DETERMINATION OF PRESENCE AND SOME PATHOGENIC CHARACTERISTICS OF MOTILE *AEROMONAS* SPECIES IN FISH AND GROUND BEEF MARKETED IN BOLU

by

RÜMEYSA KÜÇÜKSARI

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Prof. Dr. Yaşar DÜRÜST
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of the Master of Science.

Prof. Dr. Ekrem GÜREL
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Biology.

Assoc. Prof. Dr. Seza ARSLAN
Supervisor

Examining Committee Members

Assoc. Prof. Dr. Azra BOZCAARMUTLU.....

Assoc. Prof. Dr. Seyhun YURDUGÜL…….....

Assoc. Prof. Dr. Seza ARSLAN…….....

ABSTRACT

DETERMINATION OF PRESENCE AND SOME PATHOGENIC CHARACTERISTICS OF MOTILE *AEROMONAS* SPECIES IN FISH AND GROUND BEEF MARKETED IN BOLU

KÜÇÜKSARI, Rümeysa
Master of Science, Department of Biology
Supervisor: Assoc. Prof. Dr. Seza ARSLAN

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Motile *Aeromonas* species, *A. hydrophila*, *A. caviae* and *A. veronii* biovar sobria were found among the bacteria causing foodborne gastroenteritis. They are recognized as potential foodborne pathogens because of their wide spread in the environment. They have been frequently isolated from foods such as seafood and food of animal origin. In the present study, the presence and incidence of motile *Aeromonas* species were investigated in fish and ground beef. Motile *Aeromonas* spp. were isolated from 49 (44.1 %) of the 111 samples tested. Other *Aeromonas* spp. were also isolated from 89 (80.1 %) out of the 111 samples. In general, among motile *Aeromonas* spp., *A. caviae* was detected in 31 (27.9%) of the samples, followed by *A. hydrophila* in 9 (8.1%) and *A. veronii* biovar sobria in 9

(8.1%). In this research, the 138 isolated *Aeromonas* spp. were examined for extracellular enzyme activities, virulence characteristics and antimicrobial resistance. Lecithinase, protease on calcium caseinate and skim milk agar, lipase, DNase, hemolysin, siderophore and slime activity were found in 85.8%, 96%, 75.5%, 100%, 96%, 53.1%, 30.6%, and 93.9% of motile *Aeromonas* isolates, respectively. The activities of lecithinase, protease on calcium caseinate and skim milk agar, lipase, DNase, hemolysin, and siderophore and slime production were common among other *Aeromonas* spp. Besides all motile *Aeromonas* isolates showed 100% of resistance to ampicillin. In addition, the highest resistances encountered were 91.8% to vancomycin. In contrast, more than 90% of the isolates were susceptible to ceftriaxone, cefotaxime, piperacillin/tazobactam, piperacillin, imipenem, chloramphenicol, enrofloxacin, amikacin, gentamicin, polymyxin B, and ciprofloxacin. In general, all 138 *Aeromonas* isolates showed high levels of the resistance to ampicillin (94.9%) and vancomycin (88.4%). Multi-drug resistance pattern was shown in 63% of the 138 *Aeromonas* isolates to at least three or more antimicrobial agents. In this study, *Aeromonas* spp., exhibiting the extracellular enzymes and virulence factors which play role in the pathogenicity and antimicrobial resistance, were isolated mainly from fish and ground beef. The presence of *Aeromonas* spp. from these sources may be a health threat for consumers.

Keywords: *Aeromonas* spp., fish, ground beef, isolation, incidence, antimicrobial resistance, virulence factors.

ÖZET

BOLU' DA TÜKETİME SUNULAN BALIK VE KIYMALARDA HAREKETLİ *AEROMONAS* TÜRLERİNİN VARLIĞININ VE BAZI PATOJENİTE KRİTERLERİNİN BELİRLENMESİ

KÜÇÜKSARI, Rümeysa
Yüksek Lisans, Biyoloji Bölümü
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Hareketli *Aeromonas* türleri, *A. hydrophila*, *A. caviae* ve *A. veronii* biovar sobria gıda kaynaklı gastroenteritlere sebep olan bakteriler arasında bulunur. Bunlar, çevrede yaygın olarak bulunmalarından dolayı potansiyel gıda kaynaklı patojenler olarak tanımlanırlar. Deniz ürünleri ve hayvansal gıdalardan sıklıkla izole edilmektedirler. Bu çalışmada, balık ve kıymada hareketli *Aeromonas* türlerinin varlığı ve yaygınlığı araştırılmıştır. Test edilen 111 örneğin 49'unda (%44,1) hareketli *Aeromonas* izole edilmiştir. Diğer *Aeromonas* türleri 111 örneğin 89'unda (%80,1) tespit edilmiştir. Genel olarak, hareketli *Aeromonas* türleri örneklerin 31'inde (%27,9) *A. caviae*, 9'unda (%8,1) *A. hydrophila* ve 9'unda (%8,1) *A. veronii* biovar sobria olarak bulunmuştur. Bu araştırmada izole

edilen 138 *Aeromonas* izolatının enzim aktiviteleri, virulans özellikleri ve antimikrobiyal direnci incelenmiştir. Hareketli *Aeromonas* izolatlarının lesitinaz, kalsiyum kazeinat agar ve skim milk agarda proteaz, lipaz, DNaz, hemoliz, siderofor ve slime aktiviteleri sırasıyla %85,8, %96, %75,5, %100, %96, %53,1, %30,6 ve %93,9 bulunmuştur. Diğer *Aeromonas* izolatlarında ise lesitinaz kalsiyum kazeinat agar ve skim milk agarda proteaz, lipaz, DNaz, hemoliz aktiviteleri ve siderofor ve slime üretimleri yaygındır. Ayrıca tüm hareketli *Aeromonas* izolatları ampisiline %100 direnç göstermiştir. Buna ek olarak, en yüksek direnç %91,8 ile vankomisine karşıydı. Aksine, izolatların %90'ından fazlası seftriakzon, sefotaksim, piperasillin/tazobaktam, piperasillin, imipenem, kloramfenikol, enrofloksasin, amikasin, gentamisin, polimiksin B ve siprofloksasine duyarlıydı. Genelde, tüm 138 *Aeromonas* izolatı ampisilin (%94,9) ve vankomisine (%88,4) karşı yüksek seviyede direnç gösterdiler. İzolatların %63'ünde en az 3 ya da daha fazla antimikrobiyal ajana karşı çoklu direnç görüldü. Bu çalışmada, patojenlikte rol oynayan hücre dışı enzimler ve virulans faktörlere sahip, antimikrobiyal direnç gösteren *Aeromonas* türleri balık ve kıyma örneklerinin çoğundan izole edilmiştir. Bu gıdalardaki *Aeromonas* türlerinin varlığı tüketiciler için bir sağlık tehdidi olabilir.

Anahtar Kelimeler: *Aeromonas* ssp., balık, kıyma, izolasyon, identifikasyon, insidans, antimikrobiyal direnç, virulans faktörler

To my dear family…

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1. INTRODUCTION

Foodborne infection occurs as a result of the consumption of food and water contaminated with pathogenic bacteria. Microorganisms get into foods from various sources from the time of production until the time of consumption. Aeromonads are important in food for their ability to cause foodborne diseases. Because of the nature of its normal habitat, *Aeromonas* spp. is found in many foods, especially foods of animal origin (Ray, 2004).

Motile aeromonads, *A. hydrophila*, *A.caviae*, and *A. veronii* biovar sobria, those are widespread at the environment. They cause serious problems in terms of public health. They contaminate meat and meat products, fish and marine fishery, milk and dairy products, and also vegetables (İşleyici and Sancak, 2009).

The motile aeromonads are emerging as important pathogens in humans, causing a variety of extraintestinal and systemic infections, as well as gastrointestinal infections (Tomas, 2012). Extraintestinal infections caused by motile aeromonads include: septicemia, provoked by the dissemination of the organism, from the intestinal tract to systems of circulation, and in the majority observed in immunocompromised patients; wound infections, mostly superficial cutaneous infections, infections of tendons, muscles, and bones; infections of the respiratory tract, from epiglottitis to pneumonia; and less frequent, meningitis, peritonitis, eye infections and hemolytic uremic syndrome (Janda and Abbott, 2010).

Diseases are the primary constraints to the culture of many aquatic species, impeding both economic and social development and a significant constraint on aquaculture production and trade (Smith, 2006). It is recognized that bacteria are one of the important causative agents of fish diseases and motile aeromonads represent the most frequently encountered bacterial agents, associated with fish diseases in freshwater environments (Karunasagar et al., 2003; Yesmin et al., 2004).

The pathogenesis of *Aeromonas* infection is complex and multifactorial with the involvement of a number of virulence factors, including extracellular enzymes, siderophores, cytotoxic and cytotonic enterotoxins, hemolysins, endotoxins and cell associated factors such as S-layers, pili, polar and lateral flagella, outer membrane proteins, and plasmids (Martin-Carnahan and Joseph, 2005).

The aim of the present research was to investigate the occurrence of motile *Aeromonas* spp. in freshwater fish, seawater fish and ground beef and to determine extracellular enzymes and virulence factors which play role in the pathogenicity and the antimicrobial susceptibility of motile *Aeromonas* isolates to certain antimicrobial agents.

1.1. History

The first description of an organism that is now known to be an authentic *Aeromonas* species was that of *Bacillus hydrophilus fuscus* by Sanarelli (1891). Other *Aeromonas*-like organisms were also described during this period by workers studying septicemic diseases of frogs (Ernst, 1890; Sanarelli, 1891). Other strains were soon isolated from water (Zimmerman, 1890; Beijerinck, 1900), milk

(Hammer, 1917), and diseased trout (Emmerich and Weibel, 1894); (Farmer et al., 2006).

Pathogenic *Aeromonas* strains have been isolated from fish (Farmer et al., 2006). An organism that Emmerich and Weibel (1894) called 'Bacillus der Forellenseuche' was isolated from diseased trout in 1894. Since then, similar bacteria have been isolated from furunculosis lesions in fish. These bacteria were named as *Bacterium salmonicida* by Lehmann and Neumann (1896). Today this organism is known as *Aeromonas salmonicida*, an important fish pathogen (Farmer et al., 2006).

Early classifications of *Aeromonas* prior to 1920, organisms that currently belong to the genus *Aeromonas* were classified in the genera *Bacillus*, *Bacterium*, and *Aerobacter*. After 1920, species were classified in the genera *Proteus*, *Pseudomonas*, *Escherichia*, *Achromobacter*, *Flavobacterium*, *Bacterium*, *Necromonas*, *Vibrio*, and *Aeromonas* (Farmer et al., 2006).

The genus name *Aeromonas* was first used by Kluyver and van Niel (1936) in their "natural system of classification of bacteria". MacInnes et al. 1979 did the first DNA hybridization experiments with *Aeromonas* and concluded that the genus consisted of two main evolutionary lines: "a diverse group of motile aeromonads, and the genetically more homogeneous non-motile aeromonads, comprising the species *Aeromonas salmonicida*" (Farmer et al., 2006).

