REVIEW ARTICLE

Virulence Determinants of *Aeromonas* Species Implicated in Fish Diseases and Control of Infection: An overview

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Abstract

Aeromonads are halophilic, non-spore forming, Gram-negative rods which are ubiquitous in aquaculture and foodstuffs. Members of genus Aeromonas are abundant water inhabitant bacteria that were recovered from lakes, rivers, swamps, chlorinated water as well as food stuff as fish, meat, seafood, vegetables, and processed foods. *Aeromonas* species are opportunistic pathogens that affect many aquatic animals and human. These pathogens cause septicaemia, ulcerative and haemorrhagic diseases, and mortality in different fish species. They possess large number of virulence factors in addition to inherent resistance to various antimicrobials and ability to form biofilms with the help of quorum sensing. This review focuses on the pathogenic potentials of *Aeromonas* species which regarded as multifactorial and dependent on the presence of different virulence factors that enable bacteria to colonize, invade, and defeat the host's immune defences. This review also provides an update on the taxonomy, ecology, and control of Aeromonas infection in fishes.

Keywords: *Aeromonas* spp.; Virulence factors; Probiotics; Prebiotics; Genotyping.

Introduction

Aeromonas species belongs to the class Gamma-proteobacteria, order Aeromonadales and the family Aeromonadaceae [1]. These bacteria are, facultative anaerobic, motile , non-sporulating Gram negative bacilli [2]. Aeromonads are primarily aquatic organisms occurring naturally in different freshwater bodies that include rivers, water streams and lakes[3]· However, these organisms do not occur in water with a very high salinity, geothermal springs or extremely polluted rivers [4]

Members of genus *Aeromonas* are opportunistic pathogens that affect many aquatic animals [5,6]. Diseases and mortality in different fish species were attributed to *Aeromonas* species [7]. *A. caviae*, *A. hydrophila*, *A. salmonicida*, *A. sorbia*, and *A. veronii* were regarded as the most important causes of disease and mortality in fish [8–10]. Aeromonads have many virulence factors that allow bacteria to invade the host immune system and contribute to the pathogenicity of this organism. Some of these factors are serine protease (*ser*), aerolysin (*aer*), lipase (*lip*), cytotonic heat–stable enterotoxin (*ast*), hemolysin (*hly A*), cytotoxic enterotoxin (*act*) and temperature-sensitive protease (*eprCAI*). To determine the pathogenic effect of *Aeromonas* species these virulence genes have been used [11–14]. The major virulence factors correlated with *Aeromonas* species are S-layers, surface polysaccharides,

iron binding machinery, extracellular enzymes and exotoxins, secretion systems and adhesins [15]. Since the occurrence of these virulence factors is strain specific, virulence potential of different *A. hydrophila* strains was characterized by the incidence of their virulence genes [16–19]. A lot of bacterial diseases appear in fish farms due to increased stocking density of fish, which led to extensive antibiotic usage for their treatment [20, 21]. Unwise use of antibiotics led to the emergence of antibiotic resistance among pathogenic bacteria in fish farming [22,23]. *Aeromonas* species are used as a good indicator for analysing the occurrence and antimicrobial resistance of bacteria in fish farms [1, 22].

Taxonomy   and   Classification   of Aeromonas species

The genus *Aeromonas*, belonging to the class Gamma- proteobacteria and the family Aeromonadaceae, contains Gram-negative, non-sporulating, facultative anaerobic bacilli [24,25]. Until 1970, according to physiological characteristics and host range; *Aeromonas* species were classified into two main groups. Motile Aeromonads is the first group with optimum growth temperature at 35–37ºC, this group is known as *A. hydrophila* and it predicted to produce human infections. The second group is non motile aeromonad which grows at 22–28ºC, it is called *A. salmonicida* and produces infections in fishes [26]. Thereafter, new species was added to the genus *Aeromonas* followed by reclassification of pre-existing taxa [27]. Earlier, *Vibrio* species, *Aeromonas* species and *Plesiomonas shigelloides* were included in the family called Vibrionaceae but the recent genetic findings have provided enough information to change this idea and placed *Aeromonas* species in family Aeromonadaceae [28]. Based on 16S ribosomal RNA similarity and DNA-DNA hybridization; members of genus *Aeromonas* were classified [29]. DNA–DNA hybridization assay were used to classify *Aeromonas* species into multiple hybridization groups within each of the mesophilic species [24]. Until March 2016, 31 *Aeromonas* species had been discovered [10]. The genus currently includes 36 species [30].

