Australian and New Zealand Standard Diagnostic Procedures (ANZSDP) for *Aeromonas salmonicida*

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Summary

Aeromonas salmonicida subspecies *salmonicida* is the etiological agent of furunculosis, a bacterial disease affecting salmonids, and characterised by 'furuncles', a term used to describe the umbonate ulcers with abscess-like cavities in the dermis. Clinical signs of furunculosis include presence of typical furuncles, hyperpigmentation of the skin, petechiae and haemorrhagic lesions in gills, fins, vent, nares, muscles, internal organs, and septicaemia. Furunculosis was first reported in Germany in 1888 from brown trout (*Salmo trutta fario*) and brook trout (*Salvelinus fontinalis*), and since then has spread to most parts of the world, but is exotic to Australia and New Zealand.

Four other subspecies of *A. salmonicida* are recognised: *A. salmonicida* ssp. *achromogenes*, *A. salmonicida* ssp. *masoucida*, *A. salmonicida* ssp. *smithia* and *A. salmonicida* ssp. *pectinolytica*. These and other unnamed types, often termed atypical *A. salmonicida*, have been reported from salmonid and non-salmonid fish displaying clinical signs characterized by dermal ulceration, with and without septicaemia, but often devoid of the 'typical' furuncles. These subspecies and types have been reported from most parts of the world including Australia, but have not been reported from New Zealand. In Australia, strains of atypical *A. salmonicida* have been detected in imported goldfish held in quarantine and in feral goldfish and the disease is termed goldfish ulcer disease; however, in challenge experiments in Atlantic salmon these strains produced disease with similarities to furunculosis. In silver perch (*Bidyanus bidyanus*), from New South Wales, atypical *A. salmonicida* has been isolated, while in Tasmania two distinct biovars occur, atypical *A. salmonicida* biovar Acheron from farmed Atlantic salmon and an atypical *A. salmonicida* biovar from wild and farmed greenback flounder (*Rhombosolea tapirina*) consistent with the phenotype of *A. salmonicida* ssp. *smithia*.

The presence of *A. salmonicida* is confirmed by culture on routine media, and polymerase chain reaction. *A. salmonicida* ssp. *salmonicida* is phenotypically homogeneous, whereas the atypical strains are variable. Identification of typical and atypical strains is achieved by conventional biochemical tests, MALDI-TOF, specific PCR and partial *vapA* typing followed by comparison to a publicly available phylogenetic tree.

Both typical and atypical *A. salmonicida* are notifiable diseases in Australia and New Zealand. Thus, the recommendation is to submit relevant samples of all suspected cases, which may be based on clinical signs and case history, to the appropriate laboratories for culture and molecular testing, as the purpose is to definitively exclude *A. salmonicida* as a causative agent for disease. Initial testing may be done at state diagnostic laboratories, and unless there is a confirmation that *A. salmonicida* is an atypical strain, the sample should be submitted to the Australian Centre for Disease Preparedness (ACDP) in Australia or the National Animal Health Laboratory in New Zealand for confirmatory testing for the typical strain. Note that clinical signs of acute infection of *A. salmonicida* ssp. *salmonicida* may be mild and look like that of an atypical infection.

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1 Diagnostic overview

1.1 Aetiology

Aeromonas salmonicida is a Gram-negative, mainly psychrophilic, mainly oxidase-positive, non-motile, rod that belongs to the class *Gammaproteobacteria*, order *Aeromonadales* and the family *Aeromonadaceae*. Uncommon mesophilic and oxidase-negative strains have been reported.[1]

Five subspecies are recognised: *A. salmonicida* ssp. *salmonicida*, *A. salmonicida* ssp. *achromogenes*, *A. salmonicida* ssp. *masoucida*, *A. salmonicida* ssp. *smithia* and *A. salmonicida* ssp. *Pectinolytica*.[2,3,4,5,6,7,8]

Aeromonas salmonicida ssp. *salmonicida* is the aetiological agent of furunculosis, a major disease in salmonid and non-salmonid fish that usually results in high mortality and morbidity.[2,9,10,11]

The other four subspecies plus a number of strains (biovars) are referred to as 'atypical' in that their phenotypes are different to *A. salmonicida* ssp. *salmonicida*. All 'atypical' types, with the exception of *A. salmonicida* ssp*. pectinolytica,*[8] cause a range of infective conditions in aquatic animals with varying degrees of virulence according to the subspecies or biovar and the host.

Infection of different host species by *A. salmonicida* ssp. *salmonicida* results in varied clinical presentations and disease names – trout ulcer disease in trout, goldfish ulcer disease in goldfish (*Carassius auratus*) and erythrodermatitis in carp (*Cyprinus carpio*).[12] Due to a wide host range, and subsequent types of disease presentation, mortality and morbidity, the disease is commonly referred to an infection with an atypical strain.

The taxonomy of *Aeromonas* species is complex. A proposal to use the designation *Aeromonas salmonicida* ssp. *nova*[13,14] to accommodate all subspecies and other atypical strains was not formally adopted. The validity of retaining the subspecies names is in contention, as phylogenetic analysis of a universal target sequence from the *cpn60* gene indicates all subspecies, except *pectinolytica*, are identical.[15]

Aeromonas salmonicida, *A. bestiarum* and *A. piscicola*, share 99.8–100% homology across the 16S rRNA gene, and belong to the *A. hydrophila* complex.[16] Thus, sequencing the 16S rRNA gene is not recommended for identification, but the three species may be partly differentiated by performing sequencing of the partial *vapA* gene for *A. salmonicida*[17,18] and to an extent, the *gyrB* and *rpoD* genes for all three species.[19,20] Some phenotypic tests can assist in differentiation, as presented under section 2.3.

DNA:DNA reassociation (a technique that defines taxonomic status) indicates *A. salmonicida* ssp. *salmonicida* is a homogeneous genetic group, regardless of host species or geographical location. Conversely, the atypical strains are heterogeneous, yet do not represent a distinct taxonomic group. Genetic groups are recognised within the atypical strains. Subspecies *achromogenes* and *masoucida* form a distinct genetic group, while the other atypical strains comprise another genetic group and biotype causing disease in goldfish (goldfish ulcer disease). A third genetic group and biotype is the cause of disease in European carp (carp erythrodermatitis*)* while a fourth genetic group contains the remaining atypical isolates.[14] Recently, mesophilic isolates of *A. salmonicida* from a migratory bird and from humans demonstrated genetic diversity according to whole genome sequencing and diversity in pathogenicity in mice challenge.[21,22] The genetic distance, based on average nucleotide identity (ANI), indicates the isolate from the migratory bird is grouped with but distinct from the other known mesophilic strains and sits between these and the psychrophilic strains. The psychrophilic strains are all closely related at 99% nucleotide identity.[22]

A number of virulence factors may be present in strains of *A. salmonicida*, and include genes for adhesion, toxins, secretion enzymes and iron acquisition.[23] A 64–70 kiloDalton serine protease, (aspA), is responsible for haemorrhage and liquefaction of muscles which leads to the formation of furuncles.[24,25]

A paracrystalline surface array (S-layer) protein, an ordered protein structure external to the outer membrane and common to many bacteria, is a major virulence factor. In *A. salmonicida* the S-layer is a 49 kDa protein or complex of lipopolysaccharides referred to as the A-layer or A protein. With the exception of *A. salmonicida* ssp. *pectinolytica*, all other subspecies possess the A-layer protein.[17]

The A-layer protein is encoded by the virulence array protein gene *vap*A[26] and consists of conserved and variable regions.[26,27,17] Cluster analysis, based on the sequence of the hypervariable regions, groups *Aeromonas salmonicida* into different Types, with all *A. salmonicida* ssp. *salmonicida* comprising Type I, subspecies *achromogenes* as Type III, subspecies *masoucida* as Type IV, and subspecies *smithia* as an unassigned singleton. Other Types represent *Aeromonas salmonicida* strains isolated from different aquatic hosts. The nucleotide variation results in amino acid substitutions of different hydrophobicity.[17] The A-layer has novel binding capabilities and increases the bacterial surface hydrophobicity, which causes auto-agglutination of bacterial cells and adherence to fish white cells and intestinal mucus.[28,29] Also of significance for virulence is the pAsa5/pASvirA plasmid that contains Type III secretion system (TTSS) genes. Growth of the bacterium at higher temperatures (≥25°C) leads to rearrangement of the TTSS genes with resulting loss of virulence through loss of expression of the A-protein, potential loss of the plasmid, loss of auto-agglutination and loss of the ability to bind Congo Red. Virulence is retained for strains grown at <20–22°C.[30,31,32,33] The variation in the virulence array protein gene (*vapA*) that codes for the A-layer surface protein is proving to be a robust system for typing and subtyping typical and atypical *Aeromonas salmonicida* strains[17,18] (pers. comm Dr Snorre Gulla, June 2021) and is described under section 1.3.4 and section 2.4.

A brown water-soluble pigment (melanin), which is inhibited by D-glucose at a concentration of 0.1%, may be produced; however, the presence or absence of pigment is not related to pathogenesis[34,35], nor is it diagnostic of the species.

1.2 Clinical signs

Aeromonas salmonicida ssp. *salmonicida* infection in salmonids is termed furunculosis. The disease presents as peracute, acute, or chronic, or as covert infection in subclinically infected fish, which are a source of disease transmission.[36]

Infection with atypical strains occurs with varying severity and clinical signs in a wide variety of hosts both salmonids and non-salmonids.

1.2.1 Furunculosis

Salmonids infected with *A. salmonicida* ssp. *salmonicida* may present with furuncles. Furuncles, describes the umbonate ulcers or boils with abscess-like cavities in the dermis that may extend deep into the musculature and discharge pus, or expose bone.[10] Furuncles are more common in chronically infected older fish or those species with innate resistance. Symptom onset is slow, and signs of sub-acute or chronic infection include low mortality, lethargy, loss of appetite, bleeding at the base of the fins, darkened skin, foci of melanised tissue, intestinal congestion, and post death blood and/or mucus discharge from the vent.[10] In acute infection (more common in juvenile fish), furuncles are usually absent, and within two to three days mortality rates are high. Fish may show lethargy, loss of appetite and the only signs may be a darker skin pigment.[12] Importantly, furuncles may be absent regardless of the stage of the disease. Clinical signs vary according to host species,

age, and environmental factors including temperature. In some instances, disease presence may only be identified by fish mortality, an irregular dark spot just beneath the skin (often between dorsal and pelvic fins), lethargy, and dull or dark fish skin colour.[37]

1.2.2 Atypical *Aeromonas salmonicida*

Infection with *A. salmonicida* subspecies and other atypical strains can cause high mortalities, and in severe infections fish may die with no clinical signs. A range of clinical signs and disease symptoms occurs in various hosts (see section 1.3.1).[38] Skin ulceration (ranging from superficial reddened lesions of 1–2 mm to eroded areas of 10–20 mm in diameter) rather than furuncles is the most common feature of infection with atypical strains.