1.2. Description of the Genera

The genus *Aeromonas* belongs to the family *Aeromonadaceae* (Martin-Carnahan and Joseph, 2005). According to the last edition of Bergey's Manual of Systematic Bacteriology (Martin-Carnahan and Joseph, 2005), the genus *Aeromonas* includes the following species: *Aeromonas hydrophila*, *Aeromonas*

bestiarum, *Aeromonas salmonicida*, *Aeromonas caviae*, *Aeromonas media*, *Aeromonas eucrenophila*, *Aeromonas sobria*, *Aeromonas veronii* (biovars sobria and veronii), *Aeromonas jandaei*, *Aeromonas shubertii*, *Aeromonas trota*, *Aeromonas allosaccharophila*, *Aeromonas encheleia*, *Aeromonas popoffii* and two DNA homology groups: *Aeromonas* spp. (HG11) now included in *Aeromonas encheleia* (Martínez-Murcia, 1999; Huys et al., 1997) and *Aeromonas* spp. (HG13; former Enteric Group 501) that has recently been named as *Aeromonas diversa* (Miñana-Galbis, 2010). Previously defined species *Aeromonas enteropelogenes* (Schubert et al., 1990) and *Aeromonas ichthiosmia* (Schubert et al., 1990) are now considered as synonyms of *Aeromonas trota* and *Aeromonas veronii*, respectively (Collins et al., 1993, Huys et al., 2001, Huys et al., 2002, Martin-Carnahan and Joseph, 2005). Later on, thirteen aeromonads, *Aeromonas culicicola* (Pidiyar et al., 2002), *Aeromonas simiae* (Harf-Monteil et al., 2004), *Aeromonas molluscorum* (Miñana-Galbis et al., 2004), *Aeromonas sharmana* (Saha and Chakrabarti, 2006), *Aeromonas bivalvium* (Miñana-Galbis et al., 2007), *Aeromonas aquariorum* (Martínez-Murcia et al., 2008), *Aeromonas tecta* (Demarta et al., 2008), *Aeromonas piscicola* (Beaz-Hidalgo et al., 2009), *Aeromonas fluvialis* (Alperi et al., 2010a), *Aeromonas taiwanensis* and *Aeromonas sanarellii* (Alperi et al., 2010b), *Aeromonas rivuli* (Figueras et al., 2011) and *Aeromonas cavernicola* (Martínez-Murcia et al., 2013) have been described.

Members of the genus *Aeromonas* are gram-negative, facultative anaerobic, straight, rod-shaped to coccoid cells with rounded ends. They are 1.0 to 3.5 μm long and 0.3 to 1.0 μm wide, and most possess polar, usually monotrichous flagella with a wavelength of 1.7 μm. Peritrichous flagella may be formed in young cultures on solid medium (Altwegg, 1999). Occur singly, in pairs, or short chains. They are

chemoorganotrophic, displaying oxidative and fermentative metabolism of D-glucose. Nitrate is reduced to nitrite (Martin-Carnahan and Joseph, 2005). They are oxidase and catalase positive. Usually they are arginine dihydrolase positive and ornithine decarboxylase negative. Urease and phenylalanine deaminase test is negative too. Carbohydrates fermented by almost all strains include maltose, D-galactose, and trehalose (Holt et al., 1994). A variety of exoenzymes such as arylamidases, amylase, DNase, esterases, peptidases, and other hydrolytic enzymes are produced. Generally resistant to 150 μg of the vibriostatic agent 2, 4-diamino-6,7-diisopropylpteridine (O/129) (Martin-Carnahan and Joseph, 2005). Their main cellular fatty acids are hexadecanoic acid (16:0), hexadecenoic acid (16:1), and octadecenoic acid (18:1) and the guanine-plus-cytosine content of their DNA is in the range of between 57 and 63%. They grow over a wide temperature range (0 to 45°C). Human (mesophilic) strains grow at between 10 and 42°C, whereas the nonmotile, psychrophilic species have a maximum growth temperature of 37°C or lower. Some mesophilic strains are biochemically more active at 22°C than at 37°C (Altwegg, 1999).

The genus *Aeromonas* can be divided into two major groups of species, the mesophilic and motile species (*A. hydrophila*, *A. caviae*, *A. veronii* biovar sobria) and the non-motile and psychrophilic species (*A. salmonicida*) (Martin-Carnahan and Joseph, 2005). It is the mesophilic aeromonads which are of interest and concern to human public health as a cause of extra-intestinal infections and a potential agent of diarrheal disease (Palumbo et al., 2001).

1.3. Natural Habitats

Aeromonads are mainly found in aquatic ecosystems worldwide, with densities ranging from <1 to about 1000 cells per ml of groundwater, drinking water at treatment plants and in water distribution systems, clean rivers, lakes, and storage reservoirs. Higher densities, i.e., up to $>10^8$ cells per ml, are found in wastewater, treated sewage, crude sewage, and domestic sewage sludge. Occasionally, *Aeromonas* may also be found in marine environments, although these organisms seem to prefer salt waters interfacing with fresh water (Altwegg, 1999). The bacterium also occurs widely in foods, both fresh and processed, undoubtedly as result of the food's contact with a source of water containing the bacterium. Water appears to represent a major reservoir of this group because these bacteria are able to grow at low nutrient levels and they can survive for long periods of time in water, particularly at low temperatures. Motile aeromonads are present in both chlorinated and unchlorinated waters (Palumbo et al., 2001). These bacteria also reside in sink traps and drainpipes and can be recovered from tap water faucets and distilled water supplies, which are potential sources of organisms involved in nosocomial infections (Winn et al., 2006).

Motile aeromonads are not considered normal inhabitants of the human gastrointestinal tract. The fecal carriage rate of *A. hydrophila* in asymptomatic hospitalized patients varies from 0 to 8%. This suggests that motile aeromonads are transient inhabitants of the human intestinal tracts and humans are not the major contributors of the bacterium to the environment, although aeromonads that are shed can multiply in sewage lines to significant numbers prior to discharge into receiving waters. In general, there is a low rate of isolation of aeromonads in the feces of food animals such as bovine, swine, and poultry and it is unlikely that feces

of these animals represent a source of the bacteria in foods. As indicates above, water is the most likely source of these bacteria in foods, with fish and seafood most often contaminated. Motile aeromonads are not known to be part of the intestinal flora or tissue of healthy fish. However, they are easily isolated from retail market fish. Shellfish, particularly oysters, represent another known reservoir of the bacterium. Motile aeromonads are often associated with refrigerated animal products such as chicken, beef, pork, lamb, and dairy products. In addition, they can be isolated from a wide range of other foods such as vegetables, spices, and other fresh and processed foods (Palumbo et al., 2001).

1.4. Pathogenesis

The mesophilic aeromonads are emerging as important pathogens in humans, causing a variety of extraintestinal and systemic infections, as well as gastrointestinal infections (Tomas, 2012). Before the aeromonads were recognized as human pathogens, they were commonly isolated from poikilothermic animals, particularly amphibians, reptiles, and fish. One of the first diseases attributed to *Aeromonas* was 'red-leg' disease in frogs. Aeromonads are also found with some frequency to cause various types of diseases in birds and domestic animals including pneumonia, peritonitis, and various localized infections. Various species of fish develop hemorrhagic disease, ulcerative disease, furunculosis, red sore disease, and septicemia, resulting from infections with both motile and nonmotile *Aeromonas* species (Martin-Carnahan and Joseph, 2005). *A. salmonicida* occupies a limited ecological niche, producing furunculosis and bacteremia in fresh-water fish, particularly trout and salmon (Farmer et al., 2006).

Humans can suffer from a variety of extraintestinal infections including wound infections, septicemia, meningitis, osteomyelitis, septic arthritis, peritonitis, endocarditis, cholecystitis, urinary tract infections, ophthalmitis, and infections associated with leech therapy, as well as gastroenteritis, especially in children <5 years. The species most frequently isolated from clinical specimens are *A. hydrophila*, *A. veronii* biovar sobria, *A. caviae*, *A. jandaei*, *A. schubertii*, and *A. trota* (Martin-Carnahan and Joseph, 2005; Altwegg, 1999).

The varied clinical picture of *Aeromonas* infections and gastroenteric illness in particular, suggests that complex pathogenic mechanisms occur in aeromonads. For the different gastroenteric disease manifestations, it may be that strains possess multiple virulence factors in different combinations which may interact with different levels of the intestine (Galinda and Chopra, 2007). Depending on the species and strain, *Aeromonas* spp. produce an impressive array of virulence factors, including hemolysins, cytotoxic and cytotonic enterotoxins, proteases, lipases, leucocidins, endotoxin, adhesins, agglutinins, pili, various enzymes, and outer membrane arrays (Martin-Carnahan and Joseph, 2005).

The O-antigen polysaccharide, which is composed of repeating oligosaccharide units, is covalently attached to the lipid A-core complex of the LPS and extends outward from the cell surface. The genus *Aeromonas* has been classified into 96 serogroups, and the O-antigen (Galinda and Chopra, 2007). The presence of a polysaccharide capsule is presumed that it is a virulence factor that aids in complement resistance and/or in adherence to and invasion of fish cell lines (Martin-Carnahan and Joseph, 2005).

Endotoxin is probably a virulence factor in extraintestinal infections since it can cause a localized Schwartzman reaction and hemorrhagic lesions when injected into mice (Farmer et al., 2006).

Filamentous structures on aeromonads are diverse. There are two broad morphological types of pili (short, rigid and long, wavy), as well as polar and lateral flagella. Short, rigid pili are the main pilus type expressed on heavily piliated aeromonads (Martin-Carnahan and Joseph, 2005).

Most aeromonads elaborate a variety of extracellular enzymes: proteases, DNase, RNase, elastase, lecithinase, amylase, lipases, gelatinase, and chitinase. The roles of these enzymes in virulence have yet to be determined, but there is much evidence that correlates their production with increased pathogenicity (Galinda and Chopra, 2007).

Enterotoxic (aerolysin), cytotoxic (Act), and two cytotonic toxins (Alt and Ast) have been detected in *Aeromonas hydrophila* (Martin-Carnahan and Joseph, 2005). Aerolysin proteins are channel-forming proteins which produce a transmembrane channel that destroys cells by breaking their permeability barriers. Act was determined to possess a variety of biological activities, including hemolysis, cytotoxicity, and enterotoxicity, and to cause lethality in mice. Cytotonic enterotoxins produce increased levels of cyclic AMP and cause elongation of Chinese hamster ovary cells. They are thought to act similarly to cholera toxin (Galinda and Chopra, 2007).

