman No. 1 filter paper and store at 4°C.

Stacking Gel (4.5%)

H_2O	2.7 ml
3 M Tris-HCl (pH - 6.8)	1.25 ml
10% SDS	0.05 ml
10 % Ammonium persulphate	0.5 ml
30% Acrylamide mixture*	0.75 ml
TEMED	0.005 ml
Total Volume	5.255 ml

* Acrylamide with bis-acrylamide in a ratio of 30:0.8.

Preparation Of Stacking Gel Buffer Stock 3M Tris-HCl , pH (6.8).

36.5 g Tris was dissolved in 40 ml H_2O titrated to pH 6.8 with 1M HCl (~48ml) and brought up to 100 ml final volume

with distilled water. The solution was filtered through Watman No 1 filter paper and stored at 4 °C .

Procedure of Gel Electrophoresis:

Resolving Gel was carefully poured between plates and over laid with 1:1 mixture of water and isopropanol to avoid direct air contact and for smooth polymerization. After 20 - 25 minutes of polymerization the water-isopropanol mixture was drained and the surface washed with distilled water. The Stacking Gel was then poured and Teflon comb was inserted to create wells. After polymerization of Stacking Gel the comb was removed and the wells were flooded with Reservoir Buffer to remove the traces of unpolimerized acrylamide .

Composition And Preparation Method Of Reservoir Buffer. pH 8.3 for 1000 ml

Tris	30.075 gm
Glycine	144 g
SDS	10 gm

30.75 gm Tris, 144.0 glycerin, 10.0 gm SDS were dissolved in distilled water and made to 1 liter. This buffer was than filtered through Whitman No 1 filter paper and stored at 4°C.

Preparation Of Samples.

Samples were prepared for electrophoresis by mixing an equal volume of samples buffer and 2 - 4 ul of B - mercaptoethanol was added to each protein sample. The samples were boiled for 2 - 3 minutes , cooled immediately on ice for 55 minutes and then 12 ul of each sample was applied separately into the wells.

Sample buffer pH 6.8;

Tris	62.5 mM
Glycine	2%
SDS	10 %
Bromophenol blue	0.001%

The proteins were stacked and resolved in 4.5% and 12.5 % polyacrylamide gels respectively. The following standard protein markers were used in SDS - PAGE as molecular weight markers:

1. Ribose	13 kd
2. Lactalbumin	14 kd
3. Trypsinogen	24 kd

4.	Carbonic anhydride	29 kd
5.	Pepsin	35 kd
6.	Egg albumin	45 kd
7.	Bovine serum albumin	66 kd
8.	Transferrin	78 kd

A constant voltage of 80 V was applied in the stacking proteins until the bromophenol blue marker had reached the bottom and 125 V in resolving portion. Gels were fixed in fixative solution for one hour, stained in the staining reagent for 2 hours and de-stained for 3 - 4 hours at 50°C in the De-staining Reagent. These reagents were prepared as follows. The gel bands were analyzed and band size, band numbers were recorded.

Composition and Preparation Method of Fixative Solution for 500 ml

Methanol	200 ml
Glacial acetic acid	35 ml
Water	265 ml

200 ml methanol and 35 ml glacial acetic acid were mixed with 265 ml distilled water.

Composition and Preparation method of Staining reagents. For 500 ml

Methanol	200 ml
Glacial acetic acid	35 ml
Water	265 ml
Coomassie brilliant blue (G-250)	1.25 gm

1.25 gm of Coomassie brilliant blue - R 250 was dissolved in 500 ml of solution D above.

Composition and Preparation Method of De-staining Reagents, For 1000 ml

Methanol	50 ml
Glacial acetic acid	75 ml
Water	875 ml

50 ml methanol and 75 ml glacial acetic acid was mixed in 875 ml of distilled water.

5. ***BIOLOGICAL ASSAYS.***

HEMOLYSIN ASSAY

The presence of hemolysin activity in the CFCF were analyzed according to the method proposed by Gosling *et al.*, (1993). For this purpose dilution's of Cell Free Culture Filtrate were made in phosphate buffer saline and tested on rabbit erythrocytes.

Preparation of Phosphate buffer saline

Sodium Chloride	8.0 g
Dipotassium hydrogen phosphate	1.21 g
Potassium dihydrogen phosphate	0.34 g
Distilled water	1000 ml
pH	7.3

Preparation of Red Blood Cells.

The rabbit erythrocyte were prepared for the detection of hemolysin activity in the Cell Free Culture Filtrate as follows:

Collection of Defibrinated Rabbit blood:

Rabbit blood was collected aseptically from the jugular vein in a sterile flask containing sterile glass

beads. As the blood was collected the flask was swirled in a clockwise direction to avoid clotting.

Washing of Rabbit Erythrocytes:

Defibrillated rabbit blood was taken in the test tube and diluted with 5 ml saline (0.9% sodium chloride) and centrifuged at moderate speed just long enough to deposit the cells, the supernatant was discarded and the cells were re-suspended in 5 ml saline, and were again centrifuged, this process was repeated 5 times.

Preparation of 1% suspension of rabbit RBC cells:

Rabbit erythrocytes (freshly washed) 1 ml

Normal saline

Preparation of samples : 2 fold dilution (dilution factor: 2)

Doubling dilution of the CFCF in phosphate buffer saline pH 7.4 was made in microtitre plates (ICN-Flow). For this purpose 100 ul phosphate beffer saline was taken in the first row of microtiter plate having 12 wells for one

sample dilution. Then 100ul of sample was diluted in series for the preparation of two fold serial dilutions. Table- 3. Same serial dilutions were made for each sample n the same plate having 96 - wells. Two plates were used for all the 12 CFCF samples. Table -4a and 4b.

Procedure for hemolysin test:

Serial two fold dilutions of the CFCF in phosphate buffer saline pH 7.4 were made in microtitre plates (ICN Flow) and an equal volume (100 ul) of a 1% suspension of Rabbit RBCs was added. Phosphate buffer saline and broth blank were made in each tray. Trays were sealed with plastic tape and incubated for 1 hour at 37°C and then for 1 hour at 4°C. The haemolysis titer was recorded as the highest dilution giving complete haemolysis and it was taken as the end point.

Table- 3. Sample Dilutions Preparation Scheme for Haemolysin Assay In Microtiter Plate.

S.No.	Dilutions	Volumes Of Diluent	Volumes Of Sample
1.	1 : 2	100ul	100ul
2.	1 : 4	100ul	100ul
3.	1: 8	100ul	100ul
4.	1: 16	100ul	100ul
5.	1 : 32	100ul	100ul
6.	1 : 64	100ul	100ul
7.	1 : 128	100ul	100ul
8.	1 : 256	100ul	100ul
9.	1 : 512	100ul	100ul
10.	1 : 1024	100ul	100ul
11.	1 : 2048	100ul	100ul
12.	1 : 4096	100ul	100ul

Table- 4. Serial Two Fold Dilutions of CFCF for Haemolysin Assay in Microtitre Plate.

a: Pattern Of Sample Dilution In Microtiter Plate (1)

Well nos.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
Sample nos.												
1.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
2.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
3.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
4.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
5.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
7.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Blank	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096

b: Pattern Of Sample Dilution In Microtiter Plate (2)

Well nos.	1	2	3	4	5	6	7	8	9	10	11	12
Sample nos.												
8.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
9.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
10.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
11.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
12.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Blanks	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096

CYTOTOXIN ACTIVITY IN Hep 2 , MRC-5 AND Vero CELLS.

Cytotoxin activity of the CFCF was studied in separate microtitre plate cultures of Hep 2 cells, MRC-5 and Vero cells. For this purpose 25 cm^2 bottles (prepared by the procedure mentioned below were trypsinised with trypsin-EDTA solution (prepared as mentioned below. The trypsinised cells were suspended in Minimum Essential Medium (MEM medium) with 10% fetal calf serum (prepared by the procedure described under. The cell suspension was distributed in 96 well microtitre plates and the top was sealed with a plastic tape. The trays were incubated at 37°C for 24 hours. After which they were taken out and cells were inoculated with toxin .

Preparation of 25cm2 culture flask.

Cultures of Hep-2, MRC-5, and VERO cells were obtained from NIH. For each cell type a 25 cm^2 plastic pre-sterilized disposable flask was inoculated with 2ml of cell seed, and 50 ml of medium. All the flask were incubated at 37°C for seven days. The monolayer completion was observed under the inverted microscope. Completed monolayers were trypsinised for further inoculation in either microtitre plates or a subculture in more 25cm^2 flasks for continuous culture for future use.

Subculture of Hep-2, MRC-5 and Vero cells and Preparation of Microtitre plates.

1. The supernatant fluid from 7-days old cell culture bottle cultures were discarded.
2. 2ml trypsin-versene solution, pre-warmed at 37°C was added to each 25 cm^2 cell culture bottle.
3. These flasks were incubated at 37°Cfor 30 -60 seconds.
4. The bottles were turned gently over without disturbing the cell sheet; incubated for an additional 2-3 minutes at 37°C.
5. Carefully decanted the trypsin-versene solution and 2 ml pre-warmed MEM growth medium containing 10% fetal calf serum was added to suspend the cells.
6. The cell clumps were broken down by force of pipetting.
7. A 1 : 4 dilution of suspension was prepared.
8. Seeded the microtitre plates with the cells.
9. The plates were sealed and incubated for 48 hours.
10. The cells were maintained with MEM containing 2% fetal calf serum.

Cytotoxin activity Assay

Each cell monolayer type prepared as above were inoculated with CFCF's following dilution in MEM 2% ½ , 1/5, 1/10, 1/20. Was added to each well.

1. The following negative controls were used:

   i) Tryptic Soya broth supplemented with 0.6% w/v yeast extract, which was used for the preparation of CFCF.

   ii) MEM with 2% fetal calf serum

3. The plates were covered with plastic cover and then incubated at 37°C .

4. *Reading of Plates*:

   Plates were read after 5 hours and 24 hours of incubation. The readings were taken in the following manner.

Non toxic	-----	--
Slight toxic	-----	+
Weak toxic	------	++
Strong toxic	------	+++
Highly toxic	------	++++

Preparation of trypsin-EDTA solution

Sterile Trypsin-EDTA working solution was prepared as follows.

Phosphate -buffered saline (PBS).

Solution -A . (1 -liter)

NaCl	8.0	g
KCl	0.2	g
Cacl2. 2H2O	0.132	g
MgCl2 . 6H2O	0.1	g
Distilled water	800	ml

Solution -B (1 -liter)

NaHPO4	1.15	g
KH2 PO4	0.2	g
Distilled water	200	ml

1. Each salt was dissolved in de-ionized water in order listed below.
2. Solution-A and Solution-B were separately autoclaved at 15 lb. psi. for 15 minutes.
3. Solutions A and B were mixed after cooling ; Solution B was poured into Solution A, stirred slowly ; final pH - 7.0.

4. Dispensed in 500 -1000 ml amounts into sterile bottles.
5. Cultured 1-5 ml in thioglycollate broth for sterility test.
6. Stored in refrigerator.

Trypsin Solution In PBS 2.5%

PBS	-	1000 ml
Trypsin (1:300)Difco	-	25 gm

1. Trypsin was transferred to PBS and shaken to dissolve.
2. Filtered through 0.2 micron cellulose nitrate filter membranes.
3. Dispensed in 25-50 ml amounts into sterilized bottles.
4. Cultured 1-5 ml in thioglycollate broth for sterility test.
5. Stored at -20°C.

Versene Solution (0.2%)

NaCl	-	8gm
KCl	-	0.2 gm
KH2 PO4	-	0.2 gm
Na2 HPO4	-	1.15gm
Di-sodium ethylene diamine-tetraacetate (EDTA)		0.2 g
Distilled water	-	1000 ml

Each salt was separately weighed and dissolved in sequence.

Dispensed in 50 ml amounts.

Autoclaved at 15 lb. psi. for 15 minutes.

Trypsin-Versene Solution

Trypsin (2.5%)	-	5ml
Versene (0.2%)	-	45 ml

Trypsin-Versin were mixed using sterile procedures. Before use the mixture was warmed at 37°C in a water bath.

Preparation of Cell Culture Medium MEM.

MEM was prepared by emptying the packets containing pre-weighed dry powdered medium enough to make one liter of liquid medium. 2mM of Glutamine was added to the medium. Fetal calf serum was added to make the final concentration of10%. (2% when maintenance medium was prepared). pH was adjusted to 6.8.The medium was sterilized through a pre-sterilized 0.2 micron filter assembly. Sterile medium was dispensed in sterile bottles of 100 ml capacity. Medium was stored at +4°C. Sample of the medium was tested in thioglycollate broth for sterility.

ENTEROTOXIN ASSAY BY SUCKLING MOUSE TEST

Enterotoxic activity in CFCF samples was determined in infant (suckling mice) using the method of Gosling, *et al.*, (1993) and Burke, *et al.*, (1981). Suckling mouse test was performed on all the 12 samples of CFCF. An intestinal Weight/body weight (Iw/Bw) ratio of greater or equal to 0.08 was the criteria for positive results.

Three mice were used for each test , one mouse unit was defined as a minimum amount of protein that caused lw/Bw ratio greater or equal to 0.08.

Procedure:

Cell free culture filtrate 0.1 ml to which 0.02 % (w/w) trypan blue solution has been added (10 ul in 1.0 ml CFCF) were inoculated by Intraperitonial . injection using 1 ml syringes. Three Swiss albino mice (2 to 6 days old) were inoculated with each sample. Mice were then placed on a sheet of blotting paper in a partial metal tray with not more than three mice in each compartment, and incubated at 28°C for three hours.

After incubation mice were killed by cervical dislocation and the presence of dye in the small intestine and the degree of intestinal extension were recorded before the removal of the entire gut.