For goldfish ulcer disease (GUD), in the early stages of infection, small whitish areas of epithelial congestion are visible, which progress to haemorrhagic areas under the scales and at the edges of the lesion. Lesions occur on all parts of the body including eye, face and opercular cover, and are associated with areas of pigmentation on the skin. An intermediate stage is characterised by loss of scales, necrosis of the dermis and further haemorrhage. In the terminal stages, deep surface lesions erode and expose muscle tissue. Successful isolation of the bacterium is more common from lesions in the initial stages of the infection rather than the later stages when lesions become overgrown with opportunistic, colonising bacteria.[39,40]

The atypical strain isolated in feral and commercial goldfish in Australia, causes death in Atlantic salmon (*Salmo salar*) in experimental challenge with a LD_{50} of three colony forming units (CFU) per 30–40 g fish. At high bacterial concentrations (>10^5 colony forming units) death occurs overnight, whereas at a lower concentration of the bacterium, deaths occur over 19 days, with a majority of deaths at 7 to 10 days. Melanization of the skin and randomly distributed lesions occur on the skin.[41] Brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) were susceptible in challenge experiments. At higher levels of challenge, fish die without any clinical signs, but at lower doses fish develop skin ulcers, present as listless, swim slowly, have abdominal distention, produce mucoid yellow faeces, with congestion at the vent and at the base of the fins.[42]

In eels (*Anguilla rostrata*, *A. anguilla*, *A. japonica*) the disease has been referred to as head ulcer disease because of a swelling on the head as well as skin lesions, severe necrosis and erosions on the mouth and areas of haemorrhage on the snout.[38]

1.3 Epidemiology

1.3.1 Host range

Furunculosis, the disease caused by the typical strain (*A. salmonicida* ssp. *salmonicida*), primarily occurs in wild and cultured freshwater salmonids, however, other freshwater and marine fish can be infected. Brown trout and brook trout are particularly susceptible, cutthroat trout (*Oncorhynchus clarkii*) are less susceptible, whereas rainbow trout (*Oncorhynchus mykiss*) often are immune but may succumb to the disease under adverse conditions. Individuals within host species exhibit varying levels of susceptibility. Mature fish are less susceptible to the disease than juveniles, except at spawning.[43] A chronic form of infection may occur in older fish.[12]

In addition to salmonids, *A. salmonicida* ssp. *salmonicida* has been reported from a wide variety of aquatic hosts including chub (*Squalius cephalus*), dace (*Leuciscus leuciscus*), eels (*Anguilla* spp.), grayling (*Thymallus thymallus*), lake whitefish (*Coregonus clupeaformis*), paddlefish (*Polyodon spathula*), pike (*Esox lucius*), sea bass (*Dicentrarchus labrax*), sea lamprey (*Petromyzon marinus*) and sea.[9,10,44,45,46,47,48] Snapping turtles (*Chelydra tricarinata*) and painted turtles (*Chrysemys eleganz*) may be susceptible to infection as antibodies were produced during vaccine trials.[49] Furunculosis in

captive and wild fish has been reported from almost all countries in the world including Austria, Belgium, Canada, China, Denmark, France, Germany, Japan, Korea, Norway, Scotland, Spain, South Africa, UK, USA.

The disease furunculosis has not been reported in Australia or New Zealand.

The atypical strains (*A. salmonicida* ssp. *achromogenes*, ssp. *masoucida*, ssp. *smithia* and numerous unnamed or unclassified atypical strains) infect a wide host range and have been reported from more than 20 species of farmed fish and more than 30 species of wild fish, as reviewed by Wiklund and Dalsgaard (1998).[38] These include salmonids such as Atlantic salmon (*Salmo salar*), arctic char (*Salvelinus alpinus*), masu salmon (*Oncorhynchus masou*), rainbow trout, brook trout, brown trout, grayling, and other fish species including Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*), carp (*Cyprinus carpio*), eel (*Anguilla anguilla*), wolfish (Anarhichadidae), rockfish (*Sebastes schlegeli*), wrasse (Labridae), lumpsucker (*Cyclopterus lumpus*) and pike.[50,38,51,52,53,17] *A. salmonicida* (subspecies not determined) has also been isolated from shellfish and sea urchins (Echinoidea).[54,55, 56]

Recently, *Aeromonas salmonicida* was reported from the spleen, kidney and liver of a migratory bird, a pied avocet (*Recurvirostra avosetta*), found dead in Switzerland.[22] Genome sequencing revealed close similarity to the typical strain apart from lack of Type III secretion system genes and the ability to grow at 37℃. Mesophilic strains isolated from humans reportedly demonstrate diversity in the genome and pathogenicity in mouse studies.[21]

Aeromonas salmonicida ssp. *pectinolytica* is a mesophilic species that was found in a polluted river, the Matanza River in Argentina, and is non-virulent.[8]

Atypical strains have been reported from most countries in the world including Finland, Korea, Japan, USA, UK. Atypical strains of *A. salmonicida* have been reported from Australia. The presence of an atypical strain in New Zealand is uncertain, as in 2011 a suspected atypical strain was detected by PCR from lamprey (*Geotria australis*), but was not cultured. Sequencing of the amplified product did not provide conclusive identification and despite surveys in following years, no further PCR or culture positives have been detected.[57]

1.3.2 Transmission and infection reservoirs

Furunculosis spreads through contact with infected fish, carrier fish, fish that are less susceptible to infection, subclinically infected fish or fish such as wrasse that are used as cleaner fish to 'clean' farmed salmonids of external parasites, and through exposure to contaminated water; all of which are potential reservoirs for infection.[58,59] The major routes of infection are through the gills, skin and intestine.[13,60]

A. salmonicida may be difficult to detect in subclinically infected fish, but the pathogen can reside in the intestine, gills and mucus.[58] Subclinically infected fish can develop clinical disease following stressful conditions such as rapid changes in temperature, water flow, high stocking density and handling.[61] In tanks containing infected fish, the bacterium is present in faeces, effluent, water and sediment.[59] The bacterium is found in salmon lice (*Lepeophtheirus salmonis*) that feed on the mucus of Atlantic and Pacific salmon and trout, as well as zooplankton.[62] Its growth appears to be enhanced in co-culture studies with the free-living ciliate *Tetrahymena pyriformis*.[63] *A. salmonicida* can adhere to plastics, and to a lesser extent, stainless steel and fishing equipment, nets, cages and similar such fomites, which poses a risk for disease transmission.

1.3.3 Survival

The bacterium survives in an unculturable state in lake water and in a culturable state in sea water for up to ten days.[64,65,66,10] Temperature, and the presence of other bacteria, are factors that affect the viability of the bacterium to survive outside the host. In sterile tap water the bacterium can survive for more than 40 days at temperatures of 4℃ and 10℃, but only up to 17–20 days at temperatures of 20℃ and 30℃, but in the presence of sterile moist soil the bacterium can survive for more than 40 days at all temperatures tested. The presence of other bacteria such as *Pseudomonas* species may reduce the viability of *A. salmonicida*.[67] The organism is non-viable after 6 hours of drying and is killed in 1 min at 60℃. *A. salmonicida* may remain viable in distilled water from 4 days to 2 weeks, but in natural freshwater the bacterium can die in 1–3 days. In water polluted with organic matter the organism can survive for up to 23 days. In dead fish, *A. salmonicida* can remain viable in furuncles for up to 6 days at 5℃.[37]

1.3.4 Strain typing

For *A. salmonicida* typical and atypical subspecies (with the exception of *A. pectinolytica* which doesn't possess the A-layer gene), a typing scheme based on the hypervariable region of the *vapA* virulence gene, distinguished 23 A-layer types amongst 675 isolates from 50 fish species across 26 countries. Some A-layer types are highly correlated to the fish host species.[17,18] *Aeromonas salmonicida* isolates containing the A-layer came from the following hosts: goldfish, common carp, pike, smallmouth bass (*Micropterus dolomieu*), Atlantic halibut, viviparous eelpout (*Zoarces viviparus*), common dab (*Limanda limanda*), European flounder (*Platichthys flesus*), turbot (*Scophthalmus maximus*), lumpfish, and the families Gadidae, Anarhichadidae, Labridae and Salmonidae. The most common A-layer type was Type 1 from Salmonidae cultivated globally and composed of *A. salmonicida* ssp. *salmonicida*, and Type 7 that came from fish in the Pacific Ocean and composed of *A. salmonicida* ssp. *masoucida*. Two atypical isolates from cyprinids in Australia were identified as Type 10, which has been found in USA, UK, Germany and other countries. This partial *vapA* typing scheme is proving to be highly useful for differentiating typical and atypical strains and discriminates within the atypical strains. The data is available at Microreact.[18] The technique may overcome potential laboratory bias when interpreting biochemical results as reported by Dalsgaard et al., (1998)[68] (pers. comm. Dr Snorre Gulla, June 2021). Recently, a strain of *Aeromonas salmonicida* ssp. *salmonicida* from salmon cultured in North Korea that ferments sucrose has been identified using this partial *vapA* typing scheme (unpublished data, Dr Snorre Gulla, June 2021). This highlights the need to ensure correct identification of a suspect isolate.

Strain typing using pulsed field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) indicates that strains of *A. salmonicida* ssp. *salmonicida* from Atlantic salmon, brown trout and coho salmon from diverse geographical areas are genetically similar.[69]

Vaccines are available for *A. salmonicida* ssp. *salmonicida*[70] and autogenous vaccines for atypical strains,[71] and have proved highly effective in disease control. In Australia, the vaccine Tegovac® has been developed by the Centre for Aquatic Animal Health & Vaccines (DPIPWE, Tasmania) for use in Atlantic salmon in Tasmania. Tegovac® is a multivalent vaccine for the atypical *A. salmonicida* biovar Acheron, *Vibrio anguillarum* serotype O1, *Yersinia ruckeri* serotype O1 b, biotypes 1 and 2, and has proved highly effective with no evidence of the disease MAS (marine *Aeromonas* diseases of salmonids) since its routine use commencing in 2006. Depending on vaccine composition and the type of adjuvant used, abdominal adhesions can occur to varying degrees of severity[72] and may also result in skeletal deformities and other conditions that result in decreased growth.[12,73]

1.4 Occurrence and Distribution

1.4.1 *Aeromonas salmonicida* - Furunculosis

Since the first report in 1888 from Germany of furunculosis in cultured brown trout and brook trout, the disease has spread to many parts of Europe, Britain, USA[2,10] and ultimately is found worldwide with the exception of Australia, New Zealand and South America. Because of its widespread occurrence, *Aeromonas salmonicida* is no longer listed by the World Organisation for Animal Health; Office International des Épizooties (OIE).

Aeromonas salmonicida ssp. *salmonicida*, the causative organism of furunculosis, is exotic to Australia and New Zealand and is a notifiable disease in both countries.

1.4.2 Atypical *Aeromonas salmonicida*

Atypical strains of *A. salmonicida* have been reported in many countries including Australia and Chile, and the situation in New Zealand is unclear (as described below).

In Australia, the first detection of atypical *Aeromonas salmonicida* occurred in 1974 from goldfish imported from Japan for the aquarium trade and housed at a commercial goldfish-producing farm in Victoria.[74] In 1975, diseased goldfish were reported from a commercial aquarium in NSW after purchase of goldfish from the Victorian farm.[75] Infections continued to be reported including from a goldfish hatchery in New South Wales that drew water from the Lachlan River, a hatchery that drew water from the Murrumbidgee River and used goldfish from a farm dam as live feed for golden perch, and wild goldfish in Lake Burrumbeet that drains into southern Victoria.[76] In addition to hatcheries for the aquarium trade in Victoria and New South Wales, atypical strains of *A. salmonicida* were reported from roach in Lake Burrumbeet, silver perch in a NSW hatchery, koi carp at the Royal Botanic Garden, Sydney, and in Queensland from goldfish obtained from a Victorian farm.[77,75,78] Isolates from imported aquarium goldfish in Tasmania in 1981 and 1989 and in Western Australia in 1992 have been reported.[38,79] The earliest case in Tasmania was from pet shop stock, but the latter isolations in Tasmania and Western Australia were from diseased fish held in quarantine; there is no evidence of goldfish ulcer disease strains occurring in either state (N. Buller, J. Carson unpublished data). Two other biovars of atypical *A. salmonicida* have been reported from Tasmania: *A. salmonicida* biovar Flounder, which was isolated from experimentally farmed greenback flounder in 1993, and biovar Acheron in 2000 from disease outbreaks in farmed Atlantic salmon at Macquarie Harbour.[78,80]

No active surveillance program is being conducted in Australia, and given the low rates of detection by culture[81] and the possibility of subclinical transmission, the current status of atypical *A. salmonicida* strains in native, feral or cultured fish through diagnostic submissions, is unclear.

The Australian atypical strains from goldfish are pathogenic for Atlantic salmon, brown trout, rainbow trout and brook trout, as demonstrated in challenge experiments,[41,42] and atypical *A. salmonicida* biovar Acheron is pathogenic in Atlantic salmon in challenge trials.

In New Zealand, the presence of atypical *A. salmonicida* is less clear. Suspected atypical *A. salmonicida* was incidentally detected in 2011 in lamprey (*Geotria australis*) using a species-specific PCR on tissue targeting the *vap*-A gene.[57.] No isolates were cultured from the same tissue despite repeated attempts. The finding was not associated with furunculi, and the organism detected was deemed non-pathogenic based on cumulative epidemiological data.

An *Aeromonas* isolate was cultured the same year from the kidney of brown trout *Salmo trutta* (unpublished data). The isolate was recovered among light mixed growth. Although it was positive by vap-A PCR, the biochemical profile was not consistent with *A. salmonicida*: motile and growth

at 37°C. The isolate was indole positive and fermented sucrose. *A. salmonicida* ssp. *salmonicida* has not been detected in NZ before or since 2011.