Aeromonads produce at least two major classes of hemolysins, alpha and beta (Farmer et al., 2006). Act- and aerolysin-like molecules (beta-hemolysins) are enterotoxigenic cytolysins. These toxins have been proposed to contribute to the virulence of aerolysin-positive strains and to account for some of the species-

associated differences in *Aeromonas* virulence mechanisms. The second class of hemolysins, alpha-hemolysin, is elaborated during the late stationary phase of growth and causes incomplete lysis of erythrocytes and has not been associated with enterotoxic properties (Galinda and Chopra, 2007).

Aeromonas also produces a lipase, named glycerophospholipid:cholesterol acyltransferase (GCAT), which can produce cholesteryl esters and can act as a phospholipase by digesting plasma membranes (Martin-Carnahan and Joseph, 2005). Two distinct types of proteases have been isolated from aeromonads including heat labile serine protease and heat-stable metalloprotease (Galinda and Chopra, 2007). These enzymes probably function to protect the organism in its various environments, including humans, by inducing tissue destruction and assisting in the degradation of substrates for catabolic metabolism (Martin-Carnahan and Joseph, 2005).

Efficient mechanisms for iron acquisition from the host during an infection are considered essential for virulence. Siderophores, are low-molecular-weight compounds with high affinities for various forms of iron (Galinda and Chopra, 2007). Aeromonads also possess more than one type of siderophore. Mesophilic aeromonads usually produce either a unique siderophore, amonabactin, or an enterobactin-like siderophore. *Aeromonas* spp. display significantly increased siderophore production under iron limiting conditions, and clinical strains are generally more productive than environmental strains (Martin-Carnahan and Joseph, 2005).

2. MATERIALS AND METHODS

2.1. Sample Collection

Fish samples (*Oncorhynchus mykiss* and *Sparus aurata*) were purchased from fish markets and ground beef samples were collected from butcher shops in Bolu. A total of 111 samples were examined; 37 of them were seawater fish, 37 of them were freshwater fish, 37 of them were ground beef. The samples were collected individually in sterile sample bags and transported to the microbiology laboratory in a freezer bag at 4^{o}C and processed within 24 h. Aseptic procedures were strictly followed during collection, transportation and analysis.

2.2. Microbiological Analysis

2.2.1. Isolation and Identification of *Aeromonas* spp.

Twenty-five grams of the fish flesh and ground beef were weighed aseptically and homogenized for 2 minutes in stomacher bags containing 225 ml of alkaline peptone water (APW) (OXOID) (Interscience bagmixer 400). Then the homogenate was incubated at 28°C for 24 h for enrichment. To provide a suitable cultivation of *Aeromonas*, two agar media were used. Starch Ampicillin Agar was prepared according to Palumbo et al. (1985) and *Aeromonas* Selective Agar (*Aeromonas* Medium Base (RYAN) (OXOID) +Ampicillin Selective Supplement (OXOID)) was commercially available (Martin-Carnahan and Joseph, 2005). After 24 h incubation, a loopful of enriched culture from APW was streaked on both Starch Ampicillin Agar and *Aeromonas* Selective Agar plates. These plates were incubated

at 28°C for 24 h. Following incubation, suspect colonies of opaque green colonies with dark centers onto *Aeromonas* Selective Agar and suspect colonies of yellow to honey coloured colonies onto Starch Ampicillin Agar were counted as *Aeromonas*. Suspect colonies were selected and subcultured on Tryptone Soya Agar (OXOID). The presumptive colonies were examined for gram stain, oxidase and catalase, string test, motility, urease, lipase, hemolysin activity, Voges-Proskauer reaction, nitrate reduction, indol production, aesculin hydrolysis, growth in nutrient broth with 6% NaCl and without sodium chloride; H_2S production on Triple Sugar Iron Agar and H_2S formation from cysteine; resistance to a vibriostatic agent O/129 (2-4-diamino-6,7-diisopropylpteridine), arginine dihydrolase, lysine and ornithine decarboxylase, utilization of lactic acid, gas production from D-glucose, and the ability to produce acid from each of D-mannitol, myo-inositol, sucrose, arabinose, cellobiose, sorbitol and lactose (Martin-Carnahan and Joseph, 2005). All of the tests are shown in Tables 1, 2, 3, and 4.

Table 1. Biochemical characteristics of motile *Aeromonas* species[*].

Biochemical tests	*A. hydrophila*	*A. caviae*	*A. veronii* bv. sobria
Gram stain (24 h)	-	-	-
Oxidase (24 h)	+	+	+
Catalase (24 h)	+	+	+
String Test	-	-	-
Motility	+	+	+
Urease	-	-	-
Lipase	+	+	+
Nitrate Reduction	+	+	+
Indol production	+	+	+
Growth with 6% NaCl	-	-	-
Growth without NaCl	+	+	+
Resistance to O/129 (10μg)	+	+	+
Resistance to O/129 (150μg)	+	+	+
H_2S from TSI	-	-	-
H_2S from cystein	+	-	+
β-hemolysis	+	V	+
Voges-Proskauer	+	-	+
Aesculin Hydrolysis	+	V	-
Arginine dihydrolysis	+	+	+
Lysine decarboxylase	+	-	+
Ornithine decarboxylase	-	-	-
Utilization of lactic acid	+	V	-

[*] Key tests for the phenotypic differentiation of motile *Aeromonas* spp.; O/129, vibriostatic agent O/129 (2-4-diamino-6,7-diisopropylpteridine); V, variable

Table 2. Biochemical characteristics of motile *Aeromonas* species*.

Biochemical tests	*A. hydrophila*	*A. caviae*	*A. veronii* bv. sobria
Glucose, gas	+	-	+
Mannitol, acid	+	+	+
Myo-inositol, acid	-	-	-
Sucrose, acid	+	+	+
Arabinose, acid	V	+	-
Cellobiose, acid	-	+	V
Sorbitol, acid	-	-	-
Lactose, acid	V	V	-

* Key tests for the phenotypic differentiation of motile *Aeromonas* spp.; V, variable

Table 3. Biochemical characteristics of other *Aeromonas* species*.

Biochemical tests	*A. bestiarum*	*A. salmonicida*	*A. molluscorum*	*A. bivalvium*	*A. eucrenophila*	*A. schubertii*	*A. jandaei*	*A. veronii* bv. veronii	*A. simiae*	*A. encheleia*	*A. allosaccharophila*
Gram stain (24 h)	-	-	-	-	-	-	-	-	-	-	-
Oxidase (24 h)	+	+	+	+	+	+	+	+	+	+	+
Catalase (24 h)	+	+	+	+	+	+	+	+	+	+	+
String Test	-	-	-	-	-	-	-	-	-	-	-
Motility	+	V	+	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-
Lipase	+	+	+	+	+	+	+	+	+	+	+
Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+
Indol production	+	+	-	+	+	-	+	+	-	+	+
Growth with 6% NaCl	-	-	-	-	-	-	-	-	-	-	-

Growth without NaCl	+	+	+	+	+	+	+	+	+	+	+
Resistance to O/129 (10μg)	+	+	+	+	+	+	+	+	+	+	+
Resistance to O/129 (150μg)	+	+	+	+	+	+	+	+	+	+	+
H_2S from TSI	-	-	-	-	-	-	-	-	-	-	-
H_2S from cystein	+	+	-	-	+	-	+	V	-	+	+
β-hemolysis	+	V	V	-	+	V	+	+	-	V	V
Voges-Proskauer	V	V	-	-	-	V	+	V	-	-	-
Aesculin Hydrolysis	+	+	+	+	V	-	-	+	V	V	V
Arginine dihydrolysis	V	V	+	-	V	+	+	-	+	V	V
Lysine decarboxylase	+	V	-	+	-	V	+	+	+	-	+
Ornithine decarboxylase	-	-	-	-	-	-	-	+	-	-	V
Utilization of lactic acid	-	-	V	+	-	+	-	-	nd	-	V

[*] Key tests for the phenotypic differentiation of other *Aeromonas* spp.; O/129, vibriostatic agent O/129 (2-4-diamino-6,7-diisopropylpteridine); V, variable; nd, none detected

Table 4. Biochemical characteristics of other *Aeromonas* species*.

Biochemical tests	*A. bestiarum*	*A. salmonicida*	*A. molluscorum*	*A. bivalvium*	*A. eucrenophila*	*A. schubertii*	*A. jandaei*	*A. veronii* bv. veronii	*A. simiae*	*A. encheleia*	*A. allosaccharophila*
Glucose, gas	+	V	-	-	V	-	+	+	-	V	+
Mannitol, acid	+	+	+	+	+	-	+	+	-	+	+
Myo-inositol, acid	-	-	-	-	-	-	-	-	-	-	-
Sucrose, acid	+	+	+	+	V	-	-	+	+	V	+
Arabinose, acid	+	+	+	+	V	-	-	-	-	-	V
Cellobiose, acid	V	V	+	+	+	-	-	V	+	-	+
Sorbitol, acid	-	+	-	-	-	-	-	-	-	-	-
Lactose, acid	-	+	-	-	+	-	-	V	-	+	-

* Key tests for the phenotypic differentiation of other *Aeromonas* spp.; V, variable

2.2.2. Screening of extracellular enzymes and virulence characteristics of *Aeromonas* spp.

2.2.2.1. Phospholipase Activity (Lecithinase Production)

Lecithinase production was tested by adding 10% (vol/vol) egg yolk emulsion (Oxoid) to Tryptone Soya Agar (Oxoid). Plates were incubated at 37°C for 24 h. A white precipitate around or beneath of an inoculum spot indicated lecithinase formation (Esselmann and Liu, 1961).

2.2.2.2. Proteolytic Activity

Proteolytic activity was determined by two different methods. First method was performed by using calcium caseinate agar according to Frazier and Rupp (1928). The second method was taken placed on plate count agar with 10% skim milk powder (Vermelho et al., 1996). In both methods, all isolates were incubated at 37°C for 24h. Proteolytic activity was determined by the presence of clear zones around the colony.

2.2.2.3. Lipase Activity

Lipase activity of *Aeromonas* spp. was detected on tributyrin agar supplemented with 1% tributyrin. After inoculation the tributyrin agar medium was incubated at 37°C for 48 h. Lipase activity was indicated by the formation of clear zones around the colonies (Meghwanshi et al., 2006).

2.2.2.4. DNase Activity

DNase activity was assessed at 37°C on DNase test agar supplemented with 0.01% toluidine blue O. After 72 h of growth, the degradation of deoxyribonucleic acid was indicated by the formation of a pink halo surrounding the isolates (Janda and Bottone, 1981).

2.2.2.5. Hemolysin Activity

Hemolysin activity was determined using blood agar medium containing 5% sheep blood. Beta hemolytic activity was regarded as clear zones around the colony after incubation at 37°C for 24 h.

2.2.2.6. Slime Production

Slime production was detected by Congo red agar method. The medium was prepared with 37 g/l brain heart infusion broth, 50 g/l sucrose, 10 g/l agar, and 0.8 g/l Congo red. Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 min and separately from the other medium constituents, and was then added when the agar had cooled to 55°C. Plates were incubated at 37°C for 24 h. A positive result was indicated by black colonies on the surface. Non-slime producing isolates developed red colonies (Freeman et al., 1989).