The entire intestinal tract of each infant mouse was than removed. Intestinal weight (lw) and remaining body weight (Bw) for each group was recorded and ratio was worked out

The Iw and Bw ratio were calculated and scored on an arbitrary scale from 0+ to 4+ as follows:

less than	0.070	= 0
	0.070 to 0.079	= 1 +
	0.080 to 0.089	= 2 +
	0.090 to 0.099	= 3 +
greater than	0.100	= 4 +

RESULTS

The purpose of this study was to determine the toxigenic activity of the toxins produced by the 12 *Aeromonas* strains isolated from different sources at Rawalpindi / Islamabad. These isolates were identified on the bases of morphological, cultural, and biochemical characteristics.

MORPHOLOGICAL CHARACTERISTICS.

All the isolates were found to be G-ve, short and straight rods, with rounded ends. They were found in singles, pairs, and also in short chains.

CULTURAL CHARACTERISTICS.

The colonies of all the isolates on nutrient agar plates were found circular, smooth, convex, dull-white after 24 hours of incubation . On blood agar plates all the isolates were found to produce a clear zone of hemolysis. The optimum temperature of incubation was 22-28°C.

BIOCHEMICAL CHARACTERISTICS.

\All the strains were found to be oxidase +ve, catalase +ve, and beta- hemolytic. In sugar fermentation reactions Mannitol and glucose fermentation were found to be +ve

with acid and gas production (group I), a few of the isolates were found to be negative for gas production during glucose fermentation (group II).

Esculin hydrolysis capability of both the groups was checked and was seen that the group-I (gas + acid, during glucose fermentation) hydrolyzed the Esculin, while the group-II (acid with no gas production during glucose fermentation) did not hydrolyzed the Esculin (Table -5). On the basis of glucose fermentation and Esculin hydrolysis these isolates were classified on species level.

CHARACTERIZATION OF AEROMONAS TOXINS PRODUCED AS CELL FREE CULTURE FILTRATE (CFCF).

All the12 strains were used for toxin production separately. The toxin was harvested and after centrifugation and filtration Cell Free Culture Filtrate (CFCF) was produced and tested for biochemical and biological characteristics . The results are given below:

BIOCHEMICAL ASSAYS

PROTEIN ESTIMATION

The protein present in CFCF were estimated by Lowry's method using BSA standard curve (Figure -I). To obtain the standard BSA curve, different concentrations of Bovine Serum Albumin were used, and optical density was read at 650 nm on Spectronic 20 (Table - 6). A straight line of standard curve was obtained by using different concentrations of bovine serum albumin against the optical densities of these BSA concentrations. (Figure. 1).

The protein concentration in all CFCF samples was calculated by using the BSA straight line as standard curve (Figures. 2-13). The presence of protein was established in all the CFCF samples. There was no relationship between the concentration of proteins in CFCF of all *Aeromonas* strains, species and sources from where theses *Aeromonas* strains were isolated. The protein concentration of each CFCF sample of *Aeromonas* strain numbers, species, and optical densities of all CFCF samples are shown in (Table-7). The protein concentration range in all CFCF solutions, irrespective of species and sources was 144.518 - 236.0768 ug/ml. In CFCF of *Aeromonas hydrophila* (strains 1, 2, 9, 10, and 11) the protein estimate ranges between 171.98 - 198.433 ug/ ml , in *Aeromonas*

caviae strains 4, 5, 6, 7, 8, and 12 the protein estimation range was 161.086- 236. 0768 ug/ml and in *Aeromonas sobria* strain-3 it was 144.5108ug/ml. The maximum protein concentration was found in CFCF of strain number 7 and strain number 8 , both were *Aeromonas caviae*. The strain number 7, of *Aeromonas caviae* was isolated from stool sample of normal one, while the strain number 8 , *Aeromonas caviae* was isolated from a stool sample of a diarrhoeal patient .The minimum protein concentration was found in CFCF of strain number 3, which was *Aeromonas sobria*, isolated from a stool of diarrhoeal patient.

GEL ELECTROPHORESIS

Protoins bands were observed within molecular weight range 15 kda (enterotoxin), and 55 kda (hemolysin), along with many other unknown protein bands, when tested against known molecular weight markers.

BIOLOGICAL ASSAYS.

HEMOLYSIN ASSAY .

All the strains of *Aeromonas hydrophila, Aeromonas sobria, Aeromonas caviae* were found hemolytic, the hemolysin titer in CFCF of all strains was determined by method of Gosling, *et al.*, (1993).

The hemolysin titer was recorded as the highest dilution giving complete hemolysis (Figure-14) as the end point as HU/ ml (hemolytic unit/ ml) (Figure-15) and the range of hemolysin titer in the microtiter plates for *Aeromonas hydrophila* was (32-1025 HU/ml), for *Aeromonas sobria* the titer was (16 HU/ml) and for *Aeromonas caviae* it was (2-256HU/ml).

The highest amount of hemolysin titer was observed in the CFCF of *Aeromonas hydrophila* strain 9 isolated from the stool of diarrhoeal patient, but no relationship between the hemolysin production, sources of organism and species were recorded. The hemolysin titers of CFCF of all *Aeromonas* strains with strain numbers, species, and sources of organisms has been presented in Table-8 .

CYTOTOXIC ACTIVITY ASSAY

The cytopathic effects were checked on HEP-2 cell monolayer, on MRC-5 cell monolayer and Vero cell monolayer.

Cytopathogenic Effects On Hep-2 Cell Monolayer

The cytopathogenicity of toxins isolated in the form of CFCF from *Aeromonas species* were determined on the cultured HEP-2 cells monolayer. It was recorded that all the clinical and environmental isolates produced cytotoxin as evidenced by HEP-2 cell monolayer detachment, rounding, and loss of viability (Figures. 16a, 16b, 16c, and 16d), and (Figures. 17a, 17b, 17c, and 17d)

By using 1:2 dilution of CFCF, cytotoxicity for HEP-2 cells were evident as early as 30 minutes post-exposure and increased with incubation time up to 5 hours. All the CFCF caused rounding and detachment of cells except the strain 4 and 8 which exhibited low cytotoxicity.

Table-9, Shows observation after 5 hours incubation which maximized at 24 hours. Table- 10, Shows observation after 24 hours incubation. The observations are presented with following notations.

Non toxic — –

Slight toxic	—	+
Weak toxic	—	++
Strong toxic	—	+++
Highly toxic	—	++++

By using 1: 5 dilutions of the culture filtrate it was found that within 5 hour incubation, all the strains did produced cytotoxicity for Hep -2 cells, which maximized with in 24 hours except the strain nos. 4, 8 and 3. Strain number 3 slight toxic and strain no. 8 weak toxic within 24 hours incubation, while the strain no. 4 *(Aeromonas caviae)* isolated from stool of diarrheal patient was found -ve after 24 hour incubation, the results were presented in the Table-9 and Table- 10. By using 1:10 dilution of the CFCF it was observed that weak toxicity were observed as compared to 1:5 dilutions, results are presented in Table- 9 and Table - 10.

Cytopathogenic Effects On MRC - 5 Cell Monolayer.

Cytopathic effects of CFCF on MRC - 5 cells was studied using all the 12 strains. The cells showed dying by elongation and detachment from the surface .The cytotoxicity by all the CFCF samples was also same as in case of Hep-2 cell monolayer. Table- 11 and Table- 12.

Cytopathogenic Effect On Vero Cell Monolayer.

Cytopathic effects of CFCF on Vero cell line were also similar in their manifestation i.e., detachment , rounding , and loss of viability. Table -13 and Table- 14.

ENTEROTOXIGENIC ACTIVITY ASSAY .
(In Suckling mice)

The Enterotoxigenic activities were demonstrated by suckling mouse gastric distention and intestinal fluid accumulation, (Figure-18 and 19). It was found that only few strains were enterotoxigenic as determined by the ratio of intestinal weight to body weight. The results were presented in Table-15.

Enteropathogenicity of the isolates based on the sources of isolation and spp variation has been presented in Table- 16. It was found that 33% (1 out of 3) of Aeromonas hydrophila isolates from the stool of diarrheal patient were enterotoxigenic, while 66% (2 out of 3) were not enterotoxigenic.

The strains of *Aeromonas hydrophila* isolated from environmental sources were found to be 100% (2 out of 2) enterotoxigenic. Only one strain of *Aeromonas sobria* isolated from the stool of a diarrhoal patient was tested and found to be non- enterotoxigenic.

Three out of four (75%) of *Aeromonas caviae* isolated from the stool of diarrheal patient were enterotoxigenic. While 25% (1 out of 4) were non- enterotoxigenic. The *Aeromonas caviae* isolated from the stool of normal person has found to be enterotoxigenic. While the strain isolated from environmental sources were non-enterotoxigenic.

Table - 5. Biochemical Characteristics of Aeromonas Species.

CHARACTERISTICS	*Aeromonas caviae*	*Aeromonas hydrophila*	*Aeromonas sobariae*
oxidase test	+	+	+
B-Hemolysis	+	+	+
Catalase test	+	+	+
Mannitol fermentation	+	+	+
Acid/Gas from Glucose fermentation	+/-	+/+	+/+
Esculin Hydrolysis	+	+	-

Table- 6. Bovine Serum Albumin Concentrations and Optical Densities For Standard Curve.

Sample Nos.	Volumes/ Concentration of BSA(ul)	Optical Density
1	60	0.08
2	80	0.11
3	100	0.129
4	120	0.14
5	140	0.16
6	160	0.18
7	180	0.20
8	200	0.22
9	220	0.24
10	240	0.265

Table -7. ***Protein Concentrations In CFCF Of Aeromonas Strains With Species and Sources of Isolation.***

Strain . Nos.	Species	Sources	Optical. density	Concentration Of Protein. (ug/ml)
1	*A. hydrophila*	Diarrhoeal stool	0.194	171.9806
2	*A. hydrophila*	Diarrhoeal stool	0.214	192.3286
3	*A. sobria*	Diarrhoeal stool	0.167	144.5108
4	*A. caviae*	Diarrhoeal stool	0.20	178.085
5	*A. caviae*	Diarrhoeal stool	0.211	189.2764
6	*A. caviae*	Diarrhoeal stool	0.205	183.172
7	*A. caviae*	Normal stool	0.257	236.0768
8	*A. caviae*	Diarrhoeal stool	0.254	233.0246
9	*A. hydrophila*	Diarrhoeal stool	0.22	198.433
10	*A. hydrophila*	Stored water	0.20	178.085
11	*A. hydrophila*	Spring water	0.196	174.0154
12	*A. caviae*	Polluted water	0.186	161.8066

Table -8. ***Hemolysin Titer In CFCF Of Aeromonas Strains Isolated From Various Sources with Species Specificity.***

Strain nos.	Aeromonas species	Sources	Hemolysin titers (HU/ml)
1.	*A. hydrophila*	Diarrhoeal stool	64
2.	*A. hydrophila*	Diarrhoeal stool	64
3.	*A. sobria*	Diarrhoeal stool	16
4.	*A. caviae*	Diarrhoeal stool	16
5.	*A. caviae*	Diarrhoeal stool	256
6.	*A. caviae*	Diarrhoeal stool	128
7.	*A. caviae*	Normal stool	256
8.	*A. caviae*	Diarrhoeal stool	2
9.	*A. hydrophila*	Diarrhoeal stool	1024
10.	*A. hydrophila*	Stored water	128
11.	*A. Hydrophila*	Spring water	32
12..	*A. caviae*	Polluted water	64

Table-9. Cytopathogenic Effect Of CFCF On Hep-2 Cell Monolayer .
(After 5 Hours Incubation)

Con/ Strain Nos.	1	2	3	4	5	6	7	8	9	10	11	12
1:2	+++	+++	+++	++	++++	+++	++++	+	++++	+++	+++	+++
1:5	++	+++	--	--	++++	+++	++++	--	+++	+++	++	+++
1:10	+	++	--	--	++++	--	++++	--	+++	+++	+	++
C	--	--	--	--	--	--	--	--	--	--	--	--

KEY =

Non toxic	—	–
Slight toxic	—	+
Weak toxic	—	++
Strong toxic	—	+++
Highly toxic	—	++++

Con = Concentration.
Nos. = numbers.
C = Control.

Table-10. Cytopathogenic Effect Of CFCF On Hep- 2 Cell Monolayer .
(After 24 Hours Incubation)

Con/ Strain Nos.	1	2	3	4	5	6	7	8	9	10	11	12
1: 2	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
1: 5	++++	++++	++	--	++++	++++	++++	+	++++	++++	+++	++++
1: 10	++++	++++	+	--	++++	++++	++++	--	++++	++++	++++	++++
C	--	--	--	--	--	--	--	--	--	--	--	--

KEY =

Non toxic	—	–
Slight toxic	—	+
Weak toxic	—	++
Strong toxic	—	+++
Highly toxic	—	++++

Con = Concentration.
Nos. = numbers.
C = Control.

Table-11. Cytopathogenic Effect Of CFCF On MRC-5 Cell Monolayer .
(After 5 Hours Incubation)

Con/ Strain Nos.	1	2	3	4	5	6	7	8	9	10	11	12
1:2	+++	+++	+++	++	++++	+++	++++	+	++++	+++	+++	+++
1:5	++	+++	--	--	++++	+++	++++	--	+++	+++	++	+++
1:10	+	++	--	--	++++	--	++++	--	+++	+++	+	++
C	--	--	--	--	--	--	--	--	--	--	--	--

KEY =

Non toxic	—	–
Slight toxic	—	+
Weak toxic	—	++
Strong toxic	—	+++
Highly toxic	—	++++

Con = Concentration.
Nos. = numbers.
C = Control.

Table-12. <u>Cytopathogenic Effect Of CFCF On MRC-5 Cell Monolayer</u> .
(After 24 Hours Incubation)

Con/ Strain Nos.	1	2	3	4	5	6	7	8	9	10	11	12
1: 2	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
1: 5	++++	++++	++	--	++++	++++	++++	+	++++	++++	+++	++++
1: 10	++++	++++	+	--	++++	++++	++++	--	++++	++++	++++	++++
C	--	--	--	--	--	--	--	--	--	--	--	--

KEY =

Non toxic	—	–
Slight toxic	—	+
Weak toxic	—	++
Strong toxic	—	+++
Highly toxic	—	++++

Con = Concentration.
Nos. = numbers.
C = Control.