1.5 Pathology

Aeromonas salmonicida ssp*. salmonicida* causes severe septicaemia and can present as acute mortalities or chronic disease (furunculosis) in a range of finfish species. The primary pathological process of disease is necrosis.[82] An overview of clinical signs and histopathology observed from infection with typical and atypical strains in a variety of salmonid and non-salmonid hosts is reviewed by Menanteau-Ledouble *et*. al., 2016.[36]

1.5.1 Furunculosis

The use of furuncles to describe the lesions that occur with infection with *A. salmonicida* ssp. *salmonicida* is incorrect when compared to furuncles seen in some human diseases. In fish, the furuncles are areas of necrosis and do not have significant infiltration of leucocytes and other inflammatory cells normally associated with pus in mammals, but rather the furuncles in fish are boil-like abscesses or open sores on the body that consist of necrotic tissue with muscle fibres, bacteria and blood cells.[43] Although furuncles are characteristic of the disease, these may be absent. The furunculosis lesion begins from the necrotic tissue, visible as minute red spots in muscle and subcutaneous tissue and forms a swelling or blister just below the skin surface. This blister contains red, pus-like material composed of bacteria, disintegrated muscle fibres and blood, which may break through the skin to form an open sore. The furuncles tend to develop in the dorsal muscles, but other subcutaneous and muscle lesions can develop along the lateral line. Lesions at the base of the dorsal or pelvic fins may be confused with fin rot. Septicaemia occurs as the bacteria in the bloodstream collect and multiply in the capillaries that leads to destruction of the blood vessel walls and surrounding tissues. In many infections, such as occurs in fingerling trout, the fish die before the development of furuncles and the only clinical signs may be an irregular dark patch or haemorrhage at the base of the dorsal and pelvic fins or in the oral cavity.[10,43] Other signs include congested gills, congested blood vessels in the abdominal cavity, inflamed intestinal lining, diseased kidney that may break down, an enlarged, bright cherry-red coloured spleen, which is particularly evident in fingerling trout. Blood may discharge from the vent after death.[10,43]

Patchy necrosis of the liver, spleen, kidney and muscle and intralesional coccobacilli are observed in histology sections, but there is no increase in leucocytes. Numerous coccobacilli are found clumped and touching the blood vessel and capillary walls, in connective tissue spaces, neighbouring diseased areas and in clumps in the liver, spleen and kidney. Red blood cells in capillaries are lysed and may be evident by their nucleus only.[10]

The bacterium can be cultured from the blood, haemorrhagic areas in the musculature, peritoneal fluid and the internal organs, particularly heart blood, liver, kidney and spleen. The isolation rate is higher in the early stages of the disease compared to terminal stages of the disease. White clumps of bacteria can be seen in a squash preparation of the spleen. Samples from intestinal contents, pus from abscesses and mucus from the vent usually are overgrown with a variety of contaminating bacteria.[10]

Biochemistry indicates reduced blood glucose, due to the bacterium using glucose as an energy source, and in chronic infections non-protein nitrogen is increased and there may be an increase in urea reflecting kidney degeneration. An increase in creatinine level may indicate muscle degeneration. The red cell count, haemoglobin, total plasma protein, albumin and globulin are all within the normal range (Field *et al.*, 1944[83] as quoted in McCraw, 1952[37]).

1.5.2 Atypical *Aeromonas salmonicida*

In a severe infection, fish may die without any abnormalities. Pathological changes that may occur include haemorrhage, oedema, hyperaemia, granulomatous tissue with infiltration of leucocytes and fibroblast-like cells in the dermis, spleen and kidney, but there is a range of pathology that may present in salmonid and non-salmonid fish.[38]

In experimental challenge of Atlantic salmon with the atypical strain isolated from feral and commercial goldfish in Australia, intraperitoneal injection resulted in peritoneal hyperaemia and peritonitis, pale livers and occasional splenomegaly. Reddening of the muscles and skin erosions occurred in bath-challenged fish. Necrosis occurred in the liver, kidney, spleen. Progression of the disease led to renal necrosis and foci of bacteria, which were also seen in kidney and liver of some fish. Clumps of bacteria may occur in gills in the absence of inflammation. Other signs may include cutaneous lesions, loss of scales with invasion of the bacterium into the connective tissue, and lysis and degeneration of the muscle.[41]

1.6 General diagnostic tests

Identification of *Aeromonas salmonicida* initially is performed at a diagnostic laboratory, but confirmatory testing, if exotic strains of *A. salmonicida* is highly suspected (e.g. the bacteria detected from salmonid species or other hosts where *A. salmonicida* ssp. *salmonicida* has been reported), must be done in consultation with the Australian Fish Diseases Laboratory at the Australian Centre for Disease Preparedness (ACDP), previously known as the Australian Animal Health Laboratories, CSIRO, Geelong, Victoria, Australia, or in New Zealand at the Ministry of Primary Industries Laboratory, Wallaceville. Atypical strains and subspecies of *A. salmonicida* must be differentiated from *A. salmonicida* ssp. *salmonicida*.

Diagnostic tests include clinical signs, histopathology, culture and biochemical identification, specific PCRs (conventional and real time PCRs) based on the A-layer gene followed by confirmation using the partial *vapA* gene typing scheme,[17,18] and by sequencing the ISAsa4 insertion sequence and the *rpoD* house-keeping gene if required.[84,59,85,86]

In acute infections, no clinical signs may be evident, and it is only in chronic or prolonged infection outbreaks that the typical furuncles indicating *A. salmonicida* ssp. *salmonicida,* or skin lesions and ulcers indicating infection with an atypical strain, may be seen. Sampling of furuncles, lesions and ulcers for typical and atypical strains is optimal in the early stage of the infection as advanced ulcers and lesions become invaded by opportunistic bacteria and fungi and can hinder successful culture of the bacterium.[10] The isolation rate is higher in the early stages of the disease. For culture and molecular testing, appropriate sampling locations are skin and gill mucus, leading edge of new lesions and ulcers, gills, blood, haemorrhagic areas, and internal tissues including heart blood, anterior part of kidney, spleen, liver, heart, and intestine. Note that the intestine will contain normal flora that can overgrow *A. salmonicida*. Mucus and gill samples can result in a higher positive detection rate by both culture and PCR compared to kidney, spleen and intestine.[86,87] The internal tissues, lesions and ulcers are also suitable for histopathology.[36]

Suitable culture media include blood agar, tryptic soy agar, or brain heart infusion agar supplemented with 15% serum. Subculture of suspect colonies to Trypticase Soya agar (TSA) may reveal colonies with a water-soluble brown pigment, and subculture to Coomassie Brilliant Blue (CBBA) R-250 agar can enable the detection of the A-layer in virulent strains. Expression of the A-layer occurs between 15 and 22°C but not at higher temperatures.[30,31,32,33] The A-layer may be present in typical and atypical strains.[17] CCBA indicates protein is present, but it is not a specific test for *A. salmonicida* and may detect dye binding protein layers in other bacteria.

Incubation temperature and time is critical in prevention of variation in biochemical reactions.[68] When comparing results in the literature it is important to use the same format of biochemical test as used in the reference and to incubate the tests at the same temperature and time. Due to the wide variation in biochemical reactions, particularly for the atypical strains, molecular identification must also be done. A flowchart (Figure 6) for the differentiation of the typical and atypical strains was reported by Gulla *et al.*, (2016)[17] and is detailed under section 2.4. Figure 6 is to be used as a guide towards differentiation of typical and atypical strains, but specific PCR and possibly sequencing the partial *vapA* gene[17] is also recommended to accurately differentiate typical and atypical strains. *Aeromonas salmonicida* is in the matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometry database (MBT Compass library DB-6903 BioTyper, Bruker Daltonics) however, because the ribosomal proteins are so similar, the bacterium cannot be differentiated from *A. bestiarum* (and *vice versa*), although colony morphology, motility and growth at higher temperatures enable differentiation. Table 6 is included to assist differentiation of closely related species using their phenotypic characteristics. Identification of a suspect *A. salmonicida* is done by specific PCR and partial *vapA* gene sequencing. Two conventional PCR primer sets (PAAS and AP) reported by O'Brien *et al.*, (1994)[59] and Gustafson *et al.*, (1992)[84] were validated by Byers *et al.*, (2002).[86,87] Sensitivity of detection is improved by use of both primer sets. The sensitivity for the individual primer sets is 93 and 93.3%, respectively, however use of both primer sets results in a sensitivity of 99.4%.[86] The primer sets are suitable for testing colony growth or tissue. Limits of detection for the PAAS and AP primers are 10^3–10^4 and 10^4–10^5 colony forming units per gram of seeded tissue, respectively. A primer set (MIY primers) reported by Miyata et al, (1996)[85] and validated by Byers *et al.*, (2002)[86,87] are 100% specific for *A. salmonicida* ssp. *salmonicida* with a limit of detection of 10^6–10^7 CFU/gm. A higher detection rate is achieved from overt infections compared to covert infections, and detection by culture is often more successful than PCR for covert infections.[86,87]

A real time PCR using a molecular beacon to detect typical and atypical *A. salmonicida* was developed and validated by Keeling et al, (2013).[88] The specificity is 100% and the analytical sensitivity is 5 fg of DNA, and for seeded tissue it is $2.2 \times 10^4 \pm 1 \times 10^4$ CFU/g with enrichment, or 40 ± 0 CFU/g without enrichment. The PCRs are detailed under section 2.4.

If required, a strain typing scheme, that can also enable discrimination of typical and atypical strains, based on the hypervariable region of the *vapA* virulence gene for those isolates that possess the A-layer gene, has been established.[17,18] The A-layer sequence in the hyper variable region is highly correlated to host species.

Other information for identification and confirmation of *Aeromonas salmonicida* ssp. *salmonicida* can be found at The Australian Government, Department of Agriculture website under AQUAVETPLAN, Furunculosis.

1.7 Guidance on safety and biosecurity requirements

Aeromonas salmonicida ssp. *salmonicida* is a highly contagious bacterium and is exotic to Australia and New Zealand. Suspect samples with appropriate paperwork/submission form are sent to the state government veterinary laboratory for a provisional diagnosis but confirmation must be done at the Australian Centre for Disease Preparedness (ACDP) (previously Animal Health Laboratories), Geelong, Australia or in New Zealand samples are sent to the National Animal Health Laboratory, Ministry for Primary Industries (MPI), Wallaceville, Upper Hutt.

Samples are collected and packed as outlined by Handlinger (2008),[89] and all packages, equipment etc. to be disinfected following the Aquavetplan decontamination guidelines,[90] suitable disinfectants include 3% bleach, Virkon or CaviCide. The state laboratory must be notified of the suspect disease and the arrival of the samples.

For states where *A. salmonicida* is known to occur, the pathogen should be held at PC2 level unless directed otherwise by the jurisdiction's Chief Veterinary Officer. Where suspect exotic disease is likely, samples for confirmatory testing for an exotic disease agent must be sent for definitive testing at the appropriate national laboratory in each country responsible for exotic disease agent testing.

2 Test Methods

2.1 Collection and transportation of specimens for laboratory diagnosis

Moribund, freshly dead, euthanased fish or samples of tissues are collected aseptically and sent to the testing laboratory. Suitable samples for culture include haemorrhages in the musculature, kidney, heart blood, liver, spleen, and peritoneal fluid. These sites are sterile normally but become sites of concentration for *A. salmonicida* ssp. *salmonicida* as the result of haematogenous spread and proliferation. Samples from the intestine and pus-filled cavities tend to have a lot of opportunistic or normal bacterial flora that may impact on the successful outcome of culture[10] and PCR. For septicaemic conditions, kidney samples will likely prove the most rewarding for culture. A subclinical carrier state and covert infections can occur with *A. salmonicida* ssp. *salmonicida* in which case skin mucus and gill along with kidney should be sampled to increase the likelihood of detection by culture and PCR.[87]

A variety of clinical signs and symptoms occur in different hosts infected with atypical strains,[36] and collection of samples should follow those outlined for typical strains. Samples include skin lesions and ulcers, internal organs such as kidney, liver, spleen, and the eye.