2.2.2.7. Siderophore Production

Colonies were inoculated onto chrome azurol S (CAS) blue plates, which were made according to the protocol of Schwyn and Neilands (1986) with some modifications prepared by Fiss and Brooks (1991). To prepare 1 liter of blue agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$, 10 mM HCl). Under stirring this solution was slowly added to 72.9 mg hexadecyltrimethylammoniumbromide (HDTMA) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. The basal medium to which the CAS indicator solution was added consisted of (per liter): KH_2PO_4, 0.3 g; NaCl, 0.5 g; NH_4Cl, 1.0 g; PIPES (piperazine-*N*,*N*'-bis[2-ethanesulfonic acid]), 0.1 M, pH 6.8; L-

asparagine, 5.0 g; and agar, 15 g. After the basal medium was autoclaved, the sterile CAS indicator solution was added slowly, followed by 20 ml of sterile glycerol. Plates were incubated at 37°C for 5 days. The ability of the organism to extract the iron from CAS indicator caused a color change from blue to orange or yellow, which formed a halo diffusing from the colony. The siderophore test was analyzed for the presence or absence of the orange-yellow halo surrounding the colonies, which indicated the presence or absence of a siderophore, respectively.

2.2.3. Antimicrobial Susceptibility Test

Antimicrobial susceptibility was determined by the disc diffusion method of Kirby-Bauer et al. (1966) using Mueller-Hinton Agar (Oxoid). The *Aeromonas* spp. isolates were tested against the following antimicrobial discs (Oxoid): chloramphenicol, 30 µg; cephazolin, 30 µg; erythromycin, 15 µg; gentamicin, 10 µg; polymyxin B, 300 units; trimetoprim-sulphamethoxazole, 25 µg; tetracycline, 30 µg; amikacin, 30 µg; ciprofloxacin, 5 µg; piperacillin-tazobactam, 110 µg; piperacillin, 100 µg; cefotaxime, 30 µg; ceftriaxone, 30 µg; imipenem, 10 µg; amoxicillin/clavulanic acid, 30 µg; enrofloxacin, 5 µg; ampicillin, 10 µg; vancomycin, 30 µg.

Pure cultures were enriched in Brain Heart Infusion Broth (Oxoid) at 37°C for 6-8 h. These cultures were streaked onto Mueller-Hinton agar plates using sterile cotton swab. The antimicrobial agent discs were placed on the agar surface sufficiently separated so as to avoid overlapping of the inhibition zones. The plates were incubated for 24 h at 37°C. After incubation period, results were recorded by measuring the diameter of the inhibition zones and compared with Clinical and Laboratory Standards Institute (CLSI, 2006) and were classified as resistant, intermediate and susceptible.

2.3. List of culture media

Alkaline Peptone Water (APW)..... (Oxoid CM1028)

Peptone 10 g

Salt 20 g

***Aeromonas* Medium Base** ..... (Oxoid CM0833)

Protease peptone 5 g

Yeast extract 3 g

L-Lysine monohydrochloride 2 g

Inositol 2,5 g

Lactose 1,5 g

Sorbitol 3 g

Xylose 3,75 g

Bile salts No.3 3 g

Sodium thiosulphate 10.67 g

Sodium chloride 5 g

Ferric ammonium citrate 0.8 g

Bromothymol blue 0.04 g

Thymol blue 0.04 g

Agar 12.5 g

Aeromonas Selective Supplement..... (Oxoid SR136 E)

Tryptone Soya Agar..... (Oxoid CM0131)

Pancreatic digest of casein 15 g

Enzymatic digest of soya bean 5 g

Sodium chloride 5 g

Agar 15 g

Plate Count Agar.....(Merck 105463)

Peptone from casein 5 g

Yeast extract 2.5 g

D (+) Glucose 1 g

Agar-agar 14 g

Mueller-Hinton Agar .....(Oxoid CM0337)

Beef, dehydrated infusion from 300 g

Casein hydrolysate 17.5 g

Starch 1.5 g

Agar 17 g

Mueller-Hinton Broth.....(Oxoid CM0405)

Beef, dehydrated infusion from 300 g

Casein hydrolysate 17.5 g

Starch 1.5 g

SIM Medium.....(Merck 105470)

Peptone from casein 20 g

Peptone from meat 6.6 g

Ammonium iron (III) citrate 0.2 g

Sodium thiosulfate 0.2 g

Agar-agar 3 g

Urea Agar Base.....(Acumedia 7226A)

Enzymatic digest of gelatin 1 g

Dextrose 1 g

Sodium chloride 5 g

Monopotassium phosphate 2 g

Urea 20 g

Phenol red 0.012 g

Tributyrin Agar (Basis) .....(Merck 101957)

Peptone from casein 2.5 g

Peptone from meat 2.5 g

Yeast extract 3 g

Agar-agar 12 g

Glycerintributyrat (Tributyrin).....(Merck 101958)

MR-VP Broth .....(Merck 105712)

Peptone from meat 7 g

D (+) Glucose 5 g

Phosphate buffer 5 g

Bile Aesculin Agar.....(Oxoid CM0888)

Peptone 8 g

Bile salts 20 g

Ferric citrate 0.5 g

Aesculin 1 g

Agar 15 g

Nutrient Broth.....(Merck 105443)

Peptone from meat 5 g

Meat extract 3 g

Triple Sugar Iron Agar.....(Merck 103915)

Peptone from casein 10 g

Peptone from meat 10 g

Meat extract 3 g

Yeast extract 3 g

Sodium chloride 5 g

Lactose 10 g

Sucrose 10 g

D (+) Glucose 1 g

Ammonium iron (III) citrate 0.5 g

Sodium thiosulfate 0.5 g

Phenol red 0.024g

Agar-agar 12 g

Decarboxylase Medium Base .....(Difco 287220)

Peptone 5 g

Yeast extract 3 g

Dextrose 1 g

Brom cresol purple 0.02 g

Brain Heart Infusion Broth.....(Oxoid CM0225)

Calf brain infusion solids 12.5 g

Beef heart infusion solids 5 g

Proteose peptone 10 g

Glucose 2 g

Sodium chloride 5 g

di-sodium phosphate 2.5 g

Nitrate Broth .....(Fluka 72548)

Peptone 5 g

Meat extract 3 g

Potassium nitrate 1 g

Calcium-Caseinat-Agar .....(Merck 105409)

Peptone from meat 4 g

Meat extract 2 g

Peptone from casein 2 g

Calcium caseinate 3.5g

Calcium chloride dehydrate 0.2 g

tri-potassium citrate monohydrate 0.35 g

di-sodium hydrogen phosphate 0.105 g

Potassium dihydrogen phosphate 0.035 g

Sodium chloride 5 g

Agar-agar 13 g

DNase-Test Agar .....(Merck 110449)

Tryptose 20 g

Sodium chloride 5 g

Deoxyribonucleic acid 2 g

Agar-agar 15 g

2.4. List of chemicals, reagents and dyes

Agar-Agar .....(Merck 101613)

Sodium Chloride .....(Merck 106404)

Skim Milk Powder .....(Fluka 70166)

Egg Yolk Emulsion.....(Oxoid SR0047C)

L-Lysine Monohydrochloride.....(Merck 105700)

L-Ornithine Monohydrochloride.....(Merck 106906)

L-Arginine Monohydrochloride.....(Merck 101543)

L-Cysteine Hydrochloridemonohydrate .....(Merck 102839)

D-Sorbitol.....(Fluka 85532)

L(+)-Arabinose.....(Merck 101492)

Myo-Inositol.....(Merck 104728)

D (+)-Glucose-Monohydrate.....(Merck 108342)

D (-)-Mannitol.....(Merck 105982)

D (+)-Cellobiose .....(Sigma C7252)

Lactose-Monohydrate .....(Merck 107657)

Sucrose.....(Merck 107651)

Vibriostatic Agent O/129 (10µg).....(Oxoid DD14)

Vibriostatic Agent O/129 (150µg).....(Oxoid DD15)

Chrome Azurol S .....(Sigma 199532)

HDTMA (hexadecyltrimethylammoniumbromide).....(Merck 814119)

PIPES (piperazine-*N*,*N*'-bis[2-ethanesulfonic acid]) .....(Merck 110220)

L-asparagine.....(AppliChem A3721)

Griess' Reagent for Nitrite.....(Merck 109023)

Kovac's Reagent .....(Merck 109293)

2.5. List of antimicrobial agents

Imipenem (10µg).....(Oxoid CT0455B)

Piperacillin/Tazobactam (110µg).....(Oxoid CT0725B)

Cefotaxime (30µg).....(Oxoid CT0166B)

Amoxycillin/Clavulanic Acid (30µg).....(Oxoid CT0223B)

Erythromycin (15µg).....(Oxoid CT0020B)

Ceftriaxone (30µg).....(Oxoid CT0417B)

Ampicillin (10µg).....(Oxoid CT0003B)

Vancomycin (30µg).....(Oxoid CT0058B)

Chloramphenicol (30µg).....(Oxoid CT0013B)

Cephazolin (30µg).....(Oxoid CT0011B)

Ciprofloxacin (5µg).....(Oxoid CT0425B)

Gentamicin (10µg).....(Oxoid CT0024B)

Amikacin (30µg).....(Oxoid CT0107B)

Polymyxin B (300 units).....(Oxoid CT0044B)

Piperacillin (100µg).....(Oxoid CT0199B

Sulphamethoxazole/Trimethoprim (25µg).....(Oxoid CT0052B)

Tetracycline (30µg).....(Oxoid CT0054B)

Enrofloxacin (5µg).....(Oxoid CT0639B)

3. RESULTS

In this research, a total of 111, freshwater fish (*Oncorhynchus mykiss*) (n=37), seawater fish (*Sparus aurata*) (n=37) and ground beef samples (n=37) were tested to analyze motile *Aeromonas* spp. In 49 samples, motile *Aeromonas* spp. was found in a prevalence of 44.1%. The occurrence of motile *Aeromonas* isolates in the samples analyzed has been summarized in Table 5. Among the positive samples, 31 (27.9%) sample was identified as *A. caviae*, 9 (8.1%) as *A. hydrophila* and 9 (8.1%) as *A. veronii* biovar sobria. In freshwater the most frequently recovered species were *A. caviae*, *A. veronii* biovar sobria and *A. hydrophila*. In seawater the most frequently isolated species were *A. caviae* and *A. hydrophila*. *A. veronii* biovar sobria were not isolated. In ground beef samples only *A. caviae* were isolated. The distributions of percentages of motile Aeromonas isolates among motile species were shown in Figure 1. Of the 49 isolates obtained, *A. caviae* was the dominant species, followed by *A. hydrophila* and *A. veronii* biovar sobria.