Table-13. <u>Cytopathogenic Effect Of CFCF On Vero Cell Monolayer</u> .
(After 5 Hours Incubation)

Con/ Strain Nos.	1	2	3	4	5	6	7	8	9	10	11	12
1:2	+++	+++	+++	++	++++	+++	++++	+	++++	+++	+++	+++
1:5	++	+++	--	--	++++	+++	++++	--	+++	+++	++	+++
1:10	+	++	--	--	++++	--	++++	--	+++	+++	+	++
C	--	--	--	--	--	--	--	--	--	--	--	--

KEY =

Non toxic	—	–
Slight toxic	—	+
Weak toxic	—	++
Strong toxic	—	+++
Highly toxic	—	++++

Con = Concentration.
Nos. = numbers.
C = Control.

Table-14. Cytopathogenic Effect Of CFCF On Vero Cell Monolayer.
(After 24 Hours Incubation)

Con/ Strain Nos.	1	2	3	4	5	6	7	8	9	10	11	12
1: 2	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
1: 5	++++	++++	++	--	++++	++++	++++	+	++++	++++	+++	++++
1: 10	++++	++++	+	--	++++	++++	++++	--	++++	++++	++++	++++
C	--	--	--	--	--	--	--	--	--	--	--	--

KEY =

Non toxic	—	–
Slight toxic	—	+
Weak toxic	—	++
Strong toxic	—	+++
Highly toxic	—	++++

Con = Concentration.
Nos. = numbers.
C = Control.

Table- 15. Enterotoxigenic Activity with IW, BW, IW/BW Ratio.

Strain Nos.	Intestinal weight	Body weight	Fluid accumulation ratio(IW/BW)	Results
1	0.21, 0.08, 0.19	3.53, 1.49, 3.2	0.059, 0.053, 0.59.	0, 0, 0.
2	0.27, 0.1, 0.24	3.86, 1.64, 3.5.	0.0699, 0.060, 0.68.	0, 0, 0.
3	0.18, 0.08 , 0.21	3.38, 1.49, 3.7.	0.053, 0.053, 0.056.	0, 0, 0.
4	0.21, 0.10, 0.16.	2.90, 1.38, 2.2.	0.072, 0.072, 0.072.	+, +, +.
5	0.24, 0.1, 0.21.	2.97, 1.28, 2.6.	0.08, 0.078, 0.08, 0.08	++, +, ++.
6	0.2, 0.12, 0.16.	2.65, 1.7, 2.2.	0.075, 0.07, 0.072.	+, +, +.
7.	0.28, 0.1, 0.22.	3.97, 1.31, 3.1.	0.07, 0.076 , 0.07.	+, +, +.
8.	0.16, 0.08, 0.2.	2.48, 1.43, 3.0.	0.064, 0.05, 0.66.	0, 0, 0.
9.	0.19, 0.1, 0.15.	2.56, 1.29, 2.05.	0.074, 0.077, 0.073.	+, +, +.
10.	0.27, 0.13, 0.16.	2.71, 1.50, 1.8.	0.099, 0.086, 0.088	+++, ++, ++.
11.	0.43, 0.34, 0.29.	3.48, 2.71, 2.8.	0.123, 0.125, 0.103.	++++, ++++, ++++
12.	0.23, 0.11, 0.2.	2.50, 1.22, 2.23.	0.09, 0.090, 0.089.	+++, +++, ++
Blank	0.08, 0.06, 0.06	1.57, 1.63, 1.55	0.05, 0.036, 0.038	0, 0, 0,

Key =

IW/BW ratio = 0.070 ---- 0.

= 0.070 - 0.079 ---- +

= 0.080 - 0.089 ---- + +

= 0.09 - 0.099 ---- + + +

= 0.1 ---- ++++

IW = Intestinal weight.

Bw = Body weight

IW/BW = Ratio

Table- 16. ***Enterotoxigenicity, Species and Sources Of Isolation Of Strains.***

Sources	*Aeromonas hydrophila*		*Aeromonas sobria*		*Aeromonas caviae*	
	Ent +	*Ent -*	*Ent +*	*Ent -*	*Ent +*	*Ent -*
Diarrheal stool	1 /3	2/3	0 /1	1/1	3/4	¼
Normal stool	0/0	0/0	0/0	0/0	1/1	0/1
Water	2/2	0/2	0/0	0/0	0/1	1/1

KEY =

Ent + = Enteropathogenic

Ent - = Non - Enteropathogenic

Figure 1. Standard Curve of BSA for Protein Estimation by Lowery's Method.

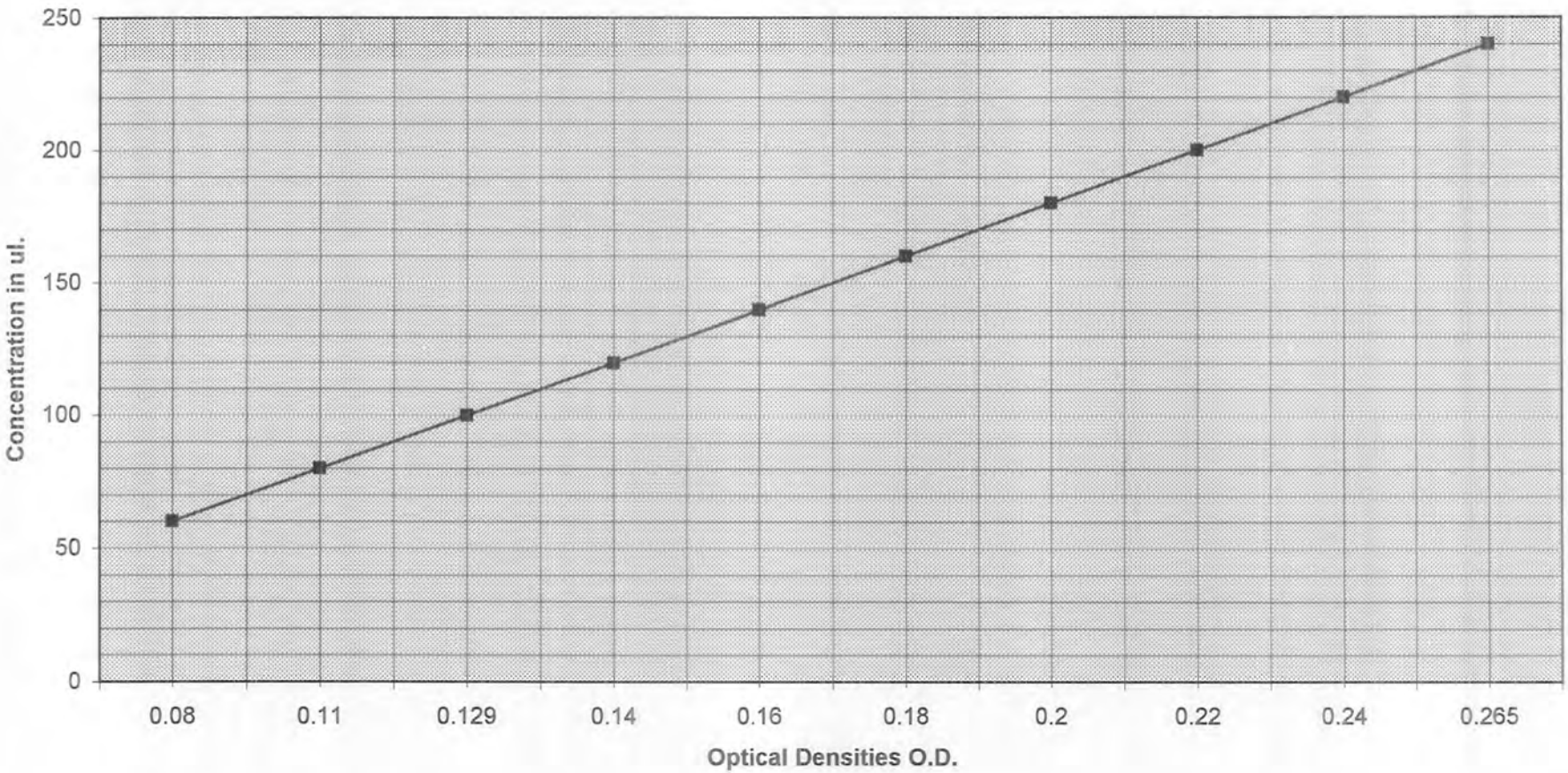


Figure 2. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 1 (A. hydrophila) using BSA Standard Curve.

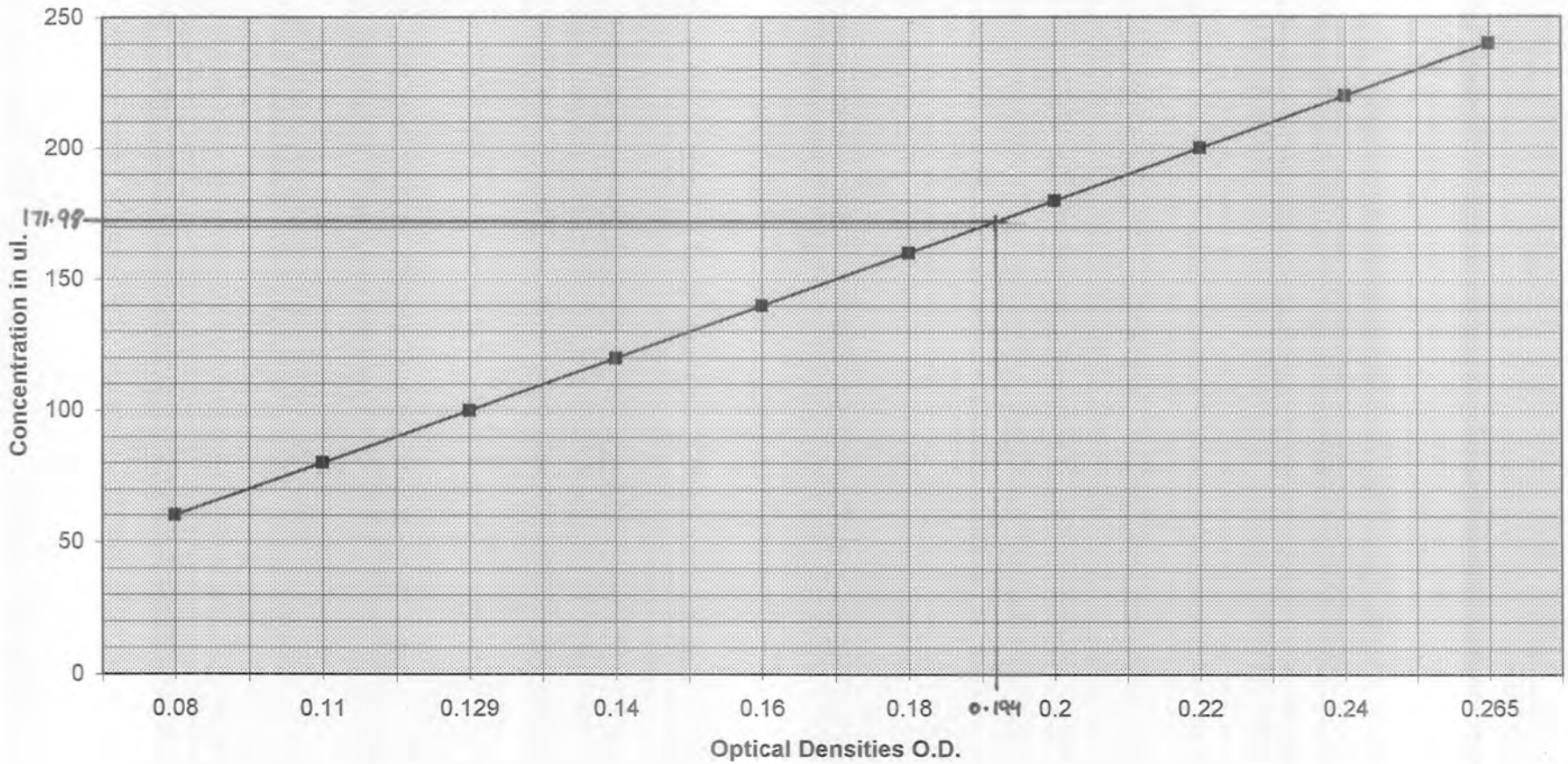


Figure 3. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 2 (A. hydrophila) using BSA Standard Curve.