Transport moribund fish in sealed plastic bags containing air and water and placed into a suitable insulated container. Dead fish are put into individual sealed plastic bags and transported on ice. If arrival at the laboratory is greater than 48 hours, then culturing on-site may provide a more consistent sample. Vacuum-packed samples of freshly dead fish or tissues may assist in retaining sample integrity.

All equipment and packaging is disinfected with Virkon or bleach to avoid transmission of any disease agent. The samples are packaged according to International Air Transport Association (IATA) regulations.

Instructions for the collection and transportation of finfish for investigation of disease is detailed in *Finfish Sampling* by J. Handlinger (2008)[89] available to download from the Australian Government Department of Agriculture, Water and the Environment website.

2.2 Culture

Aeromonas salmonicida is readily cultured on routine culture media such as blood agar, nutrient agar, tryptic soy agar, or brain heart infusion agar.[68] Of these, blood agar (BA) is recommended particularly where slow growing or fastidious strains might be involved such as from greenback flounder, flounder and turbot.[91,38]

No selective medium has been developed, however a study on the recovery of *Aeromonas* species from water and shellfish found a higher rate of recovery for some strains of *A. salmonicida* when starch ampicillin agar (SAA) was used with a pre-enrichment step, however the recovery was variable according to strains.[56] Arkwright (1912)[10] used neutral-red-lactose-bile salt (0.5%) agar to

improve isolation from intestinal contents. This medium is MacConkey agar without crystal violet as detailed by MacConkey, (1905).[92]

2.2.1 Incubation atmosphere and temperature

Plates are incubated aerobically (in air) for at least 96 hours at 15–22℃ and examined daily for the presence of colonies typical of *A. salmonicida*.

Aeromonas salmonicida grows at 5 ºC up to 32℃ with scant or no growth at 36℃. Growth does not occur at 37℃[10,43] except for mesophilic atypical strains. The optimum temperature, especially for identification of virulent strains through the production of the A-layer protein as detected on Coomassie Brilliant Blue agar, is between 20–22℃. Loss or inactivation of virulence factors may occur at temperatures >22℃. Pigment is rarely produced above 22℃ and an incubation temperature of 20℃ is recommended for both optimal pigment production and expression of the A-layer.[10,37, 30, 32,33] Biovars reported to occur in Australia have been isolated at an incubation temperature of 25°C, as have many other reports in the literature. As explained, some tests such as pigment production must be done at 20–22°C otherwise false-negative results can occur.

2.3 Phenotypic identification

2.3.1 Colony morphology

Aeromonas salmonicida is slow-growing and has a much smaller colony size compared to the motile *Aeromonas* species such as *A. hydrophila*. Typically, the colony morphology resembles *Streptococcus* species (Figure 1 and Figure 2) rather than other *Aeromonas* species though variants do occur and can have morphologies similar to mesophilic aeromonads (Figure 4).

On blood agar, colonies are pinpoint at 18–24 hours (Figure 1 and Figure 2A) and grow to 1–2 mm greyish, translucent, entire, round, raised colonies with a flattish surface after 3–7 days (Figure 2B). Beta-haemolysis occurs on horse blood agar and to a lesser extent on bovine blood agar, whereas sheep red cells tend not to be haemolysed. The production of haemolysins is influenced by a number of factors including production of proteases, incubation temperature and the growth medium. *A. salmonicida* gave a higher score of haemolytic activity against red cells when grown in nutrient broth compared to growth in tryptone soya broth.[93]

Colonies are friable and remain entire when pushed across the plate with a loop (not unlike an ice hockey puck, and similar to some *Streptococcus* species). Friable colonies persist through many subcultures.[3] Initially, colonies are a whitish grey colour and become yellowish, and after 2–4 days and over 3 weeks colonies become brownish to a dark coffee-colour, and a brown pigment diffuses into the agar.[10] On nutrient agar, colonies are 0.1 mm at 24 hours, and after 3–7 days increase to 1.0--1.5 mm, and a brown water-soluble pigment may be seen (Figure 2B and Figure 5).

Growth in nutrient broth culture is flocculent with slight turbidity, and a deposit and ring of growth occurs around the edge and bottom of the tube. When grown at 5℃ in peptone water, flocculent growth occurs but no turbidity is seen.[37,3]

Figure 1. *A. salmonicida* atypical strain from goldfish at 24°C after 24–48 hours on horse BA (A) and beta-haemolysis under the same conditions as figure 1A (B)

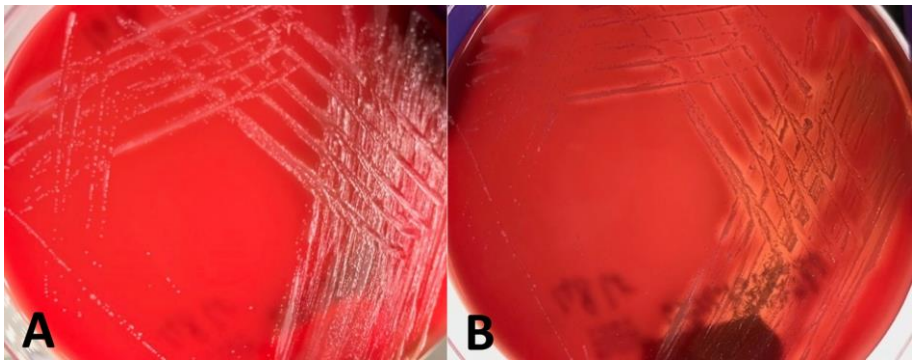


Figure 2. *A. salmonicida* atypical strain from goldfish at 24°C and 72 hours (A) and 7 days (B) on bovine BA

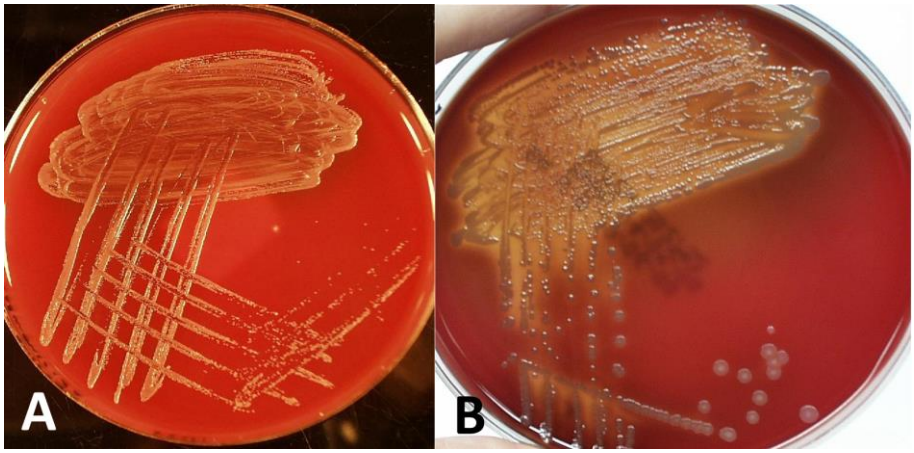


Figure 3. Gram stain of atypical *A. salmonicida* (A) and *A. salmonicida* atypical mesophilic strain (B) from goldfish at 24 hours and 24°C on horse BA

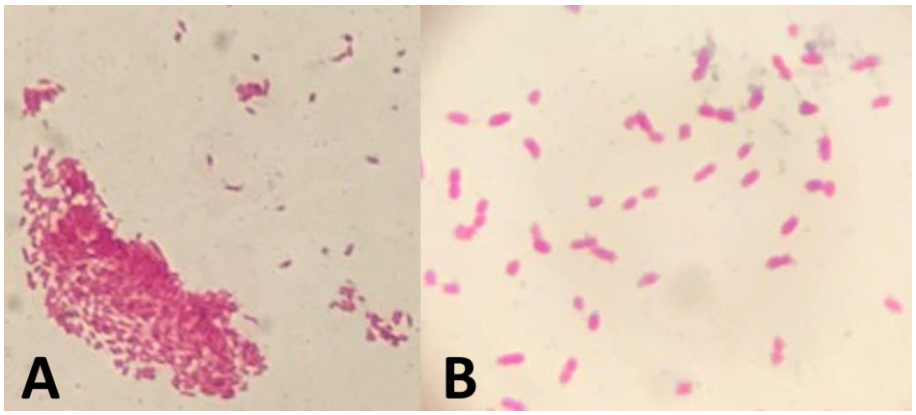
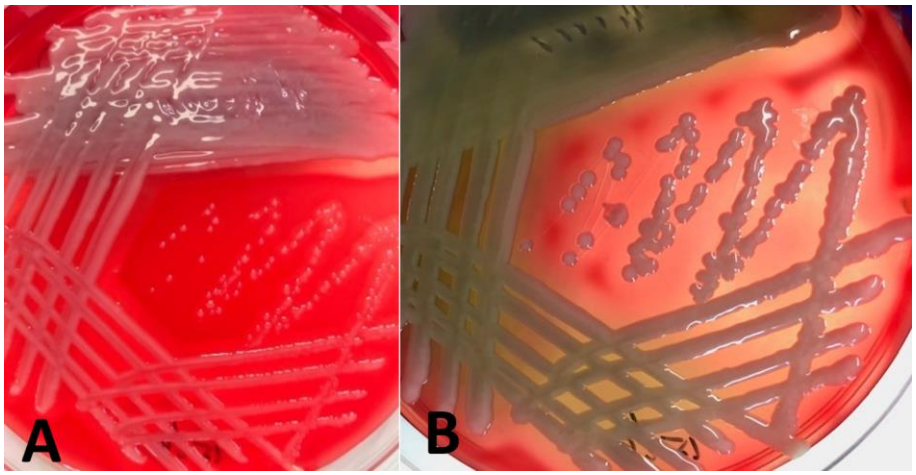


Figure 4. *A. salmonicida* atypical mesophilic strain from salmonid at 24°C and 24 hours (A) and 48 hours (B) on horse BA



2.3.2 Gram stain

Aeromonas salmonicida is a Gram-negative, short rod to coccobacillus (1–4 x 0.8–1 µm) where the length of the cell is less than twice the width (Figure 3). It often occurs in pairs but can be pleomorphic. When grown in nutrient broth short chains, clusters, or clumps composed of chains of cells may be seen, however considerable variation may occur when grown in different culture media and as cells age.[10,43,37,3] Longer rods may be seen in older cultures, and when stained with Loeffler's methylene blue or carbol-thionin blue or Leishman's stain, some may show bipolar staining.[10] Cells in smears made from blood or lesions show more bacillary forms compared to cells grown in artificial media.[37]

2.3.3 Production of pigment

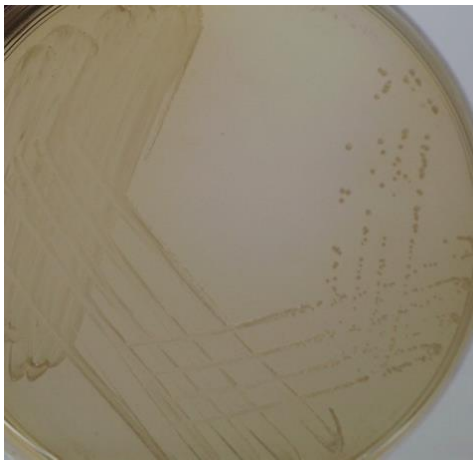
A brown, water soluble pigment is produced by all strains of *Aeromonas salmonicida* to a variable degree.[10,43,37] *Aeromonas salmonicida* ssp. *salmonicida* tends to produce pigment as do most of the atypical strains. *A. salmonicida* ssp. *achromogenes* was initially classified as a non-pigment producing species, but Dalsgaard *et al.*, (1998)[68] reported pigment from the NCMB 1110 type strain during an inter-laboratory study, and also quotes earlier papers that note pigment production from this subspecies.