Table 5. Occurrence of motile *Aeromonas* species in the different samples examined

Motile *Aeromonas* spp.	Freshwater fish (n=37)	Seawater fish (n=37)	Ground beef (n=37)	Total (n=111)
A. caviae	19 (17.1)*	8 (7.2)	4 (3.6)	31 (27.9)
A. hydrophila	8 (7.2)	1 (0.9)	nd	9 (8.1)
A. veronii biovar sobria	9 (8.1)	nd	nd	9 (8.1)
Total	**36 (32.4)**	**9 (8.1)**	**4 (3.6)**	**49 (44.1)**

*, percentage of positive isolates; nd, none detected; n, number of tested samples

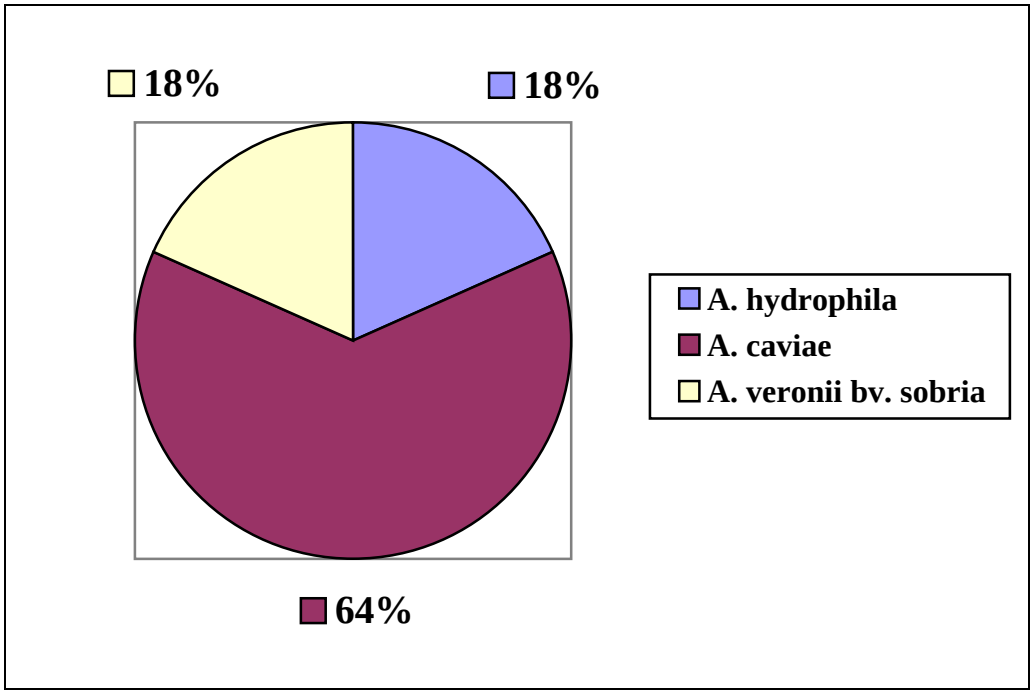

Figure 1. Distribution of percentage among motile *Aeromonas* spp.

In addition, 89 (80.1%) out of the 111 samples were found to be positive the other *Aeromonas* spp.; 40 (36%) out of 89 samples were *A. salmonicida*, 14 (12.6%) were *A. schubertii*, 10 (9%) were *A. bestiarum*, 5 (4.5%) were *A. veronii* biovar veronii, 4 (3.6%) were *A. molluscorum*, 4 (3.6%) were *A. allosaccharophila*, 4 (3.6%) were *Aeromonas* spp., 3 (2.7%) were *A. bivalvium*, 2 (1.8%) were *A. eucrenophila*, 1 (0.9%) was *A. simiae*, 1 (0.9%) was *A. encheleia*, 1 (0.9%) was *A. jandaei* and 3.6% of the *Aeromonas* spp. remained unidentified. In freshwater the most abundant species were *A. salmonicida*, *A. bestiarum* and *A. schubertii*. In seawater the most frequently recovered species were *A. salmonicida* and *A. schubertii*. In ground beef *A. salmonicida* was the most abundant. It is displayed in Table 6. The distribution of percentage among the other *Aeromonas* species has been summarized in Figure 2. Of the 89 isolates obtained, *A. salmonicida* was the

dominant species, followed by *A. schubertii, A. bestiarum*, were *A. veronii* biovar veronii, *A. molluscorum, A. allosaccharophila*, *Aeromonas* spp., *A. bivalvium, A. eucrenophila, A. simiae, A. encheleia* and *A. jandaei*.

Table 6. Occurrence of other *Aeromonas* species in the different samples examined

Other *Aeromonas* spp.	Freshwater fish (n=37)	Seawater fish (n=37)	Ground beef (n=37)	Total (n=111)
A. salmonicida	9 (8.1)*	14 (12.6)	17 (15.3)	40 (36)
A. schubertii	5 (4.5)	6 (5.4)	3 (2.7)	14 (12.6)
A. bestiarum	7 (6.3)	2 (1.8)	1 (0.9)	10 (9)
A. veronii bv. veronii	3 (2.7)	1 (0.9)	1 (0.9)	5 (4.5)
A. molluscorum	2 (1.8)	2 (1.8)	nd	4 (3.6)
A. allosaccharophila	3 (2.7)	1 (0.9)	nd	4 (3.6)
Aeromonas spp.	1 (0.9)	nd	3 (2.7)	4 (3.6)
A. bivalvium	2 (1.8)	1 (0.9)	nd	3 (2.7)
A. eucrenophila	2 (1.8)	nd	nd	2 (1.8)
A. simiae	1 (0.9)	nd	nd	1 (0.9)
A. encheleia	1 (0.9)	nd	nd	1 (0.9)
A. jandaei	1 (0.9)	nd	nd	1 (0.9)
Total	**37 (33.3)**	**27 (24.3)**	**25 (22.5)**	**89 (80.1)**

*, percentage of positive isolates; nd, none detected; n: no of tested samples;

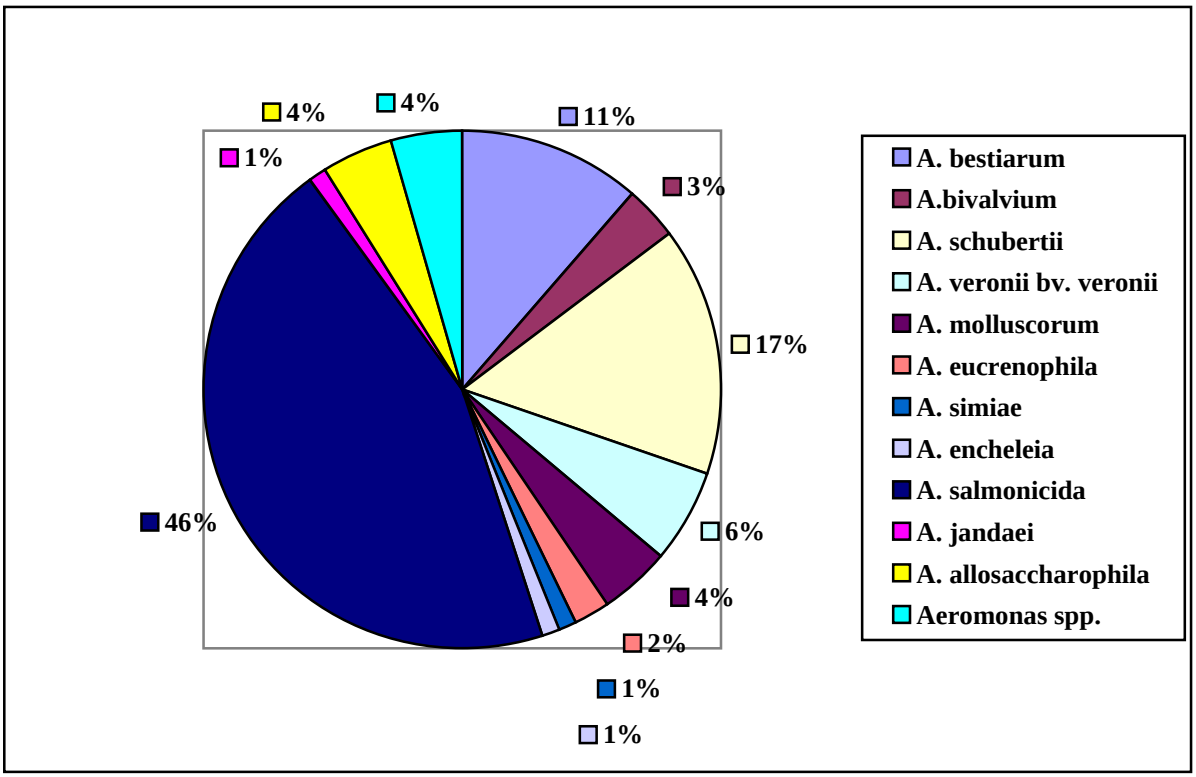

Figure 2. Distribution of percentage among other *Aeromonas* spp.

In the present study, extracellular enzyme activity and virulence characteristics of 138 *Aeromonas* isolates were studied. In general, lecithinase production was observed in 117 isolates (84.7%), being 68 isolates from freshwater, 29 from seawater and 20 from ground beef. Proteolytic activity was determined by two different methods. Proteolytic activity was different when evaluated with the calcium caseinate agar (88.4%) than with skim milk agar (71.7%). In addition, these results showed that approximately 93% of the *Aeromonas* spp. produces lipase enzymes. Besides, all the 138 isolates of *Aeromonas* were tested for DNase activity and 88.4 % of isolates were positive for DNase whereas 11.6% were negative. Of the present isolates more than 60% showed positive β-hemolytic activity on sheep blood agar. Another virulence characteristic, slime production was found in 24 (17.4 %) of 138 *Aeromonas* isolates whereas 114 (82.6%) isolates of *Aeromonas* isolates were not slime producers. Nevertheless, 138 isolates of *Aeromonas* spp. were screened for

siderophore production by the CAS assay of Schwyn and Neilands (1986) on CAS agar plates, and siderophore production was found in 133 (96.3%) of 138 *Aeromonas* isolates.

Table 7 represents the incidence of extracellular enzyme activities and virulence characteristics of the 138 *Aeromonas* isolates from freshwater fish, seawater fish and ground beef samples. The activity of DNAse, lipase, lecithinase, and protease with calcium caseinate and siderophore production was the most common among *Aeromonas* spp. Overall, the results indicated that the highest enzyme activity and virulence characteristic were observed in freshwater fish samples except the activity of protease with skim milk and siderophore production.

Table 7. Incidence of extracellular enzyme activities and virulence characteristics of the 138 *Aeromonas* isolates from freshwater fish, seawater fish and ground beef samples.