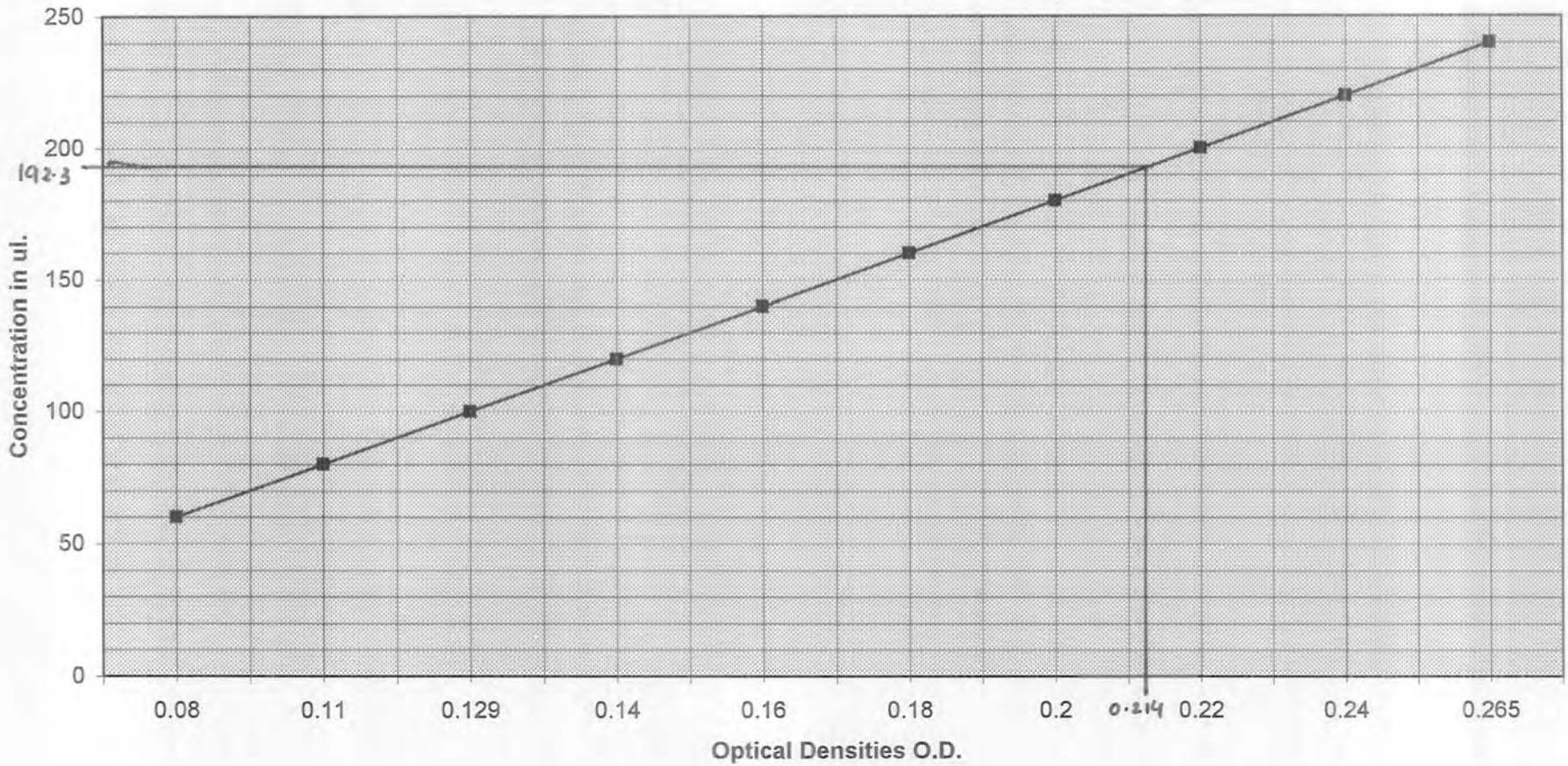


Figure 4. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 3 (A. sobria) using BSA Standard Curve.

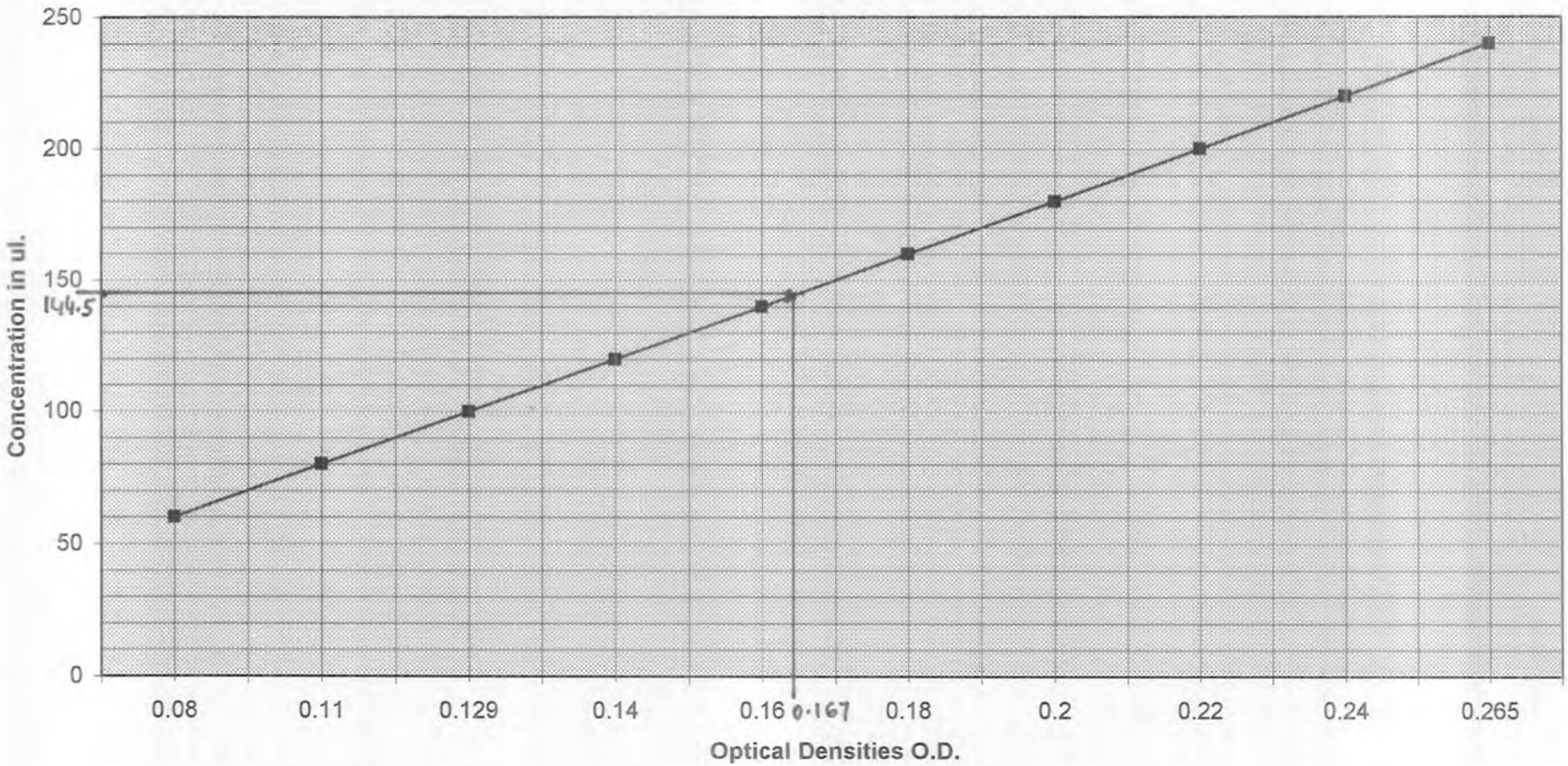


Figure 5. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 4 (A. caviae) using BSA Standard Curve.

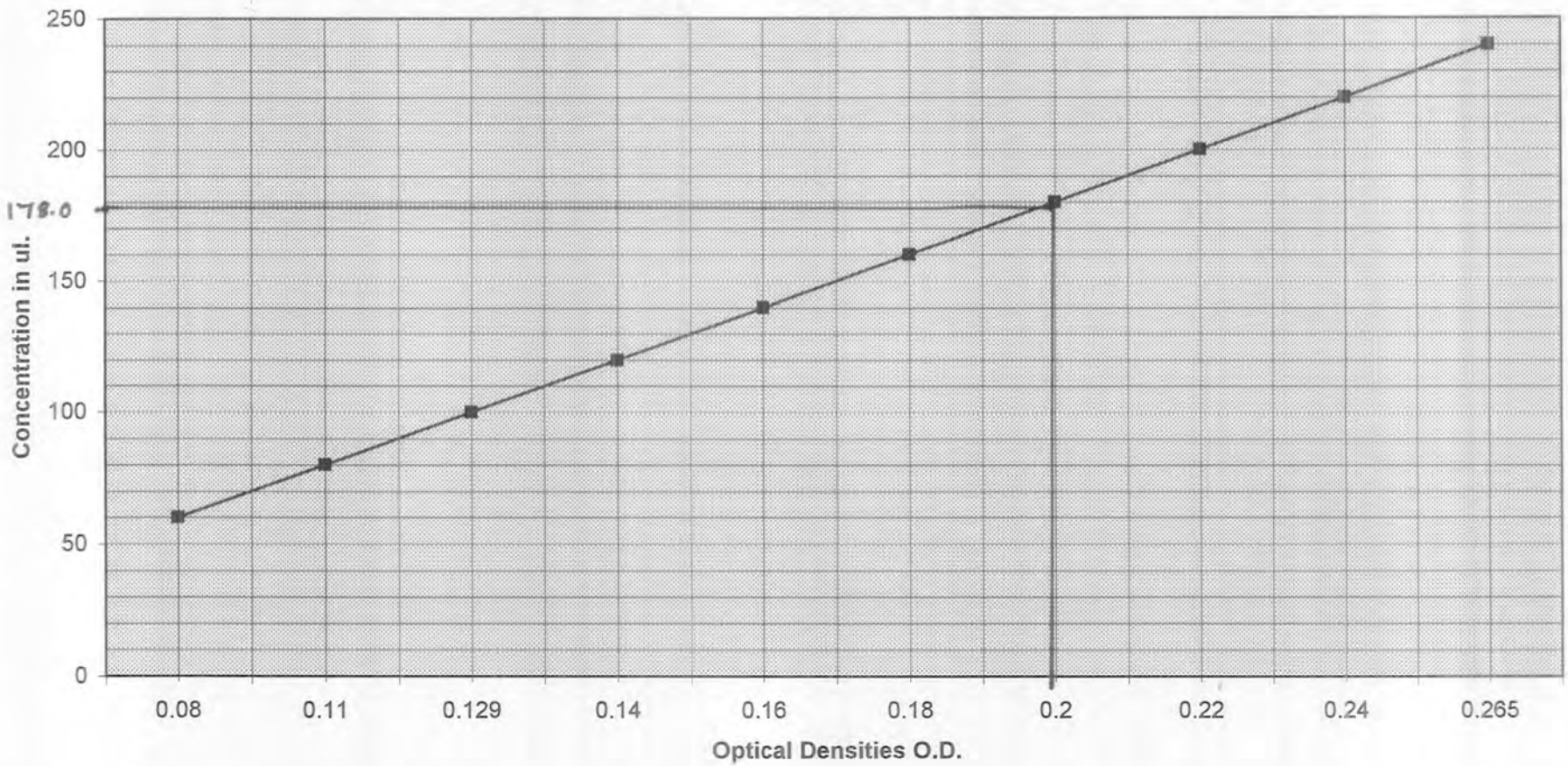


Figure 6. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 5 (A. caviae) using BSA Standard Curve.

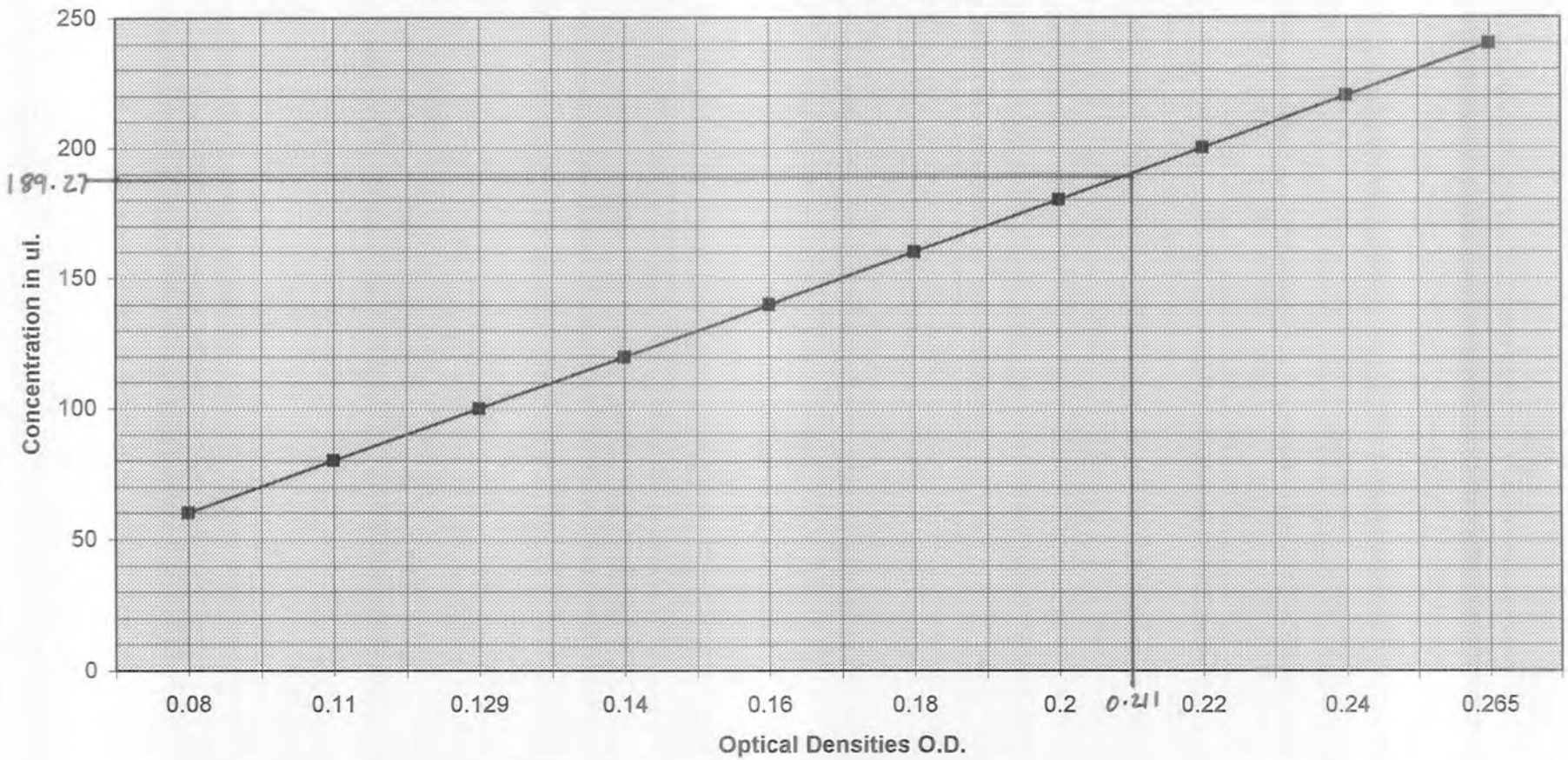


Figure 7. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 6 (A. caviae) using BSA Standard Curve.