Pigment production, melanogenesis, is influenced by growth temperature, incubation atmosphere, pH and composition of the broth or agar. The melanogenesis synthesis pathway is temperature dependent due to a critical enzyme, 4-hydroxyphenylpyruvate dioxygenase, being thermolabile at temperatures above 22°C.[94] The optimum temperature to induce melanogenesis occurs at 20–22°C, but rarely above 22°C and only under aerobic conditions and does not occur under anaerobic conditions.[37] Composition of the medium, in particular, concentrations of glucose at 0.1% w/v and greater can inhibit pigment production, but the inhibitory effect can be reversed by the addition of cyclic adenosine monophosphate (cAMP). Columbia base agar tends to give better results for pigment production.[34] Pigment production is inhibited at pH of less than 7.6.[9,37] Other bacteria can produce a brown pigment on TSA and these include *Brevundimonas diminuta*, *Stenotrophomonas* (*Xanthomonas* [*Pseudomonas*]) *maltophilia*, *Pseudomonas* species and other *Aeromonas* species such as *A. media*.[95]

Pigment production may be enhanced on media containing 0.1% tyrosine or phenylalanine, however, not all strains of *A. salmonicida* ssp. *salmonicida* produce pigment.[96] Previously, rapid production of pigment versus slow reaction or no production of pigment was included in the range

of tests to differentiate typical strains from atypical strains, however pigment production is too heavily influenced by the growth factors mentioned, to be a reliable test for differentiation.[96]

Figure 5 Atypical *Aeromonas salmonicida* from goldfish on nutrient agar showing pigment production at 7 days growth



2.3.4 Detection of the A-layer

Virulent typical and atypical strains may produce the A-layer protein. Expression of the A-layer is detected on Coomassie Brilliant Blue agar (CBBA) composed of tryptic soy agar containing 0.01% of Coomassie Brilliant Blue dye.[97,98] The A-layer protein binds Coomassie Blue, therefore colonies containing the A-layer occur as medium to dark blue colonies, whereas non-virulent strains do not have the A-layer and appear as white colonies. Colonies possessing this protein develop a blue colour after 2–7 days at 20–22℃. Fastidious strains may require prolonged incubation of 7–14 days. Some isolates of *A. salmonicida* may possess the *vapA* gene, but may be negative for the A-layer protein.[86,87] The reaction with Coomassie Blue is not specific and *Aeromonas hydrophila*, *Pseudomonas* species, *Pasteurella multocida*, *Acinetobacter* species, *Alcaligenes*, *Flavobacterium* species, *Moraxella nonliquefaciens*, *Corynebacterium* species and *Staphylococcus lentus* contain a protein layer that can bind the dye. Therefore, CBBA is not recommended as the sole primary culture medium, but is suitable as part of the screening process along with phenotyping and PCR to detect virulent strains.[95]

2.3.5 Detection of auto agglutination as a proxy for virulence

Detection of autoagglutination can be performed using broth culture or by testing hydrophobicity in a salt suspension.[98]

Aggregation in broth culture: Inoculate a tryptic soy broth or brain heart infusion broth and incubate at 20℃ for 48 hours. A smooth, stable suspension indicates non-clumping or no agglutination, whereas presence of a flocculate or sediment in the bottom of the tube indicates clumping or auto aggregation.

Hydrophobicity in ammonium sulphate: Prepare a suspension of bacterial cells in phosphate buffer, pH 7.2, 0.002M, to a density of 30% transmittance at 525 nm in a colorimeter. Centrifuge cells in 1.5 ml volumes at 1,000 g for 5 min. Resuspend pellet in 0.1 ml of 0, 10, 20, 30, 40 and 50% concentrations of $(NH_4)_2SO_4$. Incubate cells in V-bottom microtitre plates at 20°C for 24 hours. Examine visually for agglutination. Non-virulent strains are non-agglutinating, hydrophilic and will require at least 50% $(NH_4)_2SO_4$ to agglutinate the cells. Virulent isolates are hydrophobic and will agglutinate in a suspension of 10% $(NH_4)_2SO_4$.[98]

2.3.6 Biochemical tests

All *Aeromonas salmonicida* subspecies and biovars are non-motile, in contrast to other *Aeromonas* species that are mesophilic and motile. While the phenotype of *A. salmonicida* is non-motile, the species has cryptic, non-functional genes for motility[99] and some strains have been reported as being motile.[100] Reports for motility of *A. sobria* and *A. media* are variable in the literature. *A. media* is recognised as motile, however *A. veronii* biovar Sobria (motile) often has been incorrectly identified as *A. sobria* (non-motile).[1,101,102,103] Like motile *Aeromonas* species, *A. salmonicida* is resistant to the vibriostatic agent 0/129 150 µg, whereas the non-motile *A. sobria* is sensitive to the 150 µg disk.[1]

Biochemical and phenotypic tests are carried out initially to identify *Aeromonas salmonicida* before further testing such as specific PCR and partial *vapA* gene sequencing is undertaken to differentiate the typical from the atypical strains.

Aeromonas salmonicida is identified if all of the following criteria (Error! Reference source not f ound.) are present: Gram-negative coccobacillus often in clusters, non-motile, positive for oxidase and catalase, both oxidative and fermentative production of acid from glucose, resistant to vibriostat 0/129, growth at 22°C, and growth of friable colonies.[17] There are some rare A-layer-negative strains that do not produce friable colonies. Friable colonies are colonies that remain entire when pushed across the plate with a loop. The oxidase reaction can be unreliable particularly with some of the commercial methods available; as a reference method use Kovác's procedure.[89] It is important to adhere to the method for each particular reagent. In addition, uncommon oxidase-negative strains have been reported.[104,91]

Aeromonas salmonicida ssp. *salmonicida* is identified if in addition to fulfilling tests in Criterion 1, the following criteria (Error! Reference source not found.) are also fulfilled: haemolysis on horse or b ovine blood agar, production of a brown, water-soluble pigment on tryptone yeast extract agar, negative for indole, hydrolysis of gelatin and aesculin, and fermentation of mannitol. Sucrose is not fermented, however a North Korean isolate of *A. salmonicida* ssp. *salmonicida* from farmed salmon fermented sucrose (unpublished data, Dr Snorre Gulla, pers. comm. Sept 2020 & June 2021). Dalsgaard et al., (1994)[105] notes that *A. salmonicida* ssp. *salmonicida* is positive for the fermentation of L-arabinose but negative for fermentation of D-arabinose.

Isolates that fulfil Criterion 1 but not Criterion 2 can be identified as atypical *A. salmonicida*.[17] There are reports in the literature of rare strains that do not fulfil all criteria. Variable results in the literature have been reported for indole production by atypical strains.[68] When performing biochemical tests for any bacterium, it is important to use the same method (including biochemical method and incubation temperature) as those reported in the reference being consulted.

Unfortunately, biochemical identification can be unreliable and discrepancies between laboratories were reported for atypical strains during an inter-laboratory study, which found that incubation temperature and time, and method of biochemical test and preparation is important for consistency of results.[68] The recommendations for consistent results includes preparation and use of biochemical tests according to Cowan and Steel's Manual for the Identification of Medical Bacteria,[106] production of good growth in tubes by use of a heavy inoculum, which is particularly important for decarboxylases, arginine dihydrolase (ADH) and fermentation tests, reading of results after 2 days and continued incubation up to 7 days for tests negative at 2 days. Some biochemical tests are more reliable than others, with ornithine decarboxylase considered a reliable test, whereas oxidase, arginine dihydrolase, gelatin liquefaction and Voges Proskauer test (acetoin production) may be variable between laboratories. The API 20E kit (Biomerieux) is not reliable for aesculin hydrolysis or fermentation of carbohydrates. Results for gelatin liquefaction are easier to read if done using nutrient agar rather than brain heart infusion agar.[68] Tube gelatin tests may take a number of days for a positive result, but a simple plate agar method produces a result in 1–2 days.[107] It is important

to use an inoculum with the optimal cell concentration, NaCl concentration, incubation time and temperature according to the biochemical method and identification tables (Appendix A, Tables 2- 5) being consulted. Isolates from the marine environment may give different results for some tests depending upon NaCl concentration of the inoculum (and incubation temperature). Often the optimal NaCl concentration for growth for bacteria from the marine environment is 2.5% NaCl. Because of the sodium ion requirement for some enzyme pathways in the bacterium, the test results may be different when an inoculum giving a final concentration of 0.85% or 2.5% is used.[108,109] The conditions used in the reference tables will guide the inoculum concentration, and incubation temperature and time.

Matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry identifies *A. salmonicida* but cannot differentiate atypical strains from typical strains. Also, MALDI-TOF cannot distinguish *A. bestiarum* from *A. salmonicida* and will misidentify these two species. Therefore, an identification of one or the other of these species must be followed up with biochemical tests. *A. bestiarum* is motile and grows at 42°C and has a colony morphology (about 2 mm in diameter at 24 hours on BA) typical of most motile *Aeromonas* species. *A. salmonicida* is non-motile, and apart from unusual atypical mesophilic strains, does not grow at 37°C and has not been reported to grow at 42°C. Most strains are 1 mm at 48 hours on BA, although a range of morphologies may occur (J. Carson, unpublished data).

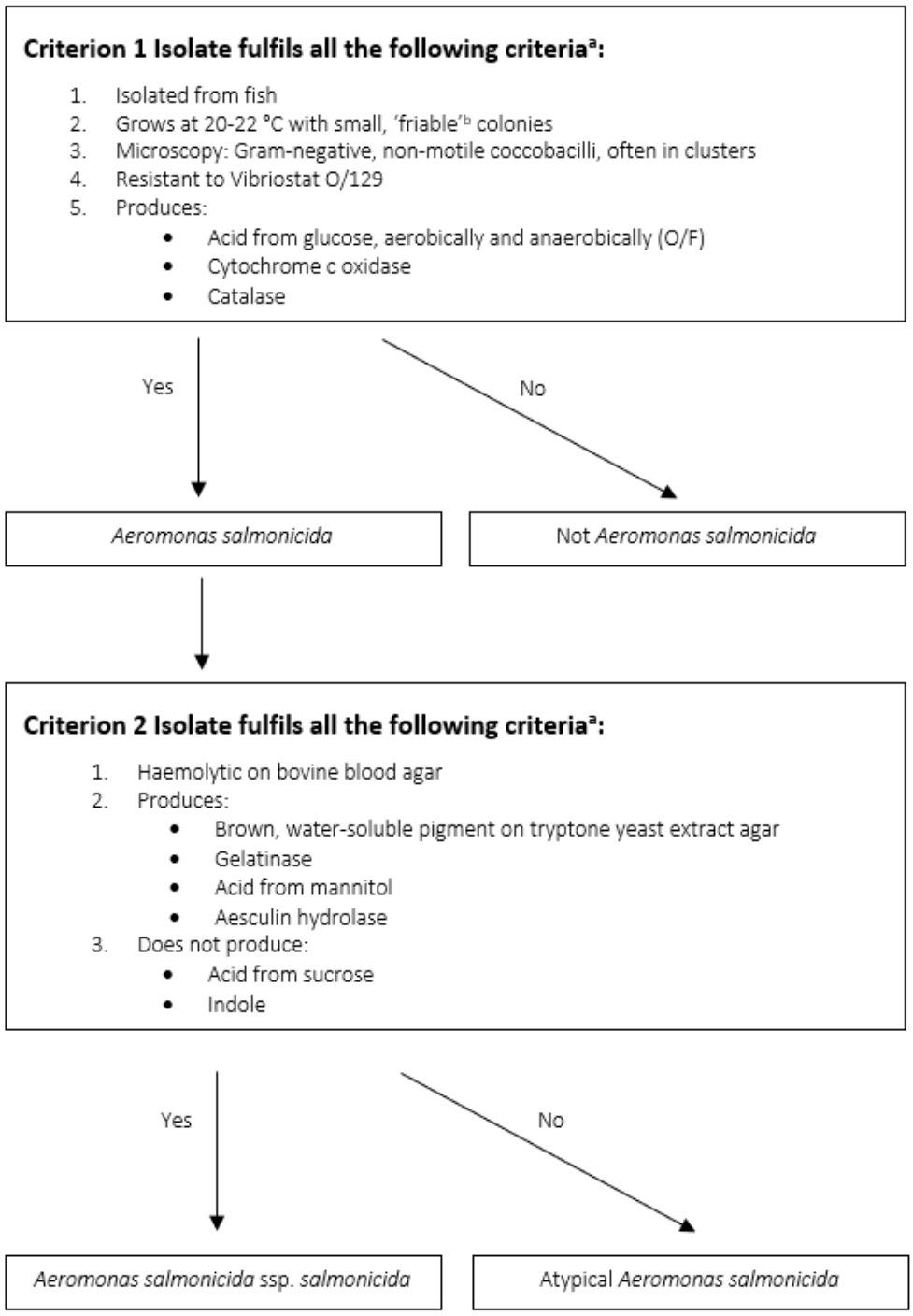
An identification strategy by phenotype is summarised in the flowchart, Figure 6. The mini-panel of tests is sufficient to achieve a presumptive identification of *A. salmonicida*. Greater confidence is achieved by increasing the panel of phenotyping tests, see Tables 2–5 in Appendix A, which should enable allocation of an unknown either to subspecies or biovar for an atypical strain, although follow-up testing with specific PCRs and partial *vapA* gene sequencing is advised.