Source	No. of isolates tested	Lecithinase activity	Protease activity		Lipase activity	DNase activity	Hemolysin activity	Slime production	Siderophore production
			Calcium caseinate	Skim milk					
Freshwater fish	73	68 (93.1)*	65 (89)	53 (72.6)	69 (94.5)	66 (90.4)	47 (64.4)	16 (21.9)	70 (95.9)
Seawater fish	36	29 (80.5)	32 (88.9)	24 (66.7)	33 (91.6)	31 (86.1)	24 (66.7)	6 (16.7)	34 (94.4)
Ground beef	29	20 (68.9)	25 (86.2)	22 (75.8)	26 (89.6)	25 (86.2)	22 (75.8)	2 (6.9)	29 (100)

* percentage of positive isolates

Potential virulence factors of motile *Aeromonas* spp. which contribute to their pathogenicity include the production of extracellular enzymes. The incidence of extracellular enzyme activities and virulence characteristics of the motile *Aeromonas* spp. isolated from freshwater fish, seawater fish and ground beef samples was exhibited in Table 8. All the *Aeromonas hydrophila* isolates produced the extracellular enzyme of lecithinase, protease, lipase, DNase, hemolysin. However, none of *A. hydrophila* isolates produced slime. Absence of slime production in the *A. hydrophila* isolates was unexpected. The present study also revealed that 100% of *A. veronii* bv. sobria exhibited the activity of lecithinase, lipase, DNase, hemolysin and siderophore production. In addition, more than 85% of the *A. veronii* bv. sobria isolates produced protease enzyme, while about 10% of the isolates synthesized siderophores. On the other hand, lipase activity was determined for all *A. caviae* isolates, as expected. The production of protease was detected in 96.7% with calcium caseinate agar and 64.5% with skim milk agar of *A. caviae* isolates. DNase and siderophore were detected in 93.5% of *A. caviae* isolates. In addition, lecithinase, slime and hemolysin production were found in 77.4%, 45.2%, and 25.8% of *A. caviae* isolates, respectively.

Table 8. Incidence of extracellular enzyme activities and virulence characteristics of the motile *Aeromonas* spp.

Motile *Aeromonas* spp.	No. of isolates tested	Lecithinase activity	Protease activity		Lipase activity	DNase activity	Hemolysin activity	Slime production	Siderophore production
			Calcium caseinate	Skim milk					
A. caviae	31	24 (77.4)*	30 (96.7)	20 (64.5)	31 (100)	29 (93.5)	8 (25.8)	14 (45.2)	29 (93.5)
A. hydrophila	9	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	nd	8 (88.9)
A. veronii bv. sobria	9	9 (100)	8 (88.9)	8 (88.9)	9 (100)	9 (100)	9 (100)	1 (11.1)	9 (100)

*percentage of positive isolates; nd, none detected

The incidence of extracellular enzyme activities and virulence characteristics of other *Aeromonas* spp. was shown in Table 9. The production of lecithinase was common among other *Aeromonas* species. The highest producers of lecithinase were *A. bestiarum* 100%, *A. veronii* bv. veronii 100%, *A. allosaccharophila* 100%, *A. eucrenophila* 100%, *A. simiae* 100%, *A. encheleia* 100%, *A. jandaei* 100%, followed by 92.5% of the *A. salmonicida*, 75% of the *A. molluscorum*, 66.7% of the *A. bivalvium*, 50% of the *Aeromonas* spp., and 21.4% of the *A. schubertii*. This study also presented protease activity which was found in 72 (80.8%) with calcium caseinate agar and 60 (67.4%) with skim milk agar of 89 other *Aeromonas* isolates. Specifically, 100% of *A. salmonicida*, *A. molluscorum*, *A. allosaccharophila*, *A. bivalvium*, *A. eucrenophila*, *A. encheleia* and *A. jandaei* were producers of protease with calcium caseinate agar. The lipase activity was also common among other *Aeromonas* species. The highest activity of lipase was 100% of *A. bestiarum*, *A. veronii* bv. veronii, *A. molluscorum*, *A. allosaccharophila*, *A. eucrenophila*, *A. simiae*, *A. encheleia*, and *A. jandaei*, followed by 97.5% of the *A. salmonicida*, 75% of the *Aeromonas* spp., 66.7% of the *A. bivalvium*, and 50% of the *A. schubertii*.

The DNase activity was positive in 40 (100%) *A. salmonicida*, 10 (100%) *A. bestiarum*, 5 (100%) *A. veronii* bv. veronii, 4 (100%) *A. allosaccharophila*, 3 (100%) *A. bivalvium* and *A. molluscorum* (75%), 2 (14.3%) were *A. schubertii*, (50%) *Aeromonas* spp. and (100%) *A. eucrenophila*, and 1 (100%) were *A. encheleia* and *A. jandaei*. Besides, *A. simiae* isolates were not DNase producers. This study also showed that 100% of *A. bestiarum*, *A. veronii* bv. veronii, *A. eucrenophila*, *A. jandaei*, 95% of *A. salmonicida*, 75% of *A. molluscorum* and *Aeromonas* spp., 50% of *A. allosaccharophila* and 7.1% of *A. schubertii* were

positive for β hemolysis. *A. bivalvium*, *A. simiae* and *A. encheleia* did not indicate β hemolytic activity. Among the other *Aeromonas* isolates, slime production was found in 50% of *A. allosaccharophila* followed by 33.3% of *A. bivalvium*, 21.4% of *A. schubertii* and 7.5% of *A. salmonicida*. On the other hand, 80% of the other *Aeromonas* isolates or more had siderophore production.

Table 9. Incidence of extracellular enzyme activities and virulence characteristics of the other *Aeromonas* spp.

Other *Aeromonas* spp.	No. of isolates tested	Lecithinase activity	Protease activity		Lipase activity	DNase activity	Hemolysin activity	Slime production	Siderophore production
			Calcium caseinate	Skim milk					
A. salmonicida	40	37 (92.5)*	40 (100)	38 (95)	39 (97.5)	40 (100)	38 (95)	3 (7.5)	39 (97.5)
A. schubertii	14	3 (21.4)	2 (14.3)	3 (21.4)	7 (50)	2 (14.3)	1 (7.1)	3 (21.4)	13 (92.8)
A. bestiarum	10	10 (100)	9 (90)	8 (80)	10 (100)	10 (100)	10 (100)	nd	10 (100)
A. veronii bv. veronii	5	5 (100)	4 (80)	1 (20)	5 (100)	5 (100)	5 (100)	nd	4 (80)
A. molluscorum	4	3 (75)	4 (100)	nd	4 (100)	3 (75)	3 (75)	nd	4 (100)
A. allosaccharophila	4	4 (100)	4 (100)	3 (75)	4 (100)	4 (100)	2 (50)	2 (50)	4 (100)
Aeromonas spp.	4	2 (50)	2 (50)	1 (25)	3 (75)	2 (50)	3 (75)	nd	4 (100)
A. bivalvium	3	2 (66.7)	3 (100)	3 (100)	2 (66.7)	3 (100)	nd	1 (33.3)	3 (100)
A. eucrenophila	2	2 (100)	2 (100)	1 (50)	2 (100)	2 (100)	2 (100)	nd	2 (100)
A. simiae	1	1 (100)	nd	nd	1 (100)	nd	nd	nd	1 (100)
A. encheleia	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	nd	nd	1 (100)
A. jandaei	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	nd	1 (100)

* percentage of positive isolates; nd, none detected

The results of antimicrobial susceptibility of 138 *Aeromonas* isolates from freshwater fish, seawater fish and ground beef are shown in Table 10. According to this, *Aeromonas* spp. had the highest resistance to ampicillin (94.9%) and vancomycin (88.4%). However, more than 90% of the isolates were susceptible to piperacillin/tazobactam, piperacillin, imipenem, enrofloxacin, ceftriaxone, cefotaxime, amikacin, gentamicin, polymyxin B, and ciprofloxacin. Multidrug-resistance pattern was observed in 63% of the isolates to at least three or more antimicrobial agents.

Table 10. Susceptibility rates of antimicrobial agents tested against the 138 *Aeromonas* isolates.

Antimicrobial Agents	Conc. (µg/disc)	No %		
		R	I	S
Piperacillin/tazobactam	110	3 (2.1)	6 (4.4)	129 (93.5)
Piperacillin	100	2 (1.5)	9 (6.5)	127 (92)
Chloramphenicol	30	7 (5.1)	13 (9.4)	118 (85.5)
Ampicillin	10	131 (94.9)	3 (2.2)	4 (2.9)
Imipenem	10	1 (0.7)	6 (4.4)	131 (94.9)
Enrofloxacin	5	1 (0.7)	10 (7.3)	127 (92)
Ceftriaxone	30	2 (1.5)	9 (6.5)	127 (92)
Vancomycin	30	122 (88.4)	9 (6.5)	7 (5.1)
Cephazolin	30	76 (55.1)	24 (17.4)	38 (27.5)
Tetracycline	30	24 (17.4)	22 (15.9)	92 (66.7)
Cefotaxime	30	2 (1.5)	10 (7.3)	126 (91.2)
Amikacin	30	0	6 (4.4)	132 (95.6)
Erythromycin	15	22 (15.9)	116 (84.1)	0
Gentamicin	10	4 (2.9)	4 (2.9)	130 (94.2)
Polymyxin B	300 units	0	1 (0.7)	137 (99.3)
Ciprofloxacin	5	0	4 (2.9)	134 (97.1)
SXT	25	11 (8)	4 (2.9)	123 (89.1)
Amoxicillin / clavulanic acid	30	19 (13.8)	25 (18.1)	94 (68.1)

Conc, concentration; R, resistant; I, intermediate; S, susceptible; SXT, Sulphamethoxazole/trimethoprim

The antimicrobial resistance and susceptible patterns obtained with the 49 motile *Aeromonas* isolates against 18 antimicrobial agents are shown in Figures 3 and 4. All isolates showed 100% of resistance to ampicillin. In addition, the highest resistance encountered was 91.8% to vancomycin. A low percentage of motile *Aeromonas* isolates was resistant to cephazolin (34.7 %), erythromycin (10.2%), tetracycline (8.1%), and trimethoprim-sulphamethoxazole (8.1%). In contrast, more than 90% of the isolates were susceptible to ceftriaxone, cefotaxime, piperacillin/tazobactam, piperacillin, imipenem, chloramphenicol, enrofloxacin, amikacin, gentamicin, polymyxin B, and ciprofloxacin.