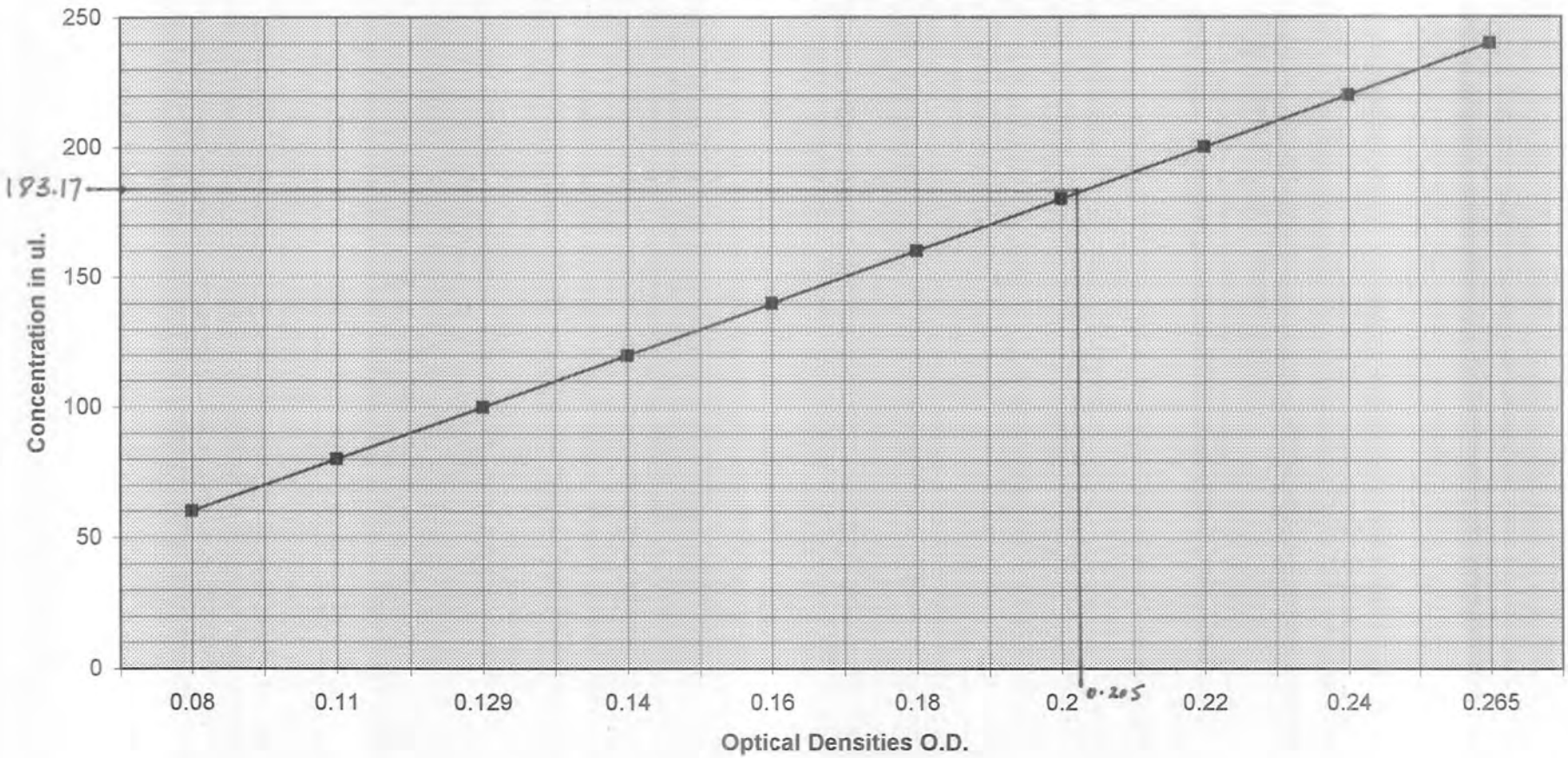


Figure 8. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 7 (A. caviae) using BSA Standard Curve.

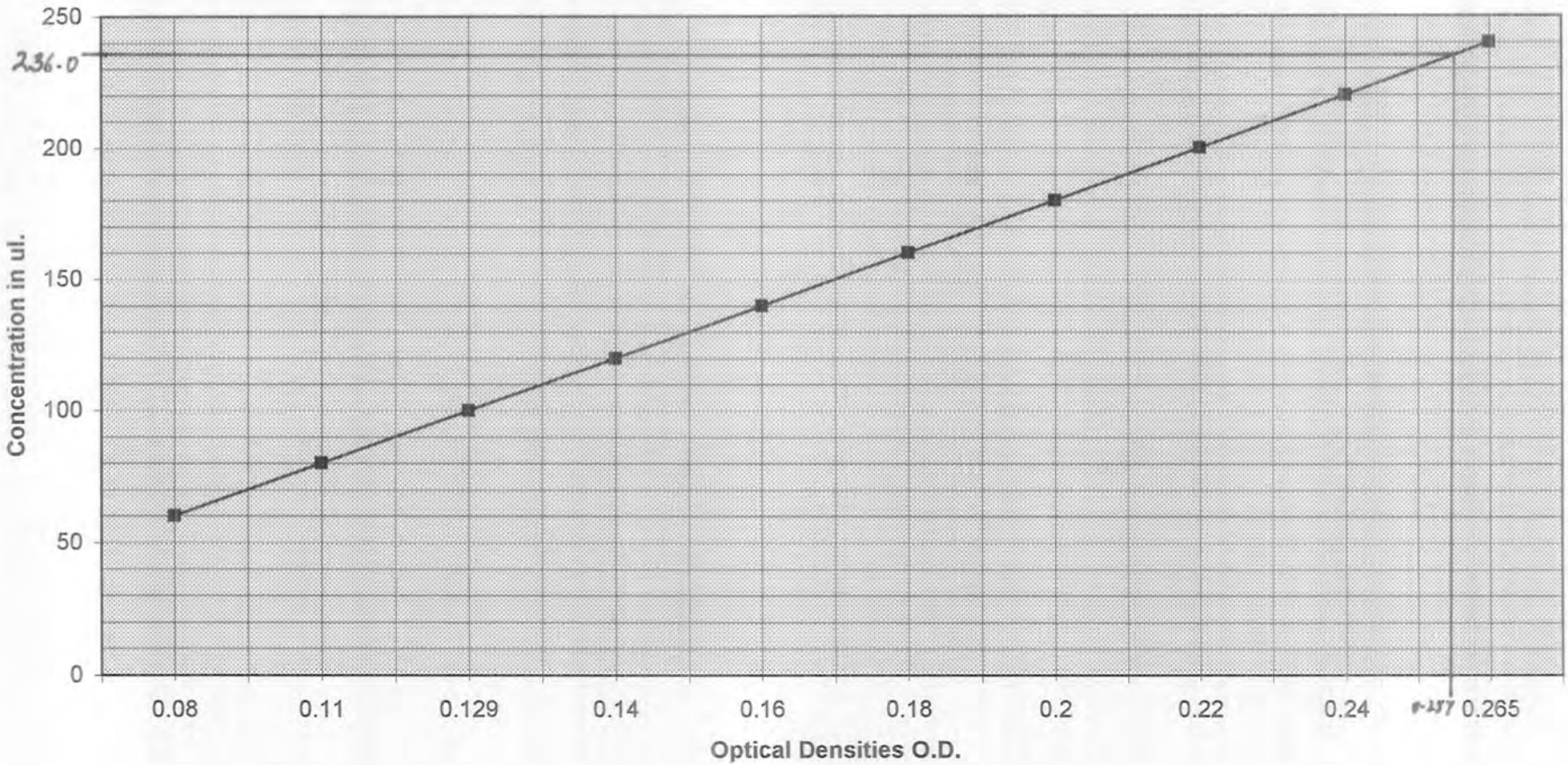


Figure 9. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 8 (A. caviae) using BSA Standard Curve.

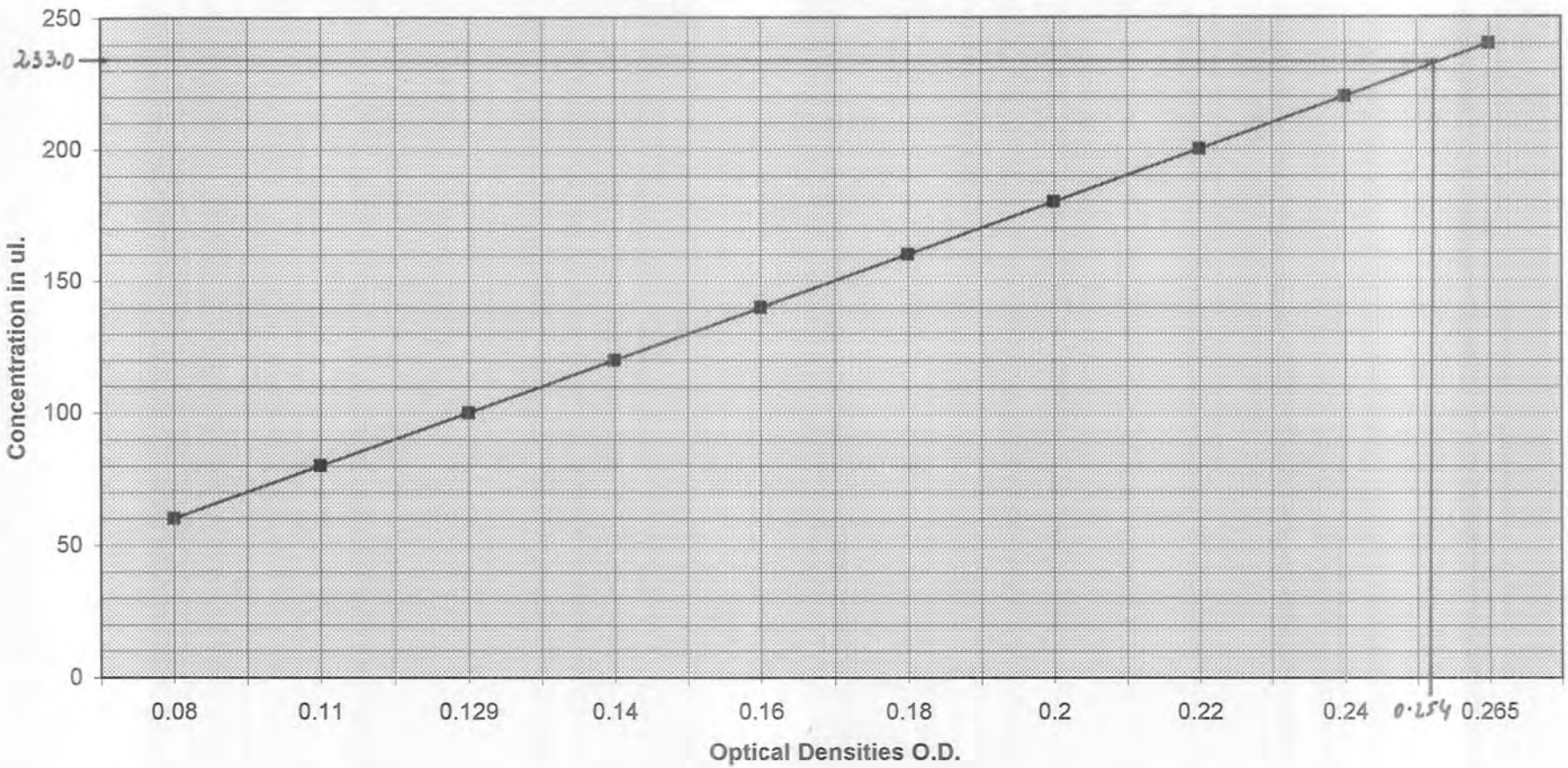


Figure 10. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 9 (A. hydrophila) using BSA Standard Curve.

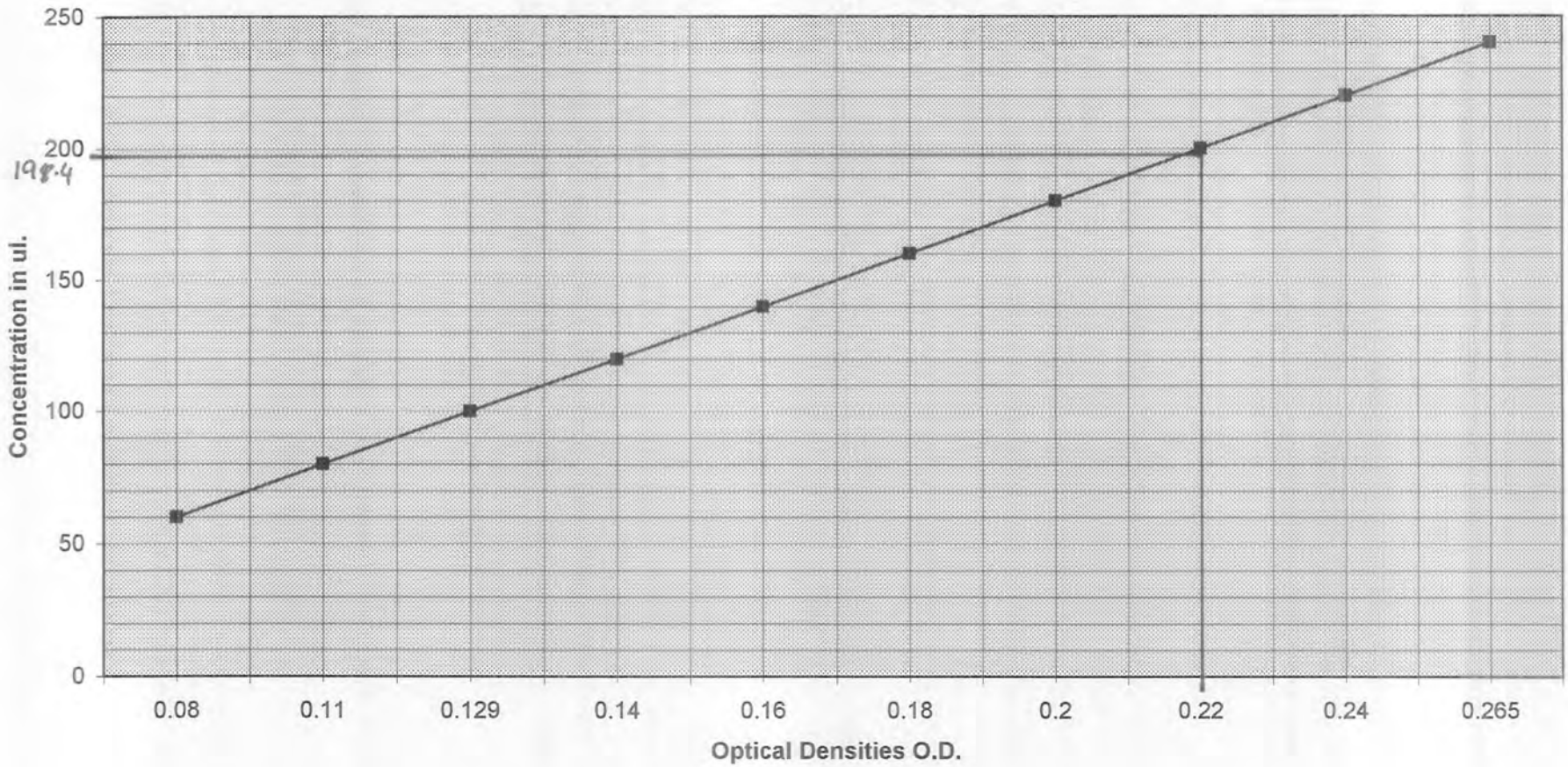


Figure 11. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 10 (A. hydrophila) using BSA Standard Curve.