Identification of Aeromonas species

Phenotypic identification

Aeromonas species are facultative anaerobes that produce characteristic colonies with hemolysis on blood agar or not. They do not require sodium ion for growth and tolerated up to 4% NaCl in the culture medium [31].

Genus Aeromonas is phenotypically identified by Gram-negative staining , oxidase positive reaction, fermentation of glucose with production of acid and gas, reduction of nitrate and growth inhibition by vibriostatic factor O/129 [32]. Because of the changing behaviour of some strains, identification to the species level using this method is complicated. Some strains recovered from diseased fish were re-identified which were identified phenotypically. The 16S rRNA PCR-restriction fragment length polymorphism (RFLP) and RNA polymerase, sigma 70 (sigma D) factor (*rpoD*) sequences were applied to reidentify the isolates and the results showed that only 35.5% were correctly identified [33]. Moreover, commercial identification kits (API 20E, Vitek, BBL Crystal Enteric/Non fermenter, and MicroScan Walk/Away systems) have frequently been utilized in laboratories, in spite of other authors decided that these kits had drawbacks [34]. Lamy et al., [35] evaluated the accuracy of six commercial kits for *Aeromonas* species identification using RNA polymerase subunit B (*rpoB*) sequencing as a reference. Molecular methods were more specific than commercial identification kits. Moreover, Soler *et al*., [36] confirmed the conclusions of the previous study that MicroScan W/A and BBL Crystal E/N systems correctly

identified 14.8% and 20.3% of Aeromonas strains that were formerly identified by PCR for 16S rRNA gene - RFLP analysis, respectively.

Molecular identification

16S rRNA gene-based techniques

Identification and comparison of bacterial species is now possible by the use of 16S rRNA gene which is highly conserved marker [37, 38]. Sequencing of 16S rRNA gene is the commonly applied molecular tool in clinical laboratory for genus and species identification [39]. When trying to identify Aeromonads using this technique wariness should be taken because of the sequence variation between 16S rRNA genes in the same strain, which may reach 1.5% [40].

One of the effective tools introduced in many clinical laboratories for the identification of bacteria is Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS basically identify proteins associated with the 16S rRNA gene [41].

MALDI-TOF MS was used to identify isolates previously characterized by sequencing of *rpoB* and results showed that 100% of isolates were properly identified at genus level, and 97% at species level [42].

Housekeeping Genes

Proteins having crucial functions for the survival of bacteria are encoded by housekeeping genes (HKG). The ideal HKG should be present in all bacteria and should not be influenced by horizontal gene transfer [40].

The first HKG that has been used to study *Aeromonas* species was *gyrB* gene which encodes the B subunit of DNA gyrase [43]. The *rpoD* gene is a similar HKG that displays a related phylogeny to *gyrB* which encodes factor sigma S70 (that enables promoter-specific transcription initiation of RNA polymerase)[34]. Other HKGs have been described: *gyrA rpoB*,

dnaJ ,recA, *dnaX*, *dnaK,cpn60*, *mdh*, *atpD*, *groL*, *gltA radA*, *metG*, *ppsA*, *tsF*, and *zipA* [44–50] for identification of *Aeromonas* species.

Amplified fragment length polymorphism (AFLP) analysis has repeatedly been shown to be a very useful method for classification and typing of Aeromonads [51].

Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) is one of the highly effective methods for genotyping of *Aeromonas* species as it is highly reproducible and easy to perform and does not need too expensive instruments. Therefore, it has been utilized in many epidemiological surveys [52-54] to investigate the clonality of Aeromonas isolates.