A range of identification strategies are available, phenotyping as well as by genomic analysis. Phenotyping offers the opportunity of strain allocation that is rapid and straightforward to implement, particularly for those laboratories where phenotyping is used routinely. Molecular approaches to identification have a specific purpose and utility. Where isolation of *A. salmonicida* occurs for the first time, then identification should be made on the basis of both phenotypic and genomic analysis; see section 2.4 for further details of targets and corresponding PCR primer sets.

The choice of approach to identification will be influenced as to the purpose of testing. For routine testing, where the pathogen is known to exist, testing may rely solely on PCR. If, however, it becomes important to assess strain variation, then phenotyping has specific advantages in terms of cost-effectiveness and speed. Strain typing using the partial *vapA* gene should be considered (see section 2.4). Ideally, a diagnosis of *A. salmonicida* is made after both phenotype and genotype analysis is performed, as variations in biochemical signatures occur between and within *Aeromonas* species can lead to misdiagnosis or underreporting.

The flowchart in Figure 6 assists in the differentiation of typical *A. salmonicida* from atypical species and was published in 2016[17] and updated in 2021[110] and is included with permission from Dr Snorre Gulla (pers.comm. June 2021). He recommends that these tests be done to determine *A. salmonicida* followed by partial *vapA* sequencing and phylogenetic tree construction with comparison to other strains available at Microreact.[18]

Figure 6 Flowchart for identification and differentiation of typical and atypical strains of *Aeromonas salmonicida*[17]



a Some rare exceptional cases have been described in the literature
b Except A-layer negative strains

2.4 Molecular identification

A number of PCR methods for the detection of typical and atypical *A. salmonicida* are reported in the literature. No PCRs have been approved for use by SCAHLS, however Byers et al., (2002)[86,87] at the Australian Centre for Disease Preparedness (ACDP) evaluated three published conventional PCR protocols; two primer sets for the detection of typical and atypical *A. salmonicida* using Probe Assay for *Aeromonas salmonicida* (PAAS) primers[59] and Array Protein (AP) primers,[84] and one primer set, MIY, specific for the detection and identification of *A. salmonicida* subspecies *salmonicida*.[85]

At MPI Wallaceville laboratories in New Zealand, Keeling et al., (2013)[88] developed and validated a real time PCR for detection of typical and atypical *A. salmonicida* that is used for routine screening. Other PCRs reported by Soler et al., (2004),[19] Nilsson et al., (2005),[122] Saavedra et al., (2006)[123] and Byers et al., (2002)[86,87] are used for further confirmation and sequencing (Sharon Humphrey, MPI, pers comm. 2020).

As per standard procedures, positive and no template controls are included with each run. Where positive control DNA is not readily available, the laboratory could consider designing artificial or synthetic positive controls.[124]

Any modifications from these published protocols such as different DNA extraction method, master mix and other reagents, thermocycler platform and change of thermocycling conditions need to undergo verification to obtain the same analytical sensitivity and specificity as found by Byers et al., (2002)[86] and Keeling et al., (2013),[88] respectively. The requirements for method validation and verification are outlined in OIE and NATA documents which are hyperlinked.[125,126]

2.4.1 Conventional PCR for detection of *A. salmonicida*

The sensitivity of detection of typical and atypical *A. salmonicida* using the PAAS[59] and AP primers[84] is 93% and 93.3%, respectively.[86,87] Neither primer set detects all *A. salmonicida* isolates, but use of both primer sets increases the sensitivity to 99.4%. The MIY primer set[85] is 100% specific for *A. salmonicida* ssp. *salmonicida*.[86,87] The PAAS and AP primers are suitable for use on tissue and the limits of detection are $10^{3-}10^{4}$ and $10^{4-}10^{5}$ colony forming units per gram of seeded tissue, respectively. The limit of detection for the MIY primers is higher ($10^{6-}10^{7}$ CFU/gm) and may not detect low numbers of organisms.[86,87] It should be noted that at these levels of bacterial loading, detection by culture would be readily achieved.

The PCRs can be used on bacterial colonies or fish tissues: gill, kidney, spleen, intestine, muscle lesion and mucus, although the limit of detection varies due to inhibitory factors in the sample. The detection rate is greater in overt infections compared to covert infections, and in covert infections detection by culture may be greater than detection by PCR.[87] Detection in mucus produces the greater number of positive PCR reactions compared to other tissues; intestine resulted in the least number of positives.

2.4.1.1 Nucleic acid extraction

The following commercial kits are suitable for DNA extraction: Puregene kit (Gentra Systems), DNAsol reagent (Life Technologies, Thermo Scientific), QIAamp Tissue kit (Qiagen).[86,87] All kits are used according to the manufacturer's instructions. Template DNA of 1–10 ng is added to the PCR mixture.

2.4.1.2 Amplification using conventional PCR

(1). PAAS PCR

The target for the PAAS primers[59] is on a 6.4 kb cryptic plasmid known to occur in 90% of *A. salmonicida* ssp. *salmonicida* isolates, all *A. salmonicida* ssp. *achromogenes*, some *A. salmonicida* ssp. *masoucida*, and some atypical *A. salmonicida* isolates.[127,128]

PAAS1 (5'- CGTTGGATATGGCTCTTCCT-3')

PAAS2 (5'- CTCAAAACGGCTGCGTACCA-3')

A 25 µl reaction mix contains 0.25 U of Platinum™ *Taq* (Life Technologies), 2.5 µl of 10 x PCR buffer, 2.5 mM $MgCl_2$, 8 pmol of the primers, and 0.2 mM of each dNTP.[86,87] Amplification is performed using an initial hold at 95℃ for 30 sec followed by 30 cycles of denaturation at 94℃ for 2 min, annealing at 57℃ for 30 sec, extension at 72℃ for 1 min 30 sec and a final extension at 72℃ for 3 min. The amplicon is 423 bp.

(2). AP PCR

The Array Protein (AP) primers[84] target the surface array protein gene (*vapA*) that encodes the A-protein or the A-layer of *A. salmonicida*.[26] Although the *vapA* gene may be present in most *A. salmonicida* ssp. *salmonicida* isolates, the A-protein may not be expressed.[84,86] This finding was also reported by Keeling et al., (2013)[88] for the molecular beacon PCR.

AP1 (5'- GGCTGATCTCTTCATCCTCACCC-3')

AP2 (5'- CAGAGTGAAATCTACCAGCGGTGC-3')

A 25 µl reaction mix contains 0.25 U of Platinum™ *Taq* (Life Technologies), 2.5 µl of 10 x PCR buffer, 2.5 mM $MgCl_2$, 8 pmol of the primers, and 0.2 mM of each dNTP.[86,87] Amplification is performed using an initial hold at 94℃ for 2 min followed by 30 cycles of denaturation at 94℃ for 15 sec, annealing at 57℃ for 30 sec, extension at 72℃ for 1 min 30 sec and a final extension at 72℃ for 3 min. The amplicon is 421 bp.

(3). MIY PCR

The MIY primers target a sequence of DNA detected through randomly amplified polymorphic DNA (RAPD) analysis that was found to be unique to *A. salmonicida* ssp. *salmonicida*.[85]

MIY-F (5'-AGCCTCCACGCGCTCACAGC-3')

MIY-R (5'-AAGAGGCCCCATAGTGTGGG-3')

The 25 µL reaction mix according to Byers et al., (2002)[86,87] is prepared with 0.6 U Platinum™ *Taq* (Life Technologies), 2.5 µL 10 x PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM of each deoxynucleotide triphosphate, 16 pmol of each primer, 5 µL DNA and nuclease-free water. Thermocycling is carried out with an initial denaturation step at 94℃ for 2 min then 35 cycles of denaturation at 94℃ for 30 sec, annealing and elongation at 68℃ for 90 sec, and a final extension at 68℃ for 3 min. The amplicon is 512 bp.

For all three primer sets, Platinum™ *Taq* (Life Technologies) produced a higher concentration of amplicon compared to *Taq* DNA polymerase (Promega).[87]

2.4.1.3 Amplification using real time PCR for detection of *A. salmonicida*

A molecular beacon real time PCR assay[88] targets the *vap*A gene that encodes for the A-layer surface array protein[129] to detect typical and atypical *A. salmonicida* from fish tissue and bacterial colonies.

2.4.1.4 Nucleic acid extraction

DNA from bacterial cells is extracted using Instagene Matrix (BioRad), DNAzol® (Life Technologies, Thermo Fisher Scientific), or the Puragene® DNA isolation kit (Gentra Systems). As a precaution for removal of an exotic pathogen from PC3 biological containment, an initial lysis step is performed by centrifuging 1 ml of an overnight Brain Heart Infusion broth culture at 10,000 g for 1 min and suspending the pellet in 200 µl of a lysis buffer composed of 50% v/v absolute ethanol, 35% v/v 6M guanidinium thiocyanate and 15% v/v water. The lysis mixture is incubated at 56℃ for 30 min, then centrifuged at 10,000 g for 1 min. After removal of the supernatant the pellet is resuspended in 200 µl of Instagene Matrix (BioRad) and DNA extracted according to the manufacturer's instructions.

For DNA extraction of *A. salmonicida* from fish tissue, the initial precautionary preparation is performed by adding the lysis buffer described for the bacterial pellet in a ratio of 25% w/v of tissue and lysis buffer. The Instagene Matrix (BioRad) is then added to the pellet to the same volume used for the lysis buffer. This avoids the need to standardise kidney weights. Following manufacturer's instructions, prior to the PCR, DNA extracted using the Instagene Matrix is re-centrifuged so there is no carryover of the Chelex resin. A DNA concentration of 1–10 ng/µL extracted from a pure culture is used in the qPCR reaction.[88]

(1). VapA qPCR

VapAF1 5′- TAAAGCACTGTCTGTTACC-3′

VapAR1 5′- GCTACTTCACCCTGATTGG-3′

AS MB 5′ d CAL Fluor Orange 560-
CGCGATC(ACATCAGCAGGCTTCAGAGTCACTG)GATCGCG-BHQ-1 3′)

The master mix is prepared with 2 x JumpStart Taq Readymix with dUTP (Sigma-Aldrich), 3.5 mM $MgCl_2$ (Sigma-Aldrich), final concentrations of 0.2 µM of each primer and 0.5 µM of the molecular beacon probe with nuclease-free water and 1 µL of DNA (1–10 ng/µL) in a final volume of 25 µL. Thermocycling is carried out on a Rotor Gene 3000 (Qiagen, previously Corbett Research) with an initial denaturation step at 94℃ for 2 min then 40 cycles of 94℃ for 15 sec, 62℃ for 30 sec and 72℃ for 20 sec with acquisition on the annealing step and the Joe fluorophore channel. Any variation from the original method must undergo verification as described in the OIE and NATA documents.[126,125]

Keeling et al., (2013)[88] verified different master mix and thermocyclers. These included use of the SsoFast Probes Supermix (BioRad) on the CFX96 thermocycler (BioRad) using the same reagent parameters but a reaction volume of 10 µL. Thermocycling was conducted at 95℃ for 2 min followed by 40 cycles of 95℃ for 5 sec and 62℃ for 5 sec with data acquisition on the Hex fluorophore channel. The ABI 7500 platform (Applied Biosystems, Life Technologies, ThermoFisher Scientific) gave the same results using the same reagent and cycling parameters as used for the Rotor Gene 3000.

2.4.1.5 Specificity and sensitivity

The analytical specificity is 100% against many motile *Aeromonas* species, *Vibrio* species, *Photobacterium damselae* and some other aquatic species pathogens.[88] For DNA from bacterial cells, the analytical sensitivity is 5 fg, and for *A. salmonicida* DNA extracted from seeded tissue with enrichment, the sensitivity is $2.2 \times 10^4 \pm 1 \times 10^4$ CFU/g, and without enrichment it is 40 ± 0 CFU/g. R2 was >0.99 for pure DNA and seeded tissue without enrichment, but for enrichment the relationship was not linear. A Ct value of <= 38 is considered positive but may need to be evaluated depending on the thermocycler used.