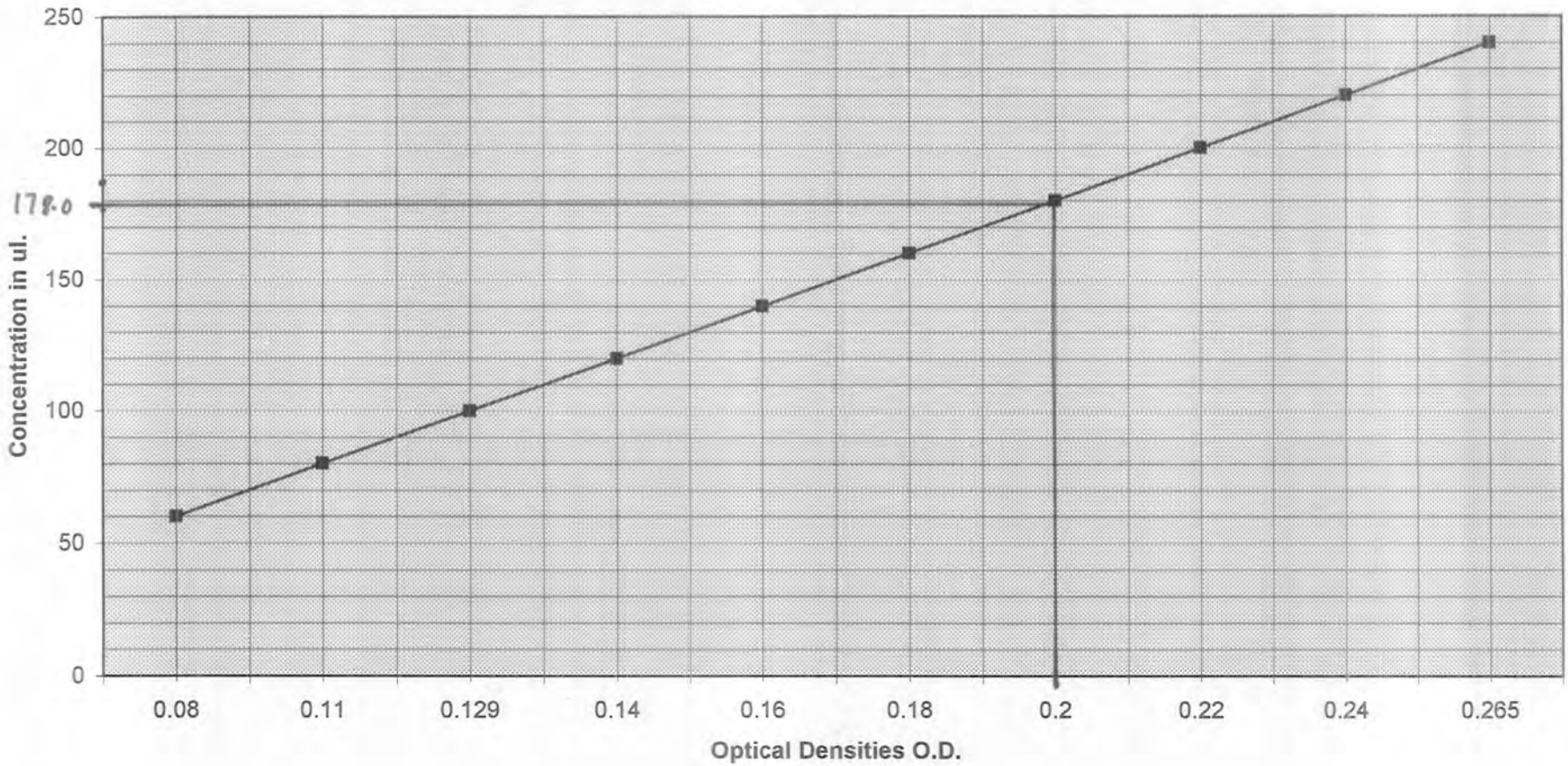


Figure 12. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 11 (A. hydrophila) using BSA Standard Curve.

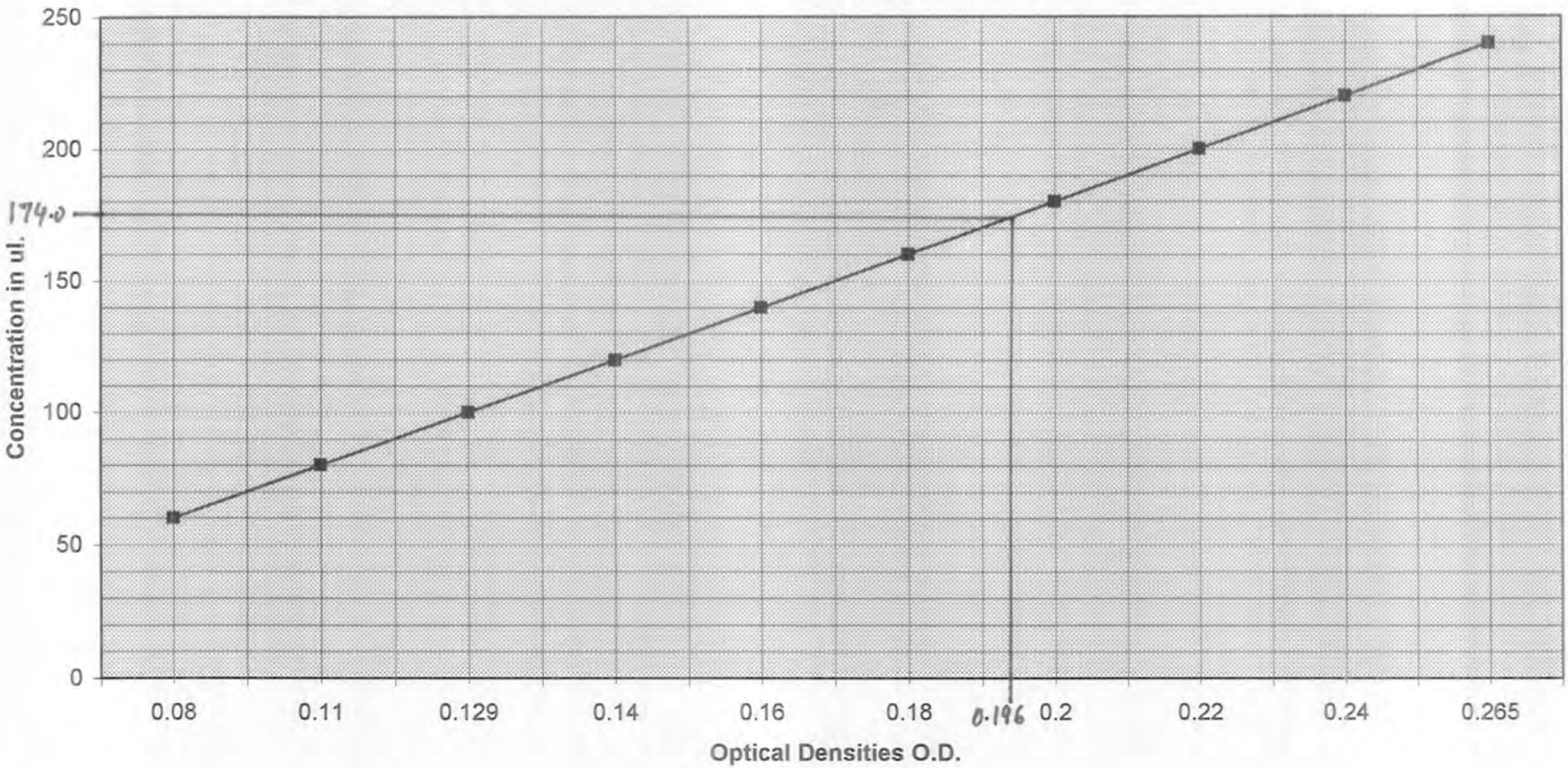


Figure 13. Estimation of Protein Concentration by Lowery's Method, in CFCF of Strain No. 12 (A. caviae) using BSA Standard Curve.