Restriction fragment length polymorphism (RFLP) assay was used for genotyping of *A. hydrophila* using *Eco*RII and *Eco3* restriction enzymes[55].

Multilocus sequence typing (MLST) is a typing method based on the sequences analysis of 5-7 housekeeping genes to identify strains by their unique allelic profiles and so facilitates the discrimination of microbial isolates [56]. This technique is highly differentiating and reproducible when compared with other molecular tools. The generated databases can guid researchers to compare the results they obtain. The bacterial isolate genome sequence database (BIGSdb) is platform that currently manages the MLST database.

Phylogenetic grouping of *Aeromonas* species rely on the analysis of two housekeeping genes (*rpoD* and *gyrB*) were consistent with the described taxonomy of *Aeromonas* species [34].

Pathogenesis and virulence factors

During host pathogen interaction, microbes multiply, survive, and combat the host's immunity. The clinical signs that are observed from *Aeromonas* infections indicate a complicated network of mechanisms forming of a multifactorial process. This hypothesis was strengthen by several

studies as the strain, infection route, and animal model affect the virulence of *Aeromonas* species [7, 14, 57]. Virulence factors of *Aeromonas* species include:

Surface structural components

Capsule

The outer membrane of the bacterial cell is enclosed by capsule which is formed of polysaccharides and water. Polysaccharides are formed by repetitions of monosaccharides which are bind to each other by glycosidic bonds forming homo- or hetero-polymers. The variation of capsule forming monosaccharides, probable change, and various linkage are responsible for the diversity and structural complexity [58].

The function of capsule as pathogenic factor is reducing opsonization and hence hindering phagocytosis [59].

S-layers

Wide range of bacteria forms S-layer which is surface protein or glycoprotein forming the outermost cell envelope. S-layers have several functions that are related to virulence. It has a major role in adhesion, and protect the bacteria from phagocytosis [60].

Adhesins

The ability of bacteria to stick and colonize the host mucosa is considered a crucial step in the infection. The first most important step in the process of infection is adhesion of bacteria to host cell. Bacteria attach to host cells and change their defence mechanisms by the initiation of colonization process. *Aeromonas* species has two class of adhesions which enable it to bind to receptors on the host cell surface [61].

Filamentous Adhesins: Fimbriae/Pili

Bacterial cell surface has filamentous structures called fimbriae or pili which consists of protein subunits called pilin. Pili have many functions rather than adhesion like cell aggregation, phage binding, biofilm formation, and transfer of DNA. [62].

Non-filamentous Adhesins

These are macromolecules on the surface of bacterial cell surface and act as adhesins, as lipopolysaccharide (LPS), S -layer and outer membrane proteins. The porins are example of outer membrane proteins which act as a lectin-type adhesins that help bacteria to attach to carbohydrate-rich surfaces as red blood cells and possibly human intestinal cells [63].

Extracellular proteins and enzymes

The process of interaction between host cells and pathogenic *Aeromonas* species is revealed by the production of enzymes and toxins and their secretion out the cells, like, lipases, proteases, enterotoxins, hemolysins and Shiga toxins [8, 15, 64]. Wide range of exotoxins is produced by *Aeromonas* species. Not all toxins are produced by all strains. Moreover, toxin genes are expressed under specific growth conditions in some strains [15].

Cytotonic (change the target cell morphologically without killing) and cytotoxic enterotoxins have been found in *Aeromonas* species 7, 15]. In *A. hydrophila* the cytotoxic enterotoxin (act) has an important role in Aeromonas infections because it inhibits phagocytosis, triggers hemolysis, and rises the level of interleukin (IL-1β) and tumour necrosis factor α (TNF-α) [65]. Proteolytic nicking near the C-terminus activates the inactive secreted protein. The active toxin attaches to a glycoprotein on the target cell surface and accumulates pores in the host cytoplasmic membrane causing cell death [66].Two classes of hemolysins at least are produced by *Aeromonas* species; α-hemolysins and ß-hemolysins. The α-hemolysins are formed in the stationary phase of growth and are responsible for incomplete lysis of erythrocytes and reversible cytotoxic effects. ß-hemolysins are formed in the exponential phase of growth cycle. They are pore forming toxins leading to

complete destruction and lysis of red blood cells [15].