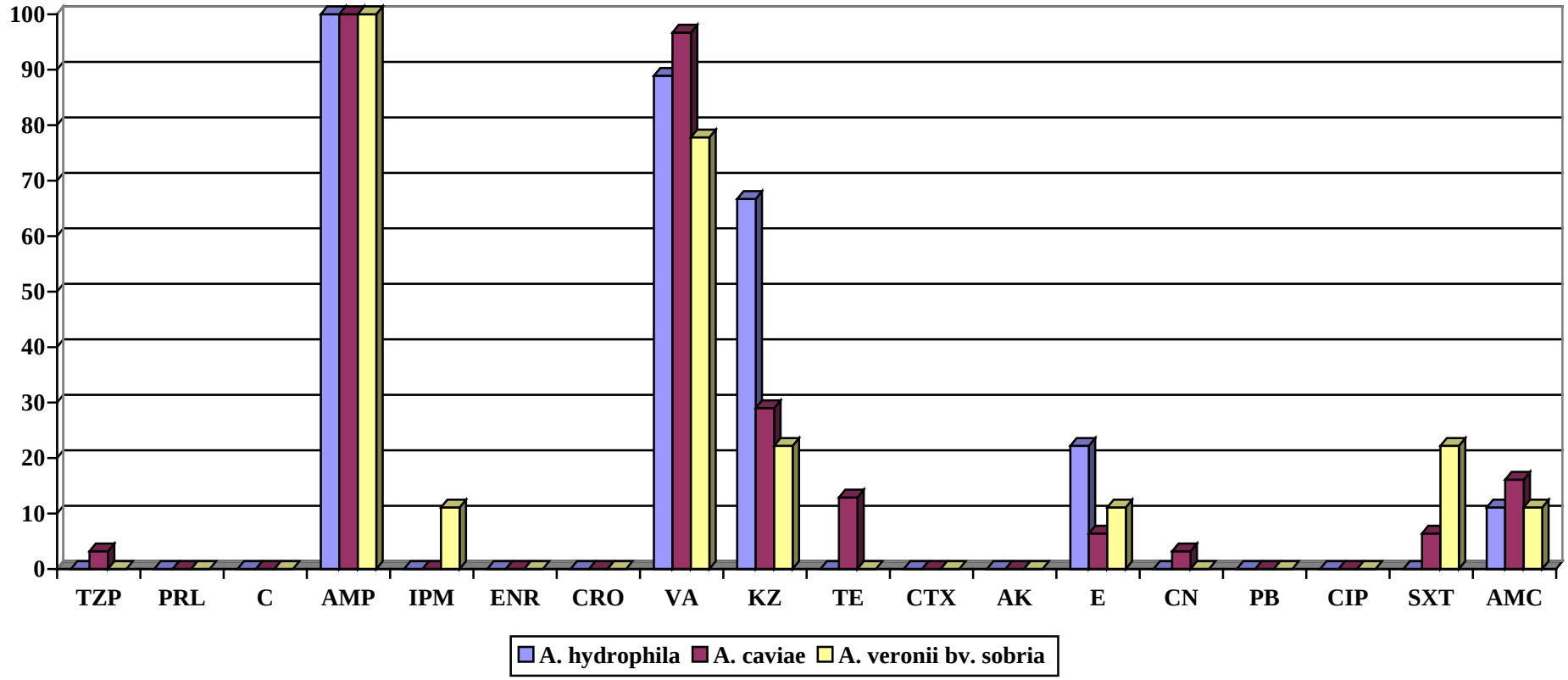

Figure 2. The percentage of antimicrobial resistance of motile *Aeromonas* spp.

TZP, piperacillin/tazobactam; PRL, piperacillin; C, chloramphenicol; AMP, ampicillin; IPM, imipenem; ENR, enrofloxacin; CRO, ceftriaxone, VA, vancomycin; KZ, cephazolin; TE, tetracycline; CTX, cefotaxime; AK, amikacin; E, erythromycin; CN, gentamicin; PB, polymyxin B; CIP, ciprofloxacin; SXT, Sulphamethoxazole/trimethoprim; AMC, amoxicillin / clavulanic acid.

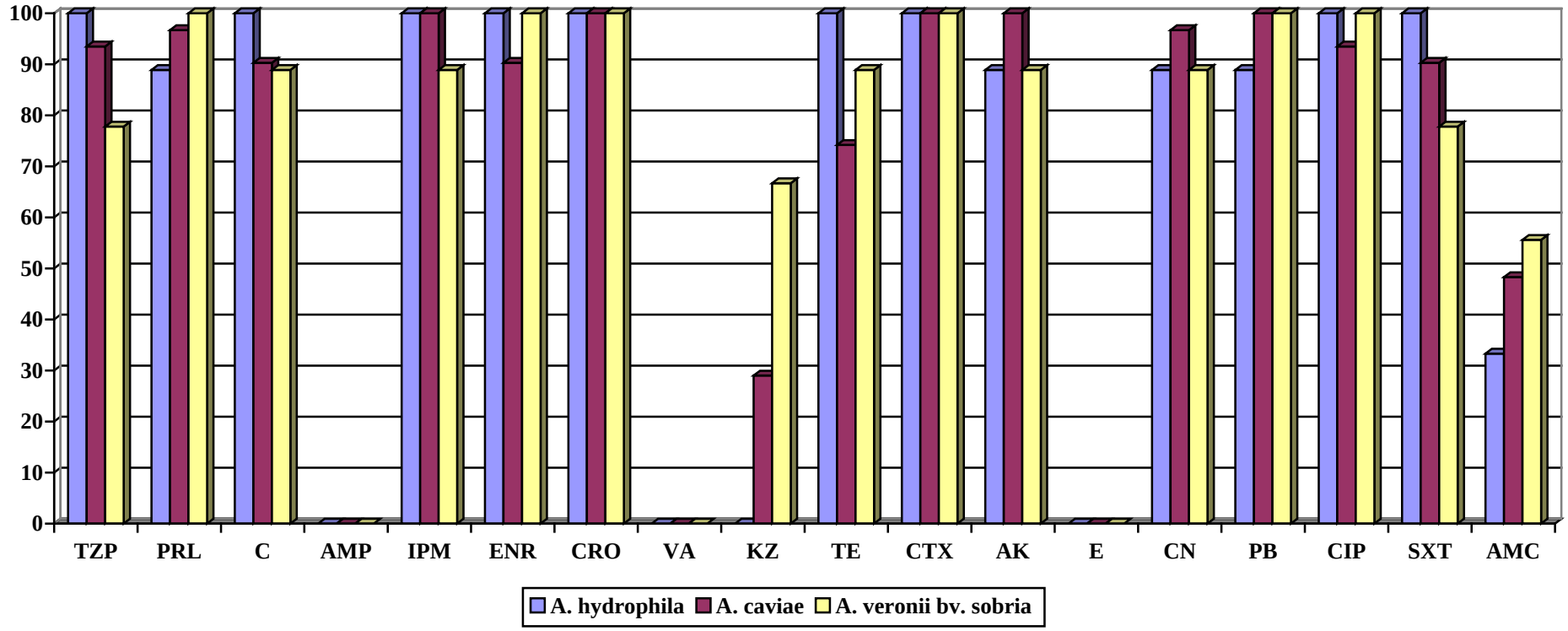

Figure 3. The percentage of antimicrobial susceptibility of motile *Aeromonas* spp.

TZP, piperacillin/tazobactam; PRL, piperacillin; C, chloramphenicol; AMP, ampicillin; IPM, imipenem; ENR, enrofloxacin; CRO, ceftriaxone, VA, vancomycin; KZ, cephazolin; TE, tetracycline; CTX, cefotaxime; AK, amikacin; E, erythromycin; CN, gentamicin; PB, polymyxin B; CIP, ciprofloxacin; SXT, Sulphamethoxazole/trimethoprim; AMC, amoxicillin / clavulanic acid.

4. DISCUSSION

The motile *Aeromonas* species including *A. hydrophila*, *A. caviae* and *A. veronii* biovar sobria are emerging as important pathogens in humans, causing a variety of extraintestinal and systemic infections, as well as gastrointestinal infections (Tomas, 2012). In this research, motile *Aeromonas* spp. were isolated from 49 (44.1%) of the 111 samples tested. In the present study the most prevalent species in freshwater fish (*Oncorhynchus mykiss*), seawater fish (*Sparus aurata*) and ground beef were *A. caviae* (27.9%), while the other species belonged to *A. hydrophila* (8.1%) and *A. veronii* biovar sobria (8.1%). The results obtained from our research agree with the fact that *A. caviae* was the most frequent species isolated from different samples in previous studies by some researchers. Altwegg et al. (1990) detected *A. caviae* as the most prevalent species in humans. Soler et al. (2002) announced that in freshwater the most frequently recovered species were *A. veronii*, *A. hydrophila* and *A. caviae*, while in seawater *A. caviae* was the most abundant. On the other hand, some researchers showed that *A. hydrophila* was the most common species from various samples including ground beef (Küplülü and Sarımehmetoğlu, 2000), fish (Durmaz and Türk, 2009), river (Paniagua et al., 1990), retail meats (Osman et al., 2012), turkey meat (Koca and Sarımehmetoğlu, 2009), freshwater fish (Hatha et al., 2005), well water (Altanlar et al., 2003), rainbow trout (Korun and Toprak; 2010). On the other hand, *A. veronii* biovar sobria has been reported as the dominant species in fish samples in another study (Radu et al., 2003).

The other *Aeromonas* species such as *Aeromonas veronii*, *Aeromonas jandaei*, *Aeromonas schubertii*, *Aeromonas bestiarum*, *Aeromonas salmonicida*, and *Aeromonas eucrenophila* are infrequently or rarely involved in human gastroenteritis (Janda and Abbott 2010). In our study, *A. salmonicida* was the dominating species among other *Aeromonas* spp., while *A. schubertii* is the second most frequently isolated species. Castro-Escarpulli et al. (2003) reported *A. salmonicida* as the most prevalent species followed by *A. bestiarum*, *A. veronii*, and *A. encheleia*. Different results were found by Razzolini et al. (2010) who reported that out of 37 isolates from collective reservoirs, *Aeromonas* spp. (67.6%) were the most frequently followed by *Aeromonas encheleia* (13.5%) and *A. allosaccharophila* (8.1%).

Aeromonas species secretes many extracellular proteins, such as lecithinase, lipase, protease, DNase, and hemolysin. These proteins are known as virulence factors that cause disease in fish and humans (Martin-Carnahan and Joseph, 2005). In the present study, most of the isolated *Aeromonas* spp. was found to produce extracellular enzymes including, protease, lipase, DNase, hemolysin and lecithinase.

Lecithinases (phospholipases) are associated with virulence in bacterial diseases. This enzyme usually acts on the animal cell membrane by insertion into the membrane, forming a pore that result in cell lysis, or by enzymatic attack on phospholipids, which destabilizes the membrane (Galinda and Chopra, 2007). In this study, lecithinase enzyme was observed in 117 *Aeromonas* isolates (84.7%), being 68 isolates from freshwater, 29 from seawater and 20 from ground beef. Among motile *Aeromonas* spp., 100% of *A. hydrophila* and *A. veronii* bv. sobria were producers of lecithinase, followed by 77.4 % of *A. caviae*. Lecithinase activity

of *A. hydrophila* (100%) has been previously reported by Saidi et al. (2011) and the results obtained support our findings.