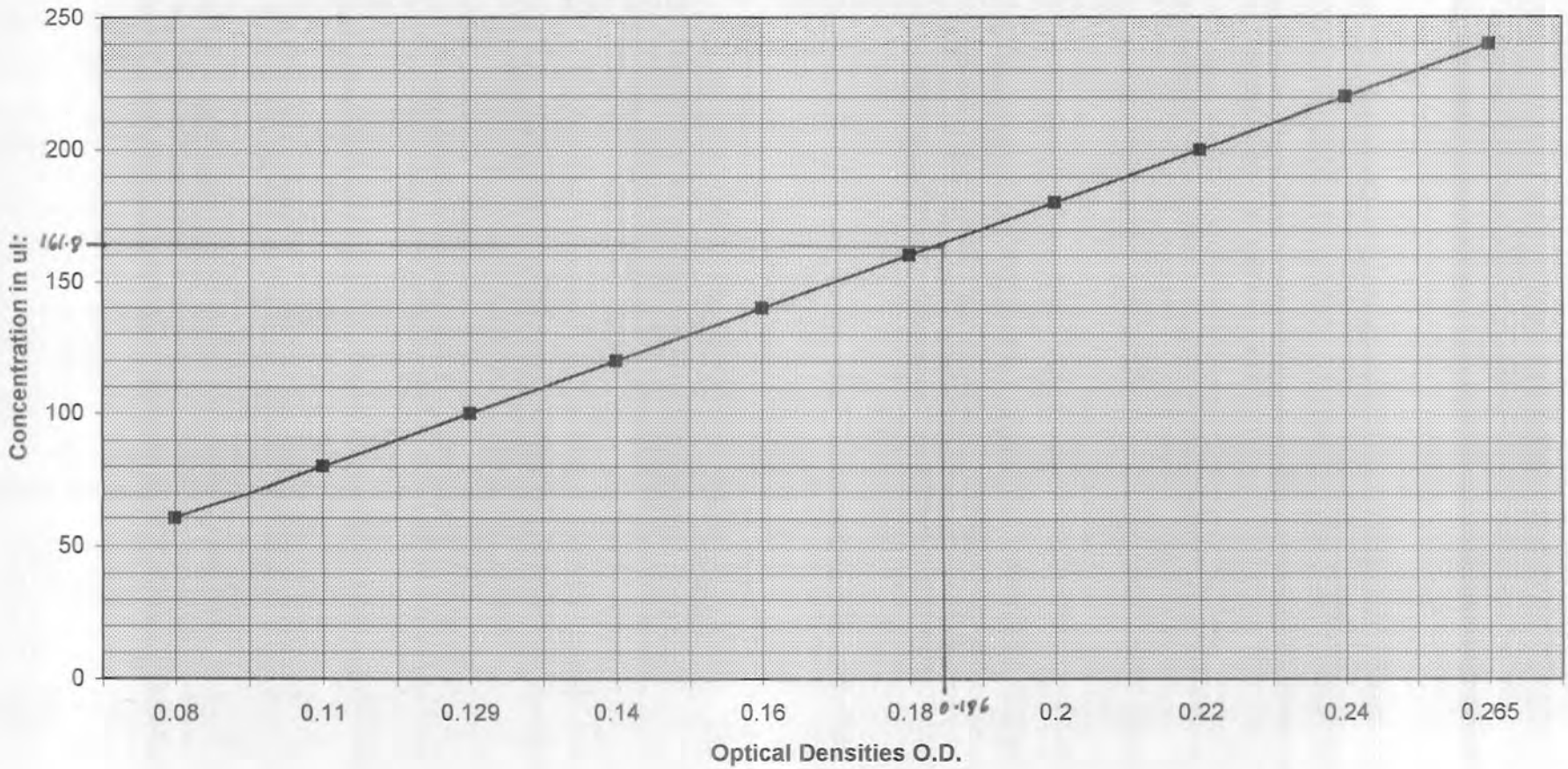
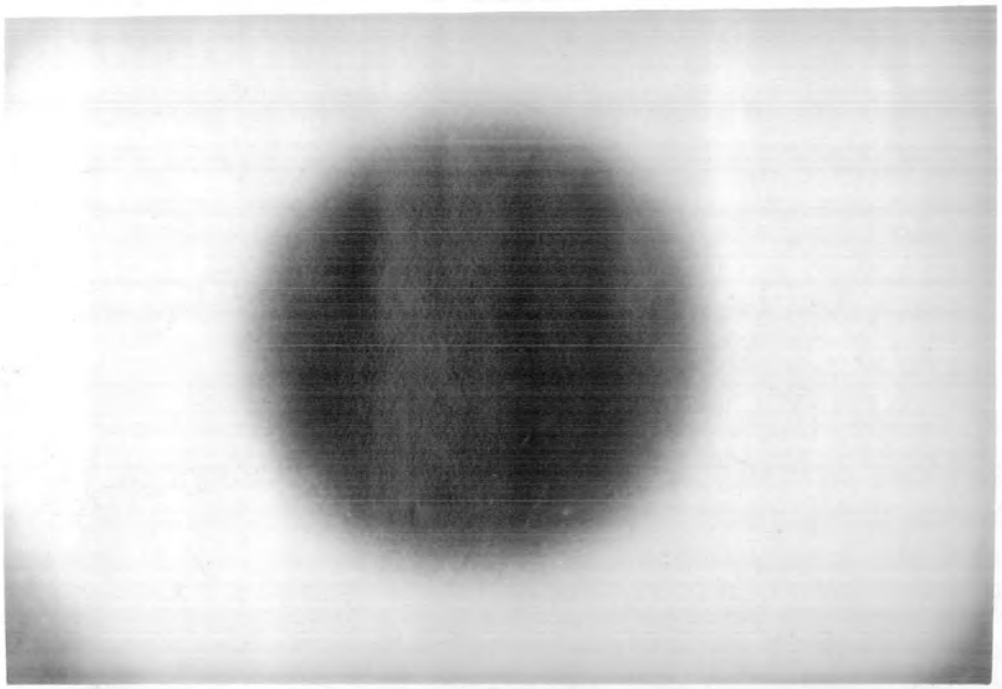
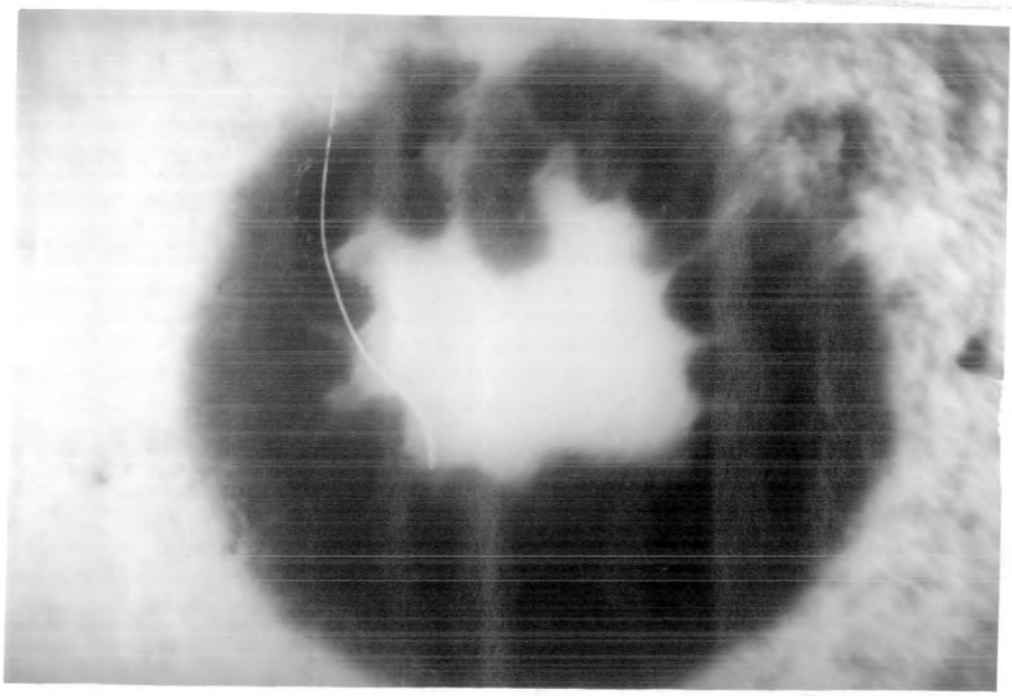


Figure 15. Photomicrograph illustrating the Haemolysin Activity of Aeromonas CFCF on 1% Rabbit Erythrocytes:

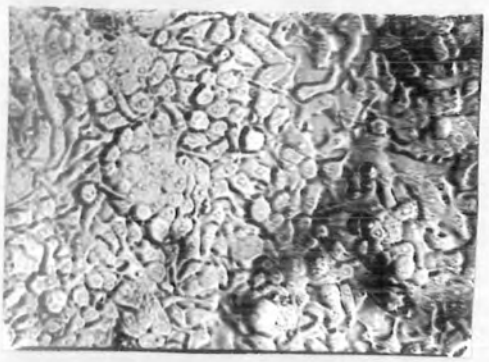


a: Negative (Button formation).

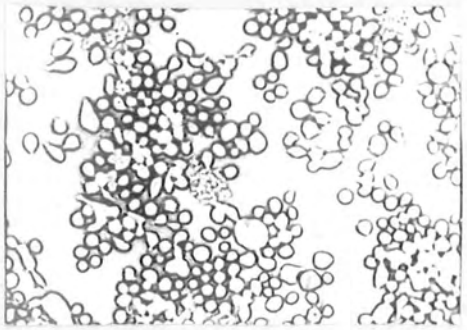


b: Partial Haemolysis.

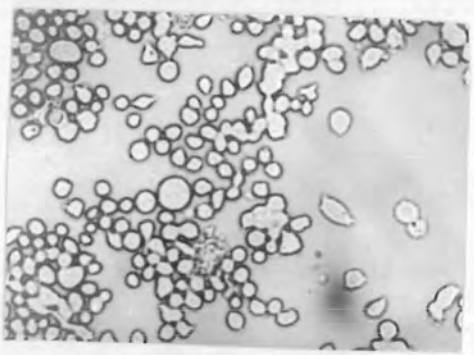
Figure 16. Photomicrograph illustrating the Cytotoxin Activity of Aeromonas CFCF in Hep-2 cell monolayer as demonstrated by cell rounding, detachment and death.



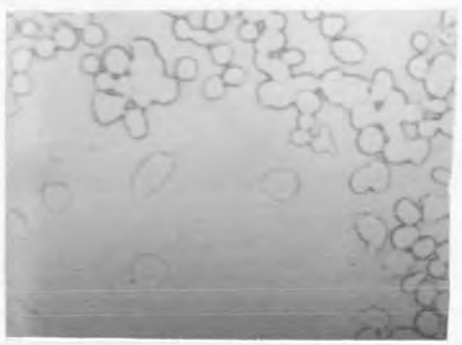
a: Negative Control.



b: Weak Cytotoxicity.

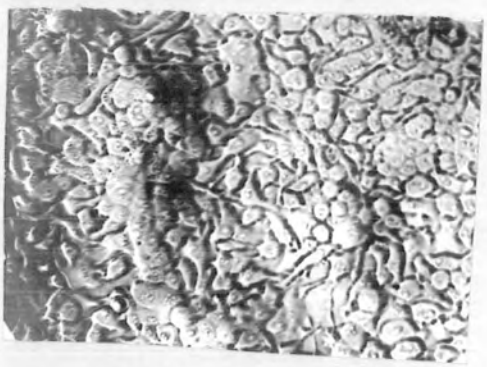


c: Strong Cytotoxicity.

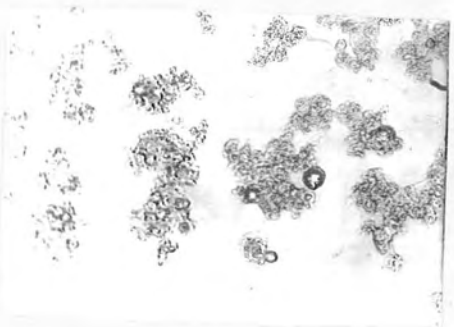


d: High Cytotoxicity.

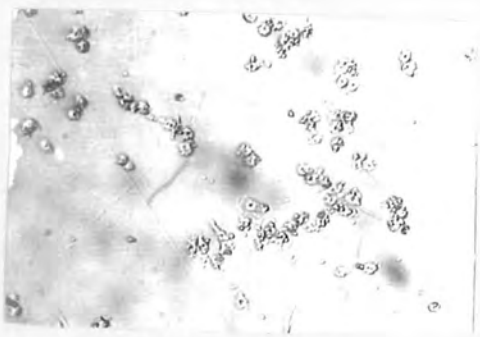
Figure 17. Photomicrograph illustrating the Cytotoxin Activity of Aeromonas CFCF in Hep-2 cell monolayer as demonstrated by cell rounding, detachment and death.



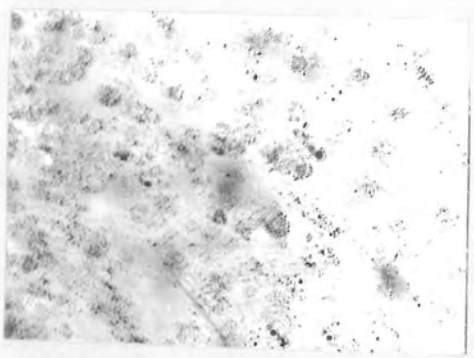
a: Negative Control.



b: Weak Cytotoxicity.



c: Strong Cytotoxicity .



d: High Cytotoxicity.

Figure 18. ***Photograph illustrating the Intraperitonial inoculation of Aeromonas CFCF for Enterotoxin Assay in Suckling mouse.***

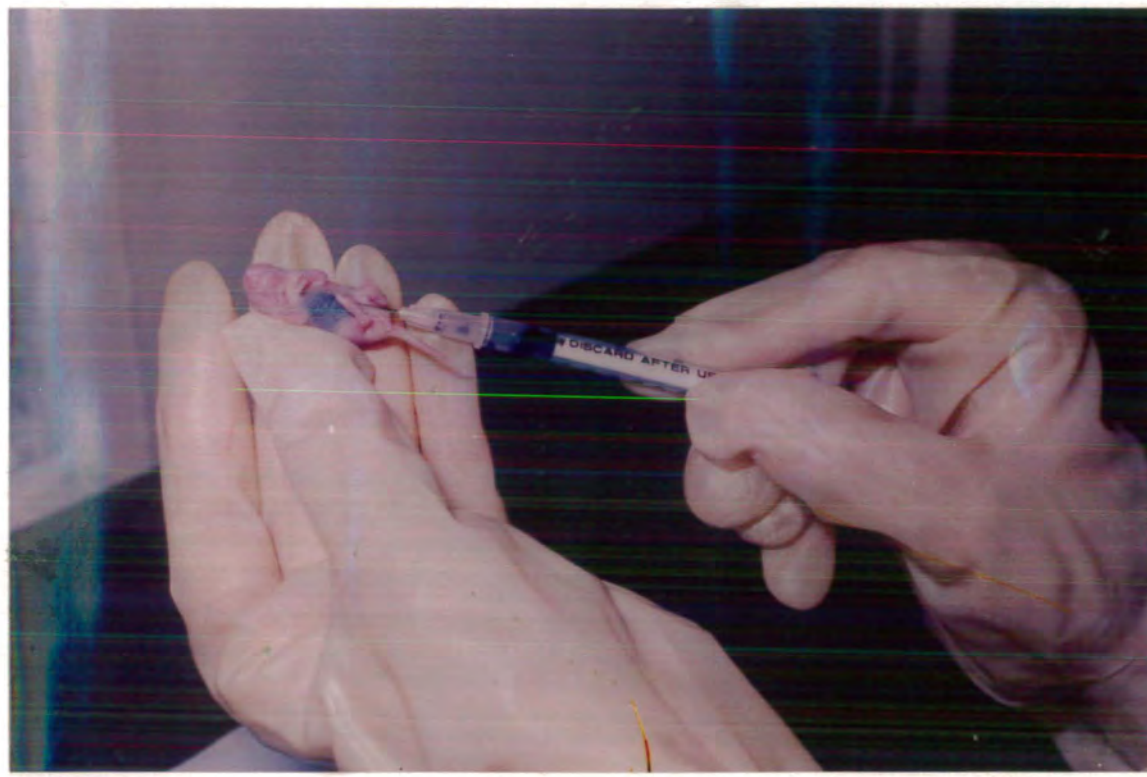
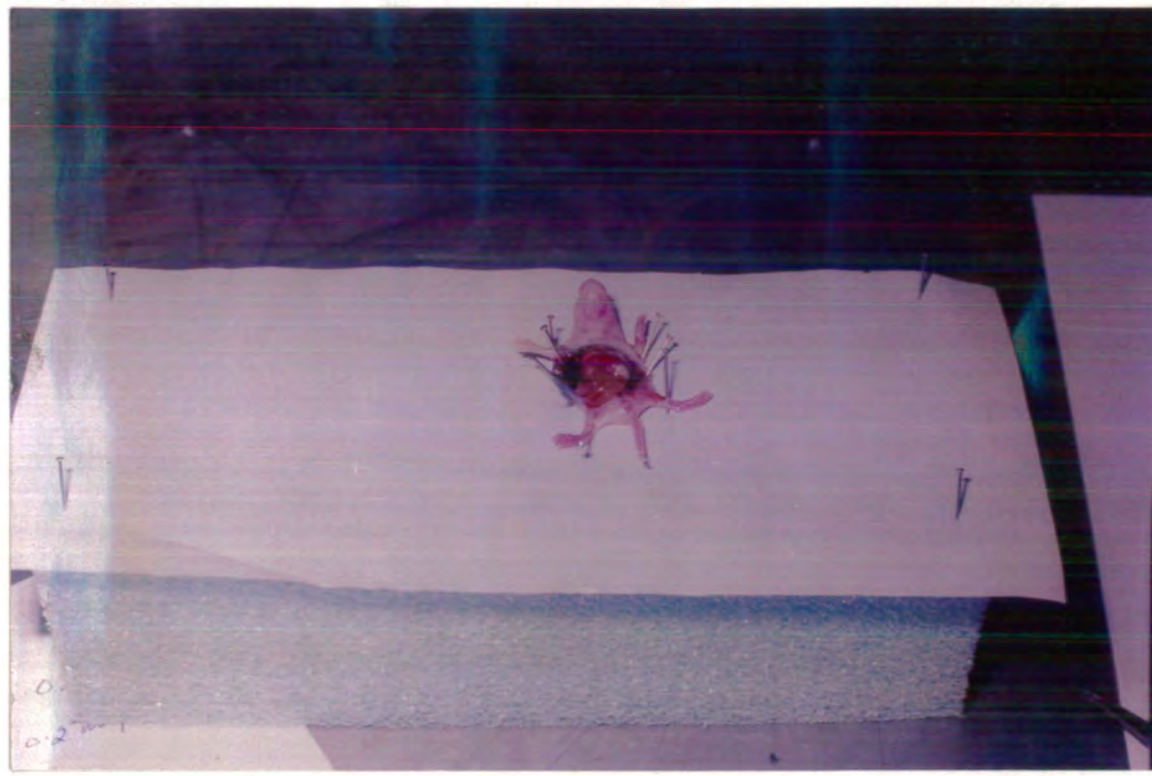


Figure 19. ***Photograph illustrating the Intestinal Distension in Suckling Mouse for Enterotoxic Activity of Aeromonas CFCF.***



DISCUSSION

Twelve strains of *Aeromonas* species from clinical and environmental sources were tested for production of different toxins, and enteropathogenic effects of the elaborated toxins were studied to determine the biological activities of these toxins.