Hemolysins make holes in the target cell membrane leading to their osmotic lysis [67]. The prototype hemolysin of the genus is aerolysin which is encoded by a gene called *aerA* [8]. Type two secretion system (T2SS) is responsible for formation of aerolysin which is known to be sec-dependent where aerolysin is transcribed as a pro-aerolysin that go through several processes of maturation before the exportation of the active aerolysin to the external environment [68].

Extracellular proteases enable *Aeromonas* species to survive in various habitats and facilitate ecological interactions with the host. Protease enhances the pathogenicity because they facilitate invasion either by toxin activation or direct host tissue damage [8,15]. Three types of proteases are secreted by *Aeromonas* species: metalloprotease (ahp, aphB), acetylcholinesterase, and serine protease (aspA) [69, 70]. Furthermore, they can assist the formation of infection by disabling the initial host defences, like, inactivating the complement, or by supplying nutrients for cell reproduction [15].

Lipases are formed by various bacterial species. *Aeromonas* species produce lipases in the surrounding environment to hydrolyse membrane lipids leading to impairment of many immune system tasks through free fatty acids produced by lipolytic activity. Lipase can digest the membranes of erythrocytes and induce their lysis [9, 16].

Secretion Systems

Gram-negative bacteria have inner cytoplasmic membrane and an outer membrane containing LPS with a thin peptidoglycan layer in between. The space between the two cytoplasmic membranes is called the periplasmic space. Gram-negative bacteria had different types of secretion systems: type I, II, III, IV, V, and VI to transfer proteins to the extracellular environment or to the cell surface [71].

Many Gram-negative bacteria have type III and VI secretion systems which deliver their toxic proteins (effectors) directly into the target host cells [16, 31, 72, 73].

Studies showed that infections by *A. hydrophila* and *A. salmonicida* strains having mutation in T3SS had a reduced virulence than the wild strains [74, 75].

Type four secretion system (T4SS) is the only known secretion system that can transport DNA in addition to proteins [76]. T4SS performs important role in the dissemination of virulence and resistance genes [8, 15].

T3SS, T4SS, and T6SS are able to insert effector proteins directly into the cytoplasm of the host cell, even though T6SS has been discovered in nonpathogenic and symbiotic organisms or [77]. After complete genome sequencing of *A. hydrophila*, T6SS was identified even though its role in virulence was undiscovered [69]. The role of T6SS in the virulence of *A. hydrophila* was then discovered [78]. T6SS was then shown to have antibacterial function in multiple bacterial infections for elimination of competing bacteria [79]. *Shigella sonnei* T6SS conferred privilege when competing with *S. flexneri* and *Escherichia coli* and this privilege was diminished in mutants defective in T6SS [80].

Metal ions

Normal biological processes of microorganisms require the presence of metal ions. They play an essential part in the interplay between host and pathogen. In the progression of an infection, the host inhibits the availability of essential metals, by disabling the metal dependent biological processes of the M.O which compensate this inadequacy by producing alternative proteins [81, 82]. Iron gain mechanisms are recognized to play a vital role in the progression of the infection. Low level of iron makes pathogenicity of bacteria more difficult [8].

Siderophore-dependent mechanism is one of the mechanisms by which *Aeromonas* species sequestrate iron from the host tissues. A functional group having high affinity to iron ions is provided by Siderophores that require certain membrane-bound receptors and a special cell- machinery to make this essential element available and incorporate the iron ions into the microbial metabolism. Other mechanisms independent on Siderophore include bacterial membrane bound protein that binds host iron [8, 15, 83].