Aeromonas spp. produce a wide range of proteases, which cause tissue damage and aid in establishing an infection by overcoming host defenses and by providing nutrients for cell proliferation (Galinda and Chopra, 2007). In the present study, proteolytic activity was determined using two different media: calcium caseinate agar and skim milk agar. Protease activity was found in 88.4% using calcium caseinate agar and 71.7% using skim milk agar of all *Aeromonas* isolates. All the motile *Aeromonas* isolates could utilize a large number of the protein substrates such as albumin, casein, fibrinogen, and gelatin, proteolytic activity is a constant property of motile *Aeromonas* spp. (Shotts et al., 1985). In the present study all isolates of *A. hydrophila* (100%) showed proteolytic activity on two agar plates. Proteolytic activity was also found in 96.7% of *A. caviae*, and 88.9 % of *A. veronii* bv. sobria isolates. Stratev et al. (2012) observed protease activity in all *A. hydrophila* and *A. veronii* biovar sobria isolates and in 81% of *A. caviae* isolates. In a study, all of the 73 isolates of *A. hydrophila* had proteolytic activity (Illanchezian et al., 2010).

In addition, our results showed that of 138 isolates tested, 128 representing approximately 93% produced lipase enzymes. Lipases are produced by a wide range of bacteria. They may provide nutrients and constitute virulence factors by interacting with human leukocytes or by affecting several immune systems functions through free fatty acids generated by lipolytic activity (Galinda and Chopra, 2007). In present research, lipase activity was detected in all motile *Aeromonas* isolates. The results of lipase activity were in line with previously

published reports (Castro-Escarpulli et al., 2003; Saidi et al., 2011 and Sreedharan et al., 2012) exhibiting 100% of lipolytic activity of motile *Aeromonas* spp.

The association of nucleases with pathogenicity has not yet been confirmed, but reports have indicated that it participates in the development of host infection (Nam et al., 2004). In our study, the DNase activity was detected in 88.4% of the 138 isolates of *Aeromonas*. Besides of the 49 motile aeromonads isolated in the present study 47 were positive for DNase activity. Of these 9 (100%) were *A. hydrophila*, 9 (100%) *A. veronii* bv. sobria and 29 (93.5%) *A. caviae*. Castro-Escarpulli et al. (2003) reported 100% of *A. hydrophila* isolates were positive for DNase activity. In a study reported by Sreedharan et al. (2012), only 48.3% of the motile *Aeromonas* isolates displayed DNase activity.

Hemolytic proteins are commonly isolated from pathogenic bacteria, and β-hemolysins are one of the important bacterial virulence factors (Pandey et al., 2010). The production of hemolytic toxins has been also regarded as strong evidence of pathogenic potential in aeromonads (Galinda and Chopra, 2007). In our study, among motile *Aeromonas* species, 100% of *A. hydrophila* and *A. veronii* bv. sobria and 77.4 % of *A. caviae* showed β-hemolysis. The present study also revealed that *A. bestiarum*, *A. veronii* bv. veronii, *A. eucrenophila*, *A. jandaei*, *A. salmonicida*, *A. molluscorum*, and *A. allosaccharophila* exhibited high rates of β hemolytic activity except *A. schubertii*. Production of β-hemolytic activity was reported by Paniagua et al. (1990), Castro-Escarpulli et al. (2003), Radu et al. (2003), Hatha et al. (2005), Razzolini et al. (2010), Singh et al. (2010), Sreedharan et al. (2012). Their results also revealed high rates of β-hemolytic activity that the presence of *Aeromonas* spp. capable of producing β-hemolysin poses a public health concern.

In the present study, slime production was found in 24 (17.4 %) of 138 *Aeromonas* isolates whereas 114 (82.6%) *Aeromonas* isolates were not slime producers. Slime is a viscous glycoconjugate material, produced by most of the gram negative bacteria. The slime is highly significant to the pathogenesis; it appears to inhibit the neutrophil, chemotaxis, phagocytosis and antimicrobial drugs (Tomas, 2012). Among the motile *Aeromonas* isolates, 45.2% of *A. caviae*, followed by 11.1% of *A. veronii* bv. sobria from fish and ground beef samples produced slime, while none of *A. hydrophila* isolates had slime. The absence of slime production in *A. hydrophila* isolates was unexpected. In contrast to our results, Illanchezian et al. (2010) observed a slime production in 78.1% of *A. hydrophila* which was also confirmed in another research that, Saidi et al. (2011) where 65% of positive isolates were *A. hydrophila*. Slime production reflects the microorganism's capacity to adhere to specific host tissues and thereby to produce invasive micro colonies and diverse illness (Galinda and Chopra, 2007).

Efficient mechanisms for iron acquisition from the host during an infection are considered essential for virulence. Siderophores are low molecular weight compounds with high affinities for various forms of iron (Galinda and Chopra, 2007). In our study, siderophore production was found in 133 (96.3%) of 138 isolates. Some investigators found that *Aeromonas* spp. showed different siderophore activity (Zywno et al., 1992; Fernandez et al., 1998; Santos et al., 1999) in their studies. The production of siderophores was common in all motile *Aeromonas* species. The highest producer of siderophores was *A. veronii* bv. sobria at a rate of 100%, followed by 93.5% of *A. caviae* and 88.9 % of the *A. hydrophila*. Erdem et al. (2010) showed that a total of 78 isolates of *Aeromonas* spp., including

A. hydrophila, *A. caviae*, and *A. veronii* bv. sobria were determined as siderophore producer.

The resistant bacteria to antimicrobial agents are a public health problem. Wide use of antimicrobial agents to treat bacterial infections and to feed food animals for promoting growth resulted in a global increase in antibiotic resistance among pathogenic bacteria. The antimicrobial resistant *Aeromonas* species can be transferred to human through contaminated food (Vivekanandhan et al., 2002). In the present study, all the isolates showed varying degree of resistance to the 18 antimicrobial agents (Table 10). High levels of resistance were against ampicillin (94.9%) and vancomycin (88.4%). This is supported by Castro-Escarpulli et al. (2003) who have observed 100% of ampicillin resistance. Moreover, in our study, around 55% of the isolates were resistant to cephazolin. None of the *Aeromonas* isolates were resistant to amikacin, polymyxin B, and ciprofloxacin in this study. In contrast, Castro-Escarpulli et al. (2003) reported 85.7 % of polymyxin B, 41.5% of ciprofloxacin, and 23% of the amikacin resistant isolates. Our findings also revealed that resistance against piperacillin/tazobactam, piperacillin, chloramphenicol, imipenem, enrofloxacin, ceftriaxone, cefotaxime and gentamicin was low (<20%). These results are partially supported by Castro-Escarpulli et al. (2003) and Matyar et al. (2010).

According to our results of the antimicrobial susceptibility, as seen in Figures 3 and 4, all motile *Aeromonas* spp. were resistant to ampicillin, due to the inherent resistance of *Aeromonas* spp. (Hatha et al., 2005). In several studies, the results of the antimicrobial susceptibility of the motile *Aeromonas* isolates were in line with our results (Hatha et al., 2005; Sreedharan et al., 2012).

In contrast, all the motile *Aeromonas* isolates were susceptible to piperacillin, chloramphenicol, enrofloxacin, ceftriaxone, cefotaxime, amikacin, polymyxin B, and ciprofloxacin in this study. Similar findings have been recorded from Sreedharan et al. (2012) who have observed 100% susceptibility to piperacillin, chloramphenicol, ceftriaxone, amikacin, ciprofloxacin, enrofloxacin. We also observed different pattern from Sreedharan et al. (2012) with respect to resistance to polymyxin B. Durmaz and Türk (2009) also found motile *Aeromonas* spp. isolated from fish samples, had no resistance to ciprofloxacin like our findings. In our research, the multidrug-resistance pattern was observed in 63% of the isolates to at least three or more antimicrobial agents. The increase in antimicrobial resistance poses a growing challenge in the treatment of *Aeromonas* infections in fish as well as in humans. If such antimicrobial resistant aeromonads, which are true human and aquatic pathogens, happen to multiply within food such as fresh water fish, seawater fish and ground beef, they obviously may turn out to be a threat to public health (Sreedharan et al., 2012).

5. CONCLUSION

Foodborne diseases that are the result of ingestion of food contaminated with microorganisms include a wide spectrum of illnesses and are considered to be a reason for important public health problem worldwide. The contamination of food may occur at any stage in the process from food production to consumption.

Fish and fish products are of great importance worldwide due to their nutritional value, clear health benefits and wholesome properties. Though seafood is nutritive, they are highly prone to contamination. They act as a vehicle for pathogenic bacteria naturally occurring in the aquatic environment referred as indigenous or derived from post harvested contamination may lead to cause human morbidities and mortalities worldwide.

Ground beef is made from meat, trimmings, and fat and has a large surface area that favors growth of aerobic bacteria. The knives of meat grinder, if not properly sanitized or washed, may be the source of contamination. Furthermore, one heavily contaminated piece of meat may be sufficient to contaminate an entire lot of ground meat. Pathogens are associated with these types of products.

Motile *Aeromonas* spp. is recognized as potential foodborne pathogens. High prevalence of *Aeromonas* spp. in the environment is considered a threat to public health, as infections caused by these pathogens are generally the result of ingestion of contaminated water or food.

In this research, a total of 111 freshwater fish, seawater fish and ground beef samples were evaluated for the presence of *Aeromonas* spp. Motile *Aeromonas*

spp. were isolated from 49 (44.1%) of the samples tested. In addition, 89 (80.1%) of the samples were positive for other *Aeromonas* spp. All *Aeromonas* spp. exhibited high rates of extracellular enzyme activity and virulence factors. Moreover, the multidrug-resistance was observed in 63% of the 138 *Aeromonas* isolates. Different species of *Aeromonas* were present in a variety of samples. The incidence of *Aeromonas* spp. may be considered remarkable.

Motile *Aeromonas* spp. is capable of expressing a number of virulence factors such as extracellular enzymes, siderophores, hemolysins, and slime layer. The increasing antimicrobial resistance among them also causes health problems in human beings. These characteristics make it to be an emerging pathogen posing several threats to humans.

Motile *Aeromonas* species are more commonly isolated from wide variety of sources including seafood and have been linked to major fish kills around the world, resulting in great economic losses. The opportunistic bacteria have also been associated with several categories of human infections. Foods of animal origin have been considered as important sources of *Aeromonas* spp. infection.

The results of the study indicate that the freshwater fish, seawater fish and ground beef creates a potential hazard to consumers. The contamination of *Aeromonas* spp. can be closely related to harvest of fish products from polluted water, low quality of beef carcass used, poor temperature control during processing and storage, inadequate cleaning and disinfection of both equipment and surfaces such as floors or poor personal hygiene and use of untrained personnel. The prevention of food contamination from these sources needs to improve the hygienic practices. Proper sanitation should be used during processing. Consumption of raw animal products or meat cooked at inadequate

temperatures should be avoided. Fish products should be harvested from unpolluted and recommended water. They should be stored properly to prevent further contamination and microbial growth.

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