The present study included *Aeromonas sobria* (1 strain), *Aeromonas caviae* (6 strains) and *Aeromonas hydrophila* (5 strains). These strains had previously been studied elsewhere for their pathogenic role in gastroenteritis.

All the twelve strains of Aeromonas were propagated in tryptic soya broth supplemented with Yeast extract 0.6% w/v, pH - 7.4, this medium has also been used by other workers like Gosling, *et al., (*1993) and Majeed and Macrae (1994) they have successfully used this medium and clamed its superiority on other mediums.

Three exotoxins (hemolysin. Cytotoxin, and enterotoxin) were elaborated by these test strains on this medium and the medium was also found to be suitable for determining the enteropathogenicity and biological activities of the elaborated exotoxins.

Aeromonas spp. are known to grow within a temperature range of 22-37°C (Kirov, *et al.,* 1993) , and are known to produce exotoxins best at 37°C (Kirov, *et al.,* 1993; Gosling, *et al.,* 1993). In the present study a toxin production procedure of (Gosling, *et al.,* (1993) and Majeed and Macrae (1994) has been used with success. However in the present study 300 oscillation per minute in 24 hours incubation has been used as proposed by Majeed and Macrae (1994) and best results were obtained.

For the production of Aeromonas exotoxins as cell free culture filtrate (CFCF) the cultures were grown in specified time and temperature as prescribed by Gosling, *et al., (* 1993). Majeed and Macrae (1994). The culture medium after propagation of seed culture were centrifuged at 10,000 g at +4°C for 30 minutes to render it cell free. The supernatants were filtered through sterile low protein binding 0.45u and 0.2u syringe filters (ICN Flow). Those cell free culture filtrates now termed as CFCF were than divided into small aliquots and stored at -20°C for further testing.

Each CFCF sample recovered by growing Aeromonas strain was tested for biological assays of exotoxin (cytotoxin activity, enterotoxin activity , and hemolysin activity) and In order to

determine the biochemical features of Cell Free Culture Filtrate protein estimation using Lowry s method and SDS-PAGE Gel electrophoresis was carried out.

In the present study the protein estimation of cell free culture filtrates was determine to correlate between the protein contents and the reaction of toxins in different tests, and it was found that a high protein contents of CFCF gave a relatively high hemolytic, cytopathic and enterotoxic effects irrespective of Aeromonas species and sources of isolation. The review literature indicates that protein estimation is only a test to varify the possible biological activities in the CFCF.

The CFCF of all the 12 strains were tested on gel electrophoresis against known molecular weight protein markers to confirm the presence of the known molecular weight toxic proteins produced by the Aeromonas strains. Proteins bands were observed within molecular weight range 15 kda (enterotoxin), and 55 kda (hemolysin), along with many other unknown protein bands. The protein profile of the exotoxins produced by Aeromonas spp. are too complex. Gosling, *et al.*, (1994) having similar view that these are not so far been fully investigated.

Aeromonas spp. produce a variety of biological active extracellular substances, including hemolysins (Asao, *et al.,* 1986), cytotoxins and enterotoxins (Chopra, *et al.,* 1986). A number of studies have concentrated on the roles of exotoxins in eteropathogenicity, and B- hemolysin is considered to be a likely pathogenic factor. However, epidemiological investigations suggest that production of hemolysin can serve as a marker for discrimination between species but does not distinguish between pathogenic and no-pathogenic species, because most strains of *A. hydrophila*, *A. caviae*, and *A. sobria* are hemolytic regardless of their source (Nishikawa, *et al.,* 1988). Aeromonas also have been known to be invasive and do posses hemagglutinins which could render them to cause diarrhea (Watson, *et al.,*1985).

The 12 strains investigated in our study and isolated from diarrheal and normal stools, and from environmental sources were 100% B-hemolytic on 5% sheep blood agar plates. Similar findings has been reported by Kindschuh, *et al.,* (1987) that all strains of the three Aeromonas species isolated from stools of children by them were B-hemolytic. In contrast Nishikawa and Kishi (1988) found that 95% of *A. hydrophila*, 86.4% of *A. sobria* and only 1.9% of

A. caviae were in their studies B-hemolytic. In another study 73% of *Aeromonas* isolates were B- hemolytic, where as the remaining were non- hemolytic among their strains (Singh and Sanyal, 1992).

The in vitro hemolysin activity (hemolysin production) in each of the 12 CFCF sample were analyzed according to the method proposed by Gosling, *et al.,* (1993) as the dilution titration using 1% rabbit erythrocytes in microtitre plates. In the present study the CFCF of all the strains of *A. hydrophila*, *A. caviae* and *A. sobria* isolated from clinical as well as environmental sources were found to cause lysis of 1% rabbit erythrocytes irrespective of the species, and sources of isolation, this has been supported by the findings of Singh and Sanyal, (1992) who also found that CFCf of all the strains of *Aeromonas* irrespective of their species designation and sources of isolation when tested for hemolytic activity only those causing B-hemolysis on sheep blood agar plates showed lysis of 2% sheep erythrocytes .

Nishikawa, *et al.,* (1988) had also found that *Aeromonas hydrophila* and *Aeromonas sobria* produced hemolysin. Nakano, *et al* , (1990) found that the strains which were hemolytic on blood

agar plates and had a high hemolytic titer value did not necessarily produce fluid accumulation in suckling mouse test. But all the strains that accumulate fluids had high hemolytic titer in microtitre method. In line with the aforementioned findings the present study revealed.

Tho cytotoxic ability of 12 strains of *Aeromonas* was tested on three different cell lines, namely Hep-2, Vero and MRC-5. In earlier studies only Hep-2 cell (Notterman and Botton, 1990) Vero cell (Majeed and Macrae, 1994) lines have been reported to be used for cytotoxicity tests.

The MRC-5 cells line (human diploid cells) has appears to be first time subjected to cytotoxic studies using Aeromonas toxins in the present study. The cytopathic effects (CPE) could be visualized in form of elongation of cells instead of rounding of cells as in case of Hep-2 and Vero cells there fore the possibility of use of MRC-5 cells for studying cytotoxic effects of bacterial toxins .There fore MRC-5 cells could also be used for studied the CPE as successfully as Hep-2 and Vero cells.

Clinical isolates of *Aeromonas spp.* are known to produce Cytotoxin (Gosting, *et al.*, 1986; Kindschun, *et al.*, 1987). In the present study it was found that all isolate of *Aeromonas* did produce cytotoxins having varying degree of toxitivity.

The cytotoxic effects observed were detachment and rounding of cell in case of Hep-2 and Vero cells resulting in cell death within next few hours. Slight toxic effects of strains no 4 and 8 of *A. caviae* isolated from stool of diarrheal patients on all the three cell lines was observed within 5 hours post inoculation . While strain 5 and 7 of *A. caviae*, (strain number 5 isolated from stool of diarrheal patient and strain number 7 isolated from the stool of normal person) showed highly toxic effects.. Strain 6 and 12 of *A. caviae* also showed highly toxic effects. Strain number 6 was isolated from diarrheal patient stool while strain no 12 was isolated from polluted water. Namdari and Bottone, (1990) used only Hep-2 monolayer cell line for the determination of cytopathic effects of CFCF produced by all three species of *Aeromonas* especially worked on CFCF *of A. caviae* and found that 1:5 dilution of culture filtrate of *A. caviae* cytotoxicity for Hep-2 cells was evident as early as 1 hour postexposure and maximized at 5 hours postinoculation. Twenty-four hours were required before cytotoxicity was evident

with the 1:10 dilution of the *A. caviae* culture filtrate. By their findings they conclude that *A. caviae* cytotoxin production is high *in vitro*, but not that of *A. hydrophila or A. sobria.*

Only one strain of *A. sobria* was included in this study which was isolated from stool of a diarrheal patient, it showed slight cytopathetic effect on Hep-2 cells after 24 hours post inoculation with 1:5 dilution. Similarly in the other two cell lines, Vero and MRC-5 the reaction was too weak to be considered as cytopathogenic.

A. hydrophila cytotoxin when tested on Hep2 cells produced rounding, detachment and death of cells which started within one hour post inoculation of 1:5 dilution of CFCF which completed within 4 -5 hours. This finding is in accordance with the report of Namdari and Bottone (1990). On Vero cells the toxin reaction was same which is also in line with the findings of Majeed and Macrae (1994). In MRC-5 the cells started showing reaction by elongation and detachment from the monolayer and than rounding off within two hours post inoculation. This cell line has not been reported before for this test. All strains of *A.hydrophila* showed strong

cytopathic effects on all the three cells monolayer irrespective of the source of isolation, thus a 100% response was observed.

A wide range of cytotoxic response of *A. hydrophila* has been reported ranging from 60% to 100% Majeed and Macrae (1994) and reported 63% cytotoxic response in *A. hydrophila*, while in the present study where 100% of *A. hydrophila* were able to produce cytotoxin supported by the observation of Plumbo, *et al., (*1989) that *Aeromonas* gastroenteritis in a spectrum of species related diseases, being associated mostly with *Aeromonas hydrophila*. In addition present study has shown that *A. hydrophila* is more likely than the other two *Aeromonas* species to be virulent. This conclusion is supported by the observation that *A. hydrophila* were able to produce a strong cytopathic reaction in cell lines as compared to the other two species (Palumbo, *et al.*, 1989).

Cytotoxic and hemolysin activities, as detected in *Aeromonas* strains, has been implicated in bacterial pathogenicity. The presence of significant levels of A*eromonas* strains with these activities may play a significant role in the epidemiology of *Aeromonas* associated gastroenteritis. The present study is also substaintiated by the a formentioned findings since most of our

isolates from diarrheal stools, thus proving them to be associated with disease and toxicity.

In the present study it was also observed that the cytotoxin activity did not correlate with the production of enterotoxin, these findings are supported by the work of (Kindschuh, *et al.,* 1987). However, some earlier investigator found complete correlation of cytotoxic activity within positive rabbit illeal loop assay, indicating almost universal production of enterotoxin like activity by cytotoxic strains.(Kindschuh, *et al.,* 1987).

Aeromonas spp. were also known to produce an enterotoxin whose identity is still under investigation due to its unstability and low production yield in vitro. Further the role of enterotoxin gets more complicated due to the presence of other exotoxins which are cytotoxic and hemolytic (Gosling, *et al.,* 1994). However, the role of *Aeromonas* spp. in gasteroentritis is associated with their being isolated from the stool of patients and the subsequent test of enterotoxin production in mice assays. Some investigators have isolated *Aeromonas spp.* from stool specimens from patients with diarrhea more frequently than from stool samples of normal patients taken as controls, while in other studies isolation from these two groups was similar (Notermans, *et al.,* 1986)

In the present study only 66% (8 of 12) CFCF of *Aeromonas* species were found to be weakly enterotoxic, 60% (3 of 5) *A. hydrophila*, 83% (5 of 6) *A. caviae* were enterotoxic. While only one sample of *A. sobria* tested was found non- enterotoxic. (Turnbull, *et al.,* 1984) have shown that enterotoxin is produced by most B- hemolytic strains of *Aeromonas*. However, (Singh and Sanyal, 1992) indicated that a significant number of B-hemolytic strains (35% of their strains) did not show any enterotoxic activity, however the B-hemolytic strains showed significantly more enterotoxic activity than the alpha-hemolytic and non-hemolytic strains, independent of their species designation. Thus Singh and Sanyal (1992) demonstrated that the capacity for enterotoxin production in *Aeromonas* spp. is not confined only to the B-hemolytic strains but that the alpha and non- hemolytic isolates also possess this property, although to a lesser extent.

Cytotoxic and hemolytic activity as detected in *Aeromonas* strains have been implicated in bacterial pathogenicity, and may play a significant role in the epidemiology of *Aeromonas* associated gastroenteritis as suggested by Majeed and Macrae (1994). In the present study 100% hemolytic and cytotoxic activity and 66% enterotoxic activity were observed which is supported by (Majeed

and Macrae, 1994) findings. In conclusion, it is inferred that *Aeromonas hydrophila* , as well as *Aeromonas caviae* and *Aeromonas sobria* are bonafide enteric pathogens as they posses the virulence factors (hemolysin , cytotoxin, and enterotoxin) that may operate in the gastrointestinal tract to provoke a diarrheal syndrome.

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