2.4.1.6 PCRs used for sequencing to assist with confirmation of identification

(1). ISAsa PCR

An insertion sequence ISAsa4 that interrupts the *tapA* gene is present in a number of copies in 93.1% of atypical *A. salmonicida* strains tested.[122] Although the *tapA* gene, which encodes for pilin subunit protein of type IV pili, is identical in both typical and atypical *A. salmonicida*, the insertion sequence ISAsa4 is not present in typical strains and may be a source of genetic diversity in atypical strains. Sequencing ISAsa4 insert may help confirm an atypical strain following PCRs using the AP, PAAS and MIY primmer sets.

ISAsa4F 5'-CCTGCACCGCCTCATTTCTC-3'

ISAsa4R 5'-GAAAACCCAGTGATCTGAGC-3'

A 25 µL master mix is prepared containing 1 x buffer (50 mM KCl, 10 mM Tris-HCl, PH 9.0 and 0.1% Triton®X-100), 2.0 mM $MgCl_2$, 400 nM of ISAsa4F and ISAsa4R primers, 100 µM of each dNTP and 1U of *Taq* DNA polymerase (Promega). Thermocycling was carried out using a Techgene thermocycler (Techne) with an initial denaturation at 94℃ for 2.5 min followed by 30 cycles of denaturation at 94℃ for 30 sec, annealing at 67℃ for 30 sec, extension at 72℃ for 30 sec and a final extension at 72℃ for 9.5 min. Atypical strains produce an amplicon of 749 bp. The limit of detection is 250 fg of DNA. The amplicon is sequenced and a Blast search conducted to compare to Accession number AY613940.[122]

(2). rpoD PCR and sequencing

Identification of *Aeromonas* species, particularly the differentiation of *A. salmonicida*, *A. bestiarum* and *A. piscicola*, has been reported using amplification and sequencing of the *rpoD* housekeeping gene that encodes the RNA polymerase sigma factor RpoD.[19,123,20] However, some state laboratories report poor amplification and amplification of indiscriminate bands.

2.4.1.7 Strain typing for confirmation of identification and discrimination of strains

(1). Partial vapA gene typing and subtyping of typical and atypical Aeromonas salmonicida strains

As stated earlier, a typing scheme based on the *vapA* gene is proving useful for the discrimination of *A. salmonicida* strains.[17,18] The method is based on 400 bp of a highly variable portion of the *vapA* gene. Basic phenotypic testing is done according to the flow charts for criterion 1 and 2. DNA is extracted from bacterial cells using a column method on the QiaCube (Qiagen) following the manufacturer's instructions. Primers *vapA* F2 and R3 are used for PCR and sequencing.

vapA-F2 5'-CTGGACTTCTCCACTGCTCA-3'

vapA-R3 5'-ACGTTGGTAATCGCGAAATC-3'

DNA template of 1 µl is added to a reaction mix consisting of 2.5 µl of 10 x ThermoPol Reaction buffer (New England BioLabs), 0.2 mM dNTP, 0.4 µM of each forward and reverse primer, 1 U *Taq* DNA polymerase (New England BioLabs) and ultrapure water to a final volume of 25 µl. Amplification is carried out on a Dyad Dual 96-well (MJ Research) thermal cycler using an initial step for 3 min at 95 ºC, then 30 cycles of 1 min at 95 ºC, 1 min at 53 ºC and 1 min at 68 ºC with a final step of 4 min at 68 ºC then held at 4 ºC indefinitely. The amplified product is checked on horizontal gel electrophoresis. A PCR clean-up is done before sending for sequencing.

PhyML v3.0[130] is used to construct a Maximum Likelihood tree that is edited in either MEGA X[131] or FigTree v1.4.3. A Maximum Likelihood tree file is publicly available.

All *A. salmonicida* ssp. *salmonicida* isolates belong to vapA Type 1. Two Australian atypical strains (NVI-03454 and L4121) from cyprinids included in the analysis belong to *vapA* Type 10.

3 Media and Reagents

3.1 Coomassie Brilliant Blue agar

Coomassie blue agar is used to detect the presence of the A-layer protein expressed by virulent isolates.[97,98]

Add Coomassie Brilliant Blue R-250 to tryptic soy agar at a final concentration of 0.01%.

Inoculate plates and incubate at 18–22℃ for three days. The presence of deep blue colonies indicates the presence of the A-layer protein, whereas white colonies indicate the absence of the A-layer protein. As discussed earlier, production of blue pigment is not specific to *A. salmonicida*.

3.2 Pigment (melanogenesis) production

The brown, water soluble, melanin pigment produced by many typical and atypical strains of *A. salmonicida* is not related to virulence[96,35] but may be involved in persistence in the environment as discussed by Koppang et al., (2000).[35] The formation of melanin pigments involves the action of tyrosinase, which is produced by many bacteria, in response to tyrosine found in the environment. Phenylalanine is another amino acid that can lead to pigment production because it is converted to tyrosine.[132]

Pigment production may be inhibited by concentrations of greater than 0.1% glucose, and enhanced by the addition of 0.1% tyrosine or 0.1% phenylalanine.[34,132] An agar for the detection of brown pigment is TSA supplemented with 0.1% 1-tyrosine or 0.1% phenylalanine.[132] Columbia base agar without glucose is also suitable for pigment production and some strains may produce pigment earlier and of a deeper colour compared to pigment production on brain heart infusion agar.[34,96,35,68]

Plates should be incubated aerobically at 20–22℃ for up to 7 days. The production of a brown diffusible pigment is positive.

3.3 Furunculosis agar

Initially, furunculosis agar was used as a primary isolation medium for *A. salmonicida* because it enabled the detection of brown pigment, and tryptone soya agar gave good growth of *A. salmonicida*. However, later reports found that it was not a reliable primary isolation medium as other bacteria can produce brown pigment and also brain heart infusion agar was a more reliable base for growth and for detection of the water-soluble, diffusible brown pigment.[133,134] The recommended primary isolation medium is blood agar, however furunculosis agar is included for reference or for those laboratories who may want to use it alongside blood agar.

Table 1 Furunculosis agar composition

Ingredients	Quantity
Bacto-Tryptone (Difco)	10 g
Yeast extract (Disco)	5 g
L-tyrosine (Merck)	1 g
NaCl (Merck)	2.5 g
Agar (Oxoid L11)	15 g
Distilled water	1000 mL

pH	7.3
Bacto-Tryptone (Difco)	10 g

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Appendix A: Biochemical reaction tables

Table 2 Biochemical reactions of *A. salmonicida* ssp. *salmonicida* and atypical species using conventional tests

Tests	*A. salmonicida* ssp. *salmonicida*	*A. salmonicida* ssp. *achromogenes*	*A. salmonicida* ssp. *smithia*	*A. salmonicida* ssp. *masoucida*	*A. salmonicida* ssp. *pectinolytica*	GUD isolates	Atypical mesophilic strain	*A. salmonicida* Atypical mesophilic strain [c]	Atypical sand eel	Other atypical *A. salmonicida* lab study [88]
Oxidase	+	+	+	+	+	+	+	+	+	+ (v)
Catalase	+	+	+	+	+	+	+	+		+
Motility	-	-	-	-	-	-	-	-	-	-
Growth at 4°C	+	+	na	na	na	NT	+	na	na	na
Growth at 22°C	+	+	+	+	+	+	+	+	na	+
Growth at 37°C	-[a]	-	-	s	+	-	+	+	na	v
Beta-haemolysis bovine RBC	+	- (v)	+	+	+ (v)	+	+	+	na	v
Growth on MacConkey agar	+	+	-	na	na	na	+	+	na	na
Growth in 3% NaCl	+w	na	-	na	na	na	+	na	na	na
Production of H_2S	- (v)	v	+	na	-	-	na	na	na	na
Nitrate reduction	+	+	na	na	+	+	na	+	na	+ (v)
Indole	-	+	-	+	+	+	-	+	+	v
CBBA for A-layer blue colonies	+	+	na	na	na	na	na	na	na	na
Brown pigment on TSA	+ (v)	- (v)	- (v)	-	+	8 days	75%+	-	5 days	+ (v)
Arginine dihydrolase	+	+ (v)	-	+	-v	-	na	+	-	+ (v)
Lysine decarboxylase	v	- (v)	-	v	-	-	-	+	-	- (v)
Ornithine decarboxylase	-	-	-	na	-	-	-	-	-	-
Phenylalanine deaminase (phenylalanine agar)	-	-	na	na	na	-	-	na	na	na
Aesculin hydrolysis	+ (v)	-	-	+	-	-	-	+	-	v
DNA hydrolysis	+	+	+	na	+	na	+	+	na	+ (v)
Urea	-	-	-	na	-	-	na	-	na	na
Gelatin hydrolysis	+ (v)	+ (v)	+	na	+	-	na	na	na	+ (-)
Lipase	+	-	na	na	na	na	na	na	na	na

Tests	***A. salmonicida* ssp. *salmonicida***	***A. salmonicida* ssp. *achromogenes***	***A. salmonicida* ssp. *smithia***	***A. salmonicida* ssp. *masoucida***	***A. salmonicida* ssp. *pectinolytica***	**GUD isolates**	**Atypical mesophilic strain**	***A. salmonicida* Atypical mesophilic strain [c]**	**Atypical sand eel**	**Other atypical *A. salmonicida* lab study [88]**
Citrate utilization (Simmons)	-	-	v	na	+	50%+	50%+	+	na	na
Methyl Red	+	+ (v)	-	+	na	na	-	-	na	na
Voges-Proskauer	-	+ (v)	-	+	+[b]	-	-	+	+	+ (v)
ONPG	v	+ (v)	+	v	+	na	na	+	na	- (v)
Acid fermentation:										
Amygdalin	-	-	na	na	na	na	na	na	na	na
Arabinose	-	-	na	na	na	na	na	na	na	na
L-Arabinose	+	-	-	+	+	-	na	+	na	na
D-Arabinose	-	na	na	na	na	na	na	na	na	na
Cellobiose	-	-	na	na	+	na	na	na	na	na
D-galactose	+	- (v)	-	+	na	na	na	na	na	na
D-Glucose	+	+	+	+	+	+	+	+	na	+
Gas from glucose	+	-	+	+	+	na	na	+	-	-
Inositol	-	-	-	-	na	-	-	-	na	na
Lactose	-	- (v)	-	na	+	-	50%+	+	na	na
Maltose	+	v	- (v)	+	na	+	25%+	+	na	na
D-Mannitol	+	v	-v	+	+	+	+	+	+	+ (v)
Mannose	+	-	na	na	na	na	na	+	na	na
Melezitose	na	na	na	na	na	na	na	na	na	na
Raffinose	-w	-	na	na	na	-	na	na	na	na
L-Rhamnose	-	-	na	na	-	-	na	na	na	na
Salicin	+ (v)	-	-	-	na	-	na	+	na	- (+)
D-sorbitol	-	-	-	na	+	-	62%+	+	na	na
Sucrose	- (v)	+	v	+	+	+	+	+	+	+ (v)
Trehalose	+ (v)	+ (v)	-	+	na	-	-	+	na	na
Xylose	-	-	na	na	na	-	na	-	na	na
Vibriostatic agent 0/129	R	R	R	na	R	R	na	na	na	R
Flocculent growth in broth culture	+	na	na	na	na	Granular	na	-	na	na
Autoagglutination in 0.9% NaCl	+	+	+(v)	na	na	+	na	-	+	na

Tests	*A. salmonicida* ssp. *salmonicida*	*A. salmonicida* ssp. *achromogenes*	*A. salmonicida* ssp. *smithia*	*A. salmonicida* ssp. *masoucida*	*A. salmonicida* ssp. *pectinolytica*	GUD isolates	Atypical mesophilic strain	*A. salmonicida* Atypical mesophilic strain [c]	Atypical sand eel	Other atypical *A. salmonicida* lab study [88]
Inoculum	na	na	na	na	na	na	na	0.85% NaCl	na	na
Test incubation temperature	22°C	na	na	na	25°C	na	na	24°C, 20°C for pigment	na	20°C
Test incubation time (days)	7 days	na	na	na	2 days	5 days	na	2 days	na	7–14 days (14 for ADH)

a Isolates capable of growth at 37°C have been reported from a bird and humans. **b** negative at 35 ºC but positive at 25 ºC. **c** *A. salmonicida* atypical mesophilic strain from salmonid (unpublished data). **GUD** Goldfish Ulcer disease isolates. **na** test not applicable. **NT** not tested. **R** resistant. **RBC** red blood cells. **s** slow. **v** variable reactions reported in the literature.[12,6,8,88,111,112,4,113,114,115,68,3,116]

Note: When using biochemical identification tables, the tests must be carried out using the same formulations and incubation conditions as detailed in the respective tables.