Mesophilic *Aeromonas* produce either enterobactin siderophores or amonabactin siderophores, but never produce both. The enterobactinis are discovered in various Gram-negative bacteria, but the amonabactin is identified only in *Aeromonas* species [84].

Quorum sensing

Quorum sensing (QS) is a bacterial communication system for organizing genetic expression in response to cell population, this system enables bacteria to evade the host immune system [85,8]. Expression of virulence genes, antibiotic production, plasmid conjugation and biofilm formation in *Aeromonas* species, can be induced by QS system [86- 89]. Bacteria produce substances which act as a chemical signal. In Gram-negative bacteria, these chemicals are basically acylated homoserine (AHLs), AHL in *Aeromonas* can change the host immune response [90, 89, 8, 91].

Chan *et al.*, [92] reported 159 sequences of QS-related genes in *A. veronii*, which increase its virulence. Nowadays, Liu *et al.*, [93] demonstrated that the formation of biofilm in *A. salmonicida* is affected by the infection with the asaI-mutant (failed to produce the short chain AHLs signal). Recently, Blöcher *et al.*, [94] developed anti-QS compounds to inhibit biofilm formation of the resistant *A. caviae* strain Sch3. This study helps control the bacterial antibiotic resistance problem.

Fish diseases caused by *Aeromonas* species

Aeromonas species have an important role in fish diseases, and this has been known for decades, mainly there are two types of *Aeromonas* species responsible for fish disease. *A. salmonicida* is considered the causative agent of furunculosis, which has been considered the most important fish diseases in aquaculture [95, 96]. It was thought in the past, the infection affected salmonids only, but by the time it is known to affect fresh and marine water fishes [97,-96]. Fish suffer from furunculosis show some symptoms like skin hyperpigmentation, lack of appetite, lethargy, presence of the typical furuncles, septicaemia, exophthalmia, petechiae, anaemia, ascites and haemorrhagic lesions in the gills, nares, fins, vent, muscles, and internal organs [97,96]. Mesophilic motile *Aeromonas* species cause another fish disease known as 'motile Aeromonas septicaemia or epizootic ulcerative syndrome which shows similar clinical signs as furunculosis, but some time injury may be seen only in the internal organs or skin [97-,98].

Control of Aeromonas infection in fish

To control the extensive use of antibiotics in fish farms and their possible negative impacts on the environment and public health, a continuous search for other alternative strategies is required. Even when the antibiotic concentrations in fish diet are below the minimum inhibitory concentration, the prolonged existence of antibiotics in water, combined with high numbers of bacteria in the polybacterial environments as the sediment, pond, or biofilm put selective pressure on bacteria and allow the exchange of resistance genes between them [99,100]. *Aeromonas* species may continue being adhered to biofilms on abiotic or biotic surfaces, and its existence with *E. coli* in mixed biofilms encourages the exchange and distribution of

antimicrobial resistance genes [101]. The alternative strategies include:

Probiotics

Probiotics are combination of living microorganisms (bacteria and yeasts) that, when administered in sufficient amounts, confer a health benefit on the host [102]. The beneficial activities of probiotics were attributed to modification of intestinal microbiota, production of antitoxin substances or antibacterial (bacteriocins and organic acids), immune system modulation and competition with pathogens for nutrients, and adhesion to intestinal mucosa [103]. There was an increase of the survival rate and protective effect of probiotic against Aeromonas species. The amplitude of the survival rate between the probiotic and control groups varied considerably and depended on the probiotic species, the feeding dosages, and durations [104]. Relatively high level of protection against *A. hydrophila* was recorded in Nile tilapia for each probiotic agent *Bacillus pumilus* or mixture of *Lactobacillus acidophilus*, *Bacillus subtilis*, Saccharomyces *cerevisiae*, and *Aspergillus oryzae* at the end of the second month more than that obtained at the end of the first month of the feeding trial [105].

Furthermore, it has been shown that the combination of multispecies probiotics of *S. cerevisiae, B. subtilis,* and *Lactococcus lactis* [106] or *B. subtilis, L. plantarum,* and *Pseudomonas. aeruginosa* [107] improves health status more effectively than the incorporation of a monospecies probiotic in the diet.