Table 3 Biochemical reactions of *A. salmonicida* ssp. *salmonicida* compared to atypical *A. salmonicida* Australian strains using conventional tests

Tests	*A. salmonicida* spp. *salmonicida* ATCC 14174	Atypical Australian strain 402 (1980)	Atypical Australian strain B7/1 (Victoria, 1985)	Atypical Australian 1987	FD 1334 Import WA[a]	81.4326 (from Victorian hatchery)[b]
Short GNR	+	+	+	+	+	+
Motility	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	na
Growth at 37°C	-	-	-	-	NT	na
Growth at 22 ºC	+	+	+	+	+	+
Friable	+	+	+	na	+	+
Auto-agglutinates[c]	+	+	na	na	+	na
Beta haem	na	na	na	na	-	-
Brown pigment[d]	+	>4 days	+	+	+	+
ADH	+	V	-	-	-	+
LDC	-	-	-	-	-	-
ODC	na	na	na	na	-	-
Nitrate	+	+	+	+	+s	+
Indole	-	+w	+	+	+s	+
Citrate	-	-	-	-	-	-

Tests	*A. salmonicida* spp. *salmonicida* ATCC 14174	Atypical Australian strain 402 (1980)	Atypical Australian strain B7/1 (Victoria, 1985)	Atypical Australian 1987	FD 1334 Import WA[a]	81.4326 (from Victorian hatchery)[b]
Urea	-	-	-	-	-	-
MR	+	-	-	-	+s	na
VP	-	-	-	-	-	na
Aesculin	+	-	-	-	+s	-
Gelatin	+	-	-	-	w	-
ONPG	+	-	+	-	w	na
Acid fermentation:						
L-Arabinose	-	-	-	-	-	-
Glucose	+	+	+	+	+	+
Inositol	-	-	-	-	-	na
Lactose	-	-	-	-	-	-
Maltose	+	+	+	+ (v)	+	na
Mannitol	+	v	+	+	+	+
Mannose	+	-	-	-	-	na
Salicin	+	-	-	-	-	-
Sorbitol	-	-	-	-	-	na
Sucrose	-	+	+	+	+	+
Trehalose	+	-	-	-	-	-
Xylose	-	-	-	na	-	na
DNAse	na	na	na	na	+s	na
H_2S in TSI	-	-	-	-	na	na
Growth in 0% NaCl	+	-	-	v	na	na
Growth in 3% NaCl	w	+	+	+	na	na
Resistance to Vibriostatic agent 0/129	R	R	R	R	R	na
Inoculum	na	na	na	na	na	na
Incubation temperature	28°C	25°C	28°C	28°C	24°C	20°C
Incubation time (days)	6	7	6	6	4–6	na

a imported goldfish in quarantine in WA. **b** goldfish in Tasmania imported from Victorian hatchery. **c** Virulent strains auto-agglutinate. **d** an incubation temperature of 20–22°C is recommended for pigment production **na** test not applicable. **NT** not tested. **R** resistant. **s** slow. **v** variable reactions reported in the literature. **w** weak reaction.[76,117,41,118]

Note: The incubation temperatures and times used in these reports from the literature are detailed in the table. When using these tables, the same methods and conditions must be followed.

Table 4. Biochemical reactions of *A. salmonicida* subspecies and known Australian biovars by biochemical profile based on conventional test methods in a microtray format[103]

Tests	*A. salmonicida* ssp. *salmonicida*	*A. salmonicida* ssp. *achromogenes*	*A. salmonicida* ssp. *masoucida*	*A. salmonicida* ssp. *smithia*	*A. salmonicida* biovar Acheron	*A. salmonicida* biovar GUD
No. isolates	59	9	4	2	9	27
Percentage isolates positive						
Arginine dihydrolase	+	+	+	+	+	+
Lysine decarboxylase	12	-	50	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-
Acid from:						
L-arabinose	-	-	75	-	+	-
Cellobiose	-	-	+	-	-	-
Glycerol	86	+	75	-	+	15
Lactose	-	-	-	50	-	-
Mannitol	+	+	+	-	+	+
Mannose	20	+	+	-	-	26
α-methyl-D-glucoside	85	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-
Salicin	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-
Sucrose	7	+	+	+	-	+
Aesculin hydrolysis	+	50	25	-	+	37
Sole carbon utilisation:						
Citrate	-	-	-	-	-	-
DL-lactate	-	-	-	-	-	-
Urocanate	-	-	-	-	-	-
Gluconate oxidation	-	-	-	-	22	-
Pyrazinamidase	-	17	-	-	-	-
Phenylalanine deaminase	90	+	+	-	+	+
Acetoin (Voges-Proskauer)	-	-	+	-	+	-
Indole	-	17	75	-	+	70
Resistance to:						
Cephalothin 30µg	10	67	25	50	+	11
Ampicillin 10µg	12	+	50	-	+	74
Vibriostat 0/129 150µg	85	+	+	-	+	+

Motility	-	-	-	-	-	-

Note: Tests incubated at 25°C for 48 hours.[103] Each of the named subspecies includes the profile of the type strain. Data from unpublished study M. Higgins, J. Carson (DPIPWE), N. Gudkovs (CSIRO), 2006.

Table 5. API 20E results for *Aeromonas salmonicida* and atypical species according to incubation temperature reported in the literature cited

Tests	*A. salmonicida salmonicida* NCIMB 1102[119]	*A. salmonicida salmonicida* ATCC 14174[120]	*A. salmonicida salmonicida* ATCC 14174[120]	*A. salmonicida* ssp. *achromogenes* NCIMB 1110[119]	*A. salmonicida* ssp. *achromogenes* NCIMB 1110[120]	*A. salmonicida* ssp. *achromogenes* NCIMB 1110[120]	*A. salmonicida* ssp. *masoucida*[120]	*A. salmonicida* ssp. *masoucida*[120]	Atypical *A. salmonicida*[119]	Atypical *A. salmonicida*[119]	Atypical *A. salmonicida* imported goldfish, WA[118]
ONPG	+	+	-	-	-	-	+	+	-	+	-
ADH	+	+	-	-	-	-	+	+	-	+	w
LDC	+	-	-	-	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	+	-	-	+	+	+	-	+
VP	-	-	-	+	-	+	-	+	+	-	-
Gelatinase	+	+	-	-	-	+	-	+	-	+	-
Glucose	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	-	+	+	+	+	-
Inositol	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	+	+	+	+	+	+	-	w
Melibiose	-	-	-	-	-	-	-	-	-	-	-

Amygdalin	+	+	-	-	-	-	+	-	-	+	-
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+
NO_2	+	+	+	+	+	+	+	+	+	+	+
N2	na	na	na	na	na	na	na	na	na	na	na
Motility	-	-	-	-	-	-	-	-	-	-	-
MCA	+	+	+	+	+	+	+	+	+	+	
Inoculum	DW/NS	DW/NS	DW/NS	DW	DW/NS	DW/NS	DW/NS	DW/NS	DW	DW	NS
Incubation temperature	20°C	18°C	28°C	20°C	18°C	28°C	18°C	28°C	20°C	20°C	24°C
Incubation time	48 hours	48 hours	48 hours	48 hours	48 hours	48 hours	48 hours	48 hours	48 hours	48 hours	72 hours
Bionumber	700610517	300610517	000410417	004512417	000412417	000702417	304402517	304702417	004512417	300610517	204402417

ADH arginine dihydrolase. **DW** distilled water. H_2S hydrogen sulphide. **LDC** lysine decarboxylase. **MCA** MacConkey agar. **na** test not applicable. **N2** nitrite. NO_2 nitrate. **NS** normal saline. **ONPG** ortho-nitrophenyl-β-galactosidase. **ODC** ornithine decarboxylase. **TDA** tryptophan deaminase. **VP** Voges Proskauer.

Note: If using biochemical tables, the interpretation must be done using the same condition as those reported.

Table 6 Tests to assist differentiation of *A. salmonicida* species from closely related *Aeromonas* species

Tests	*A. salmonicida* ssp. *salmonicida*	*A. salmonicida* ssp. *achromogenes*	*A. salmonicida* ssp. *smithia*	*A. salmonicida* ssp. *masoucida*	*A. salmonicida* ssp. *pectinolytica*	GUD isolates	Atypical mesophilic strain	Atypical sand eel	Other atypical *A. salmonicida* lab study [88]	*A. salmonicida* Atypical mesophilic strain [a]	*A. hydrophila*	*A. bestiarum*	*A. piscicola*	*A. sobria* [b]
Motility	-	-	-	-	-	-	-	-	-	-	+	+	+	-
Growth at 22°C	+	+	+	+	+	+	+	na	+	+	+	+	+	+
Growth at 37°C	-[c]	-	-	s	+	-	+	na	v	+	+	+	+	na
Maximum growth temperature	na	na	<30°C	na	na	na	39°C	na	na	41°C	41°C	39°C	39°C	na
Indole	-	+	-	+	+	+	-	+	v	+	+	+	+	+
Lysine decarboxylase	v	- (v)	-	v	-	-	-	-	- (v)	+	+	-[c]	+	+
Brown pigment	+ (v)	- (v)	- (v)	-	+	na	na	na	na	-	-	-	-	-
Aesculin hydrolysis	+ (v)	-	-	+	-	-	-	-	v	+	+	v	+	-
Citrate utilization (Simmons)	-	-	v	na	+	50%+	50%+	na	na	+	v	v	na	+
Voges-Proskauer	-	+ (v)	-	+	+[c]	-	-	+	+ (v)	+	+	v	+	-(w)
Acid fermentation:														
L-Arabinose	+	-	-	+	+	-	na	na	na	+	v	+	-	-
Lactose	-	- (v)	-	na	+	-	50%+	na	na	+	v[d]	v[d]	-	-
D-sorbitol	-	-	-	na	+	-	62%+	na	na	+	-	-	v	-
Sucrose	- (v)	+	v	+	+	+	+	+	+ (v)	+	+	+	+	+

Tests	***A. salmonicida* ssp. *salmonicida***	***A. salmonicida* ssp. *achromogenes***	***A. salmonicida* ssp. *smithia***	***A. salmonicida* ssp. *masoucida***	***A. salmonicida* ssp. *pectinolytica***	**GUD isolates**	**Atypical mesophilic strain**	**Atypical sand eel**	**Other atypical *A. salmonicida* lab study [88]**	***A. salmonicida* Atypical mesophilic strain [a]**	***A. hydrophila***	***A. bestiarum***	***A. piscicola***	***A. sobria* [b]**
Autoagglutination in 0.9% NaCl	+	+	+(v)	na	na	+	na	+	na	-	-	-	-	-
Test incubation temperature	22 ºC	na	na	na	25°C	na	na	na	20 ºC	24°C	35°C	35°C	35°C	25°C
Test incubation time (days)	7	na	na	na	2	5	na	na	7–14 (14 for ADH)	2 (6 for pigment)	2	2	2	2 (7 for LDH)

a *A. salmonicida* atypical mesophilic strain from salmonid (unpublished data). **b** *A. veronii* biovar Sobria is often mis-identified in the literature as *A. sobria*, which is found in fish and the environment, but is not found in human clinical samples; optimum growth of *A. sobria* is 22–30 ºC.[1] **c** negative at 35ºC but positive at 25 ºC. **d** 26–74% of strains positive at 35 ºC but negative at 25 ºC. **na** test not applicable. **v** variable reactions reported in the literature. **w** weak reaction.[8,7,6,121].

Note: all *Aeromonas* species are negative for urease and, with the exception of *A. sobria*, are resistant to vibrio static agent pteridine 0/129 150 µg disc.