Prebiotics

Prebiotics are indigestible fibers that are selectively used by host microflora to confer health benefits and enhance growth performance due to the by-products generated from their fermentation by gut beneficial bacteria, such as altering the composition of the microbiota, inhibiting pathogens, stimulating immune responses, and improving resistance to stress factors

[108-109]. ß-glucan (ß-1,3-glucan or ß-1,6-glucan) which is extracted from the cell wall of *S. cerevisiae* was one of the most important prebiotics used to prevent disease in freshwater fish by *Aeromonas* species. when added to the basal diet [110-111]. Feeding fish with ß glucan at 1 to 2 g/ kg diet for at least 2 weeks appeared to be ideal to induce high protection and stimulate immune response in different Aeromonas infected freshwater fishes including rainbow trout, common carp, and Nile tilapia [112-113]. The immunomodulatory mechanisms of prebiotics in augmentation of fish immunity need to be further investigated. Some studies shown that a ß-glucan supplemented diet could display variable gene expression levels of some immune and inflammation-related cytokines in fish infected with *Aeromonas* species. The response depended on the organ, with downregulation in the gut and an upregulation in the spleen and kidney [112,114,115]. Despite a preventive effect against Aeromonas infection in some investigations, no significant effect of dietary ß-glucan on immune parameters (leucocyte subpopulations, lysozyme activity, alternative complement activity (ACH50)) assessed in serum of rainbow trout and Nile tilapia has been proved [112, 114,116] .

Synbiotics

Synbiotics are feed supplements consist of a mixture of probiotics and prebiotics that beneficially affect the health of the host. In aquaculture, synbiotics were used to improve growth performance and feed utilization as well as increasing resistance to diseases, digestibility, and modulation of the immune system [117-119].

Different formulas of synbiotics were investigated with the purpose of studying their beneficial role to protect freshwater fish against Aeromonas infections like, *L. plantarum* JCM1149 and Fructooligosaccharides (FOS) [120], *B. subtilis* and Mannan Oligosaccharides

(MOS)) [121], inactivated *E. faecalis* and MOS [122], Bacillus spp. (*B. coagulans or B. subtilis*) and Chitooligosaccharide (COS) [123].

Conflict of interest

The authors declare that there is no conflict of interest.

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الملخص العربي

محددات الضراوة لأنواع الأيروموناس المتورطة في أمراض الأسماك والتحكم في العدوي: نظره عامه

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الأيروموناد هي عصيات، غير بوغية، سالبة الجرام وتنتشر على نطاق واسع في البيئة المائية والمواد الغذائيةأنواع الأيروموناس منتشرة في كل مكان تنتقل عن طريق الماء. يتم عزلها من الأنهار والبحيرات والمستنقعات ومياه المعقم بالكلور وأنواع مختلفة من الطعام، مثل اللحوم والأسماك والمأكولات البحرية والخضروات والأطعمة المصنعة. تعتبر أنواع الأيروموناس من مسببات الأمراض الانتهازية التي تصيب العديد من الحيوانات المائية والبشر. هذه العوامل الممرضة هي المسؤولة عن الأمراض التقرحيه وتسمم الدم والوفيات في الأسماك المختلفة. لديهم عدد كبير من عوامل الضراوة بالإضافة إلى المقاومة المتأصلة للمضادات الحيوية المختلفة والقدرة على تكوين الأغشية الحيوية بمساعدة استشعار النصاب. تركز هذه المراجعة على الإمكانات المسببة للأمراض اللإيروموناس والتي تعتبر متعددة العوامل وتعتمد على وجود العديد من عوامل الضراوة التي تسمح للبكتيريا باستعمار وغزو وهزيمة جهاز المناعة. تهدف هذه المراجعة أيضًا إلى تحديث للمعرفة المكتسبة مؤخرًا حول التصنيف والبيئة والتحكم في عدوي الايروموناس في الاسماك.

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