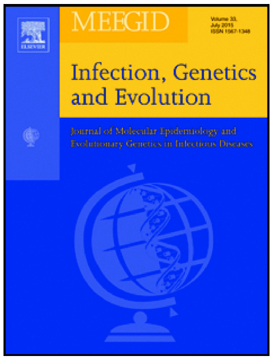

Investigation of the virulence and genomics of Aeromonas salmonicida strains isolated from human patients

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Research article

Investigation of the virulence and genomics of *Aeromonas salmonicida* strains isolated from human patients

Antony T. Vincent[1]*, Ana Fernández-Bravo[2]*, Marta Sanchis[2], Emilio Mayayo[2,3],
María Jose Figueras[2#] and Steve J. Charette[1#]

1. Université Laval, Quebec City, QC, Canada
2. Universitat Rovira i Virgili, IISPV, Reus, Spain.
3. University Hospital Joan XIII, Tarragona, Spain.

* These authors contributed equally to this article.

Corresponding authors:

Steve J. Charette, Institut de Biologie Intégrative et des Systèmes, Charles-Eugène-Marchand,
1030 avenue de la Médecine, Université Laval, Quebec City, QC, Canada, G1V 0A6.
Steve.Charette@bcm.ulaval.ca; Phone: 1-418-656-2131 ext. 6914 ; Fax: 418 948-5487

Maria J. Figueras, Unitat de Microbiología, Departament de Ciències Mèdiques Bàsiques,
Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Spain.
mariajose.figueras@urv.cat

Running Title

Pathogenic *A. salmonicida* from human patients

Abstract

The bacterium *Aeromonas salmonicida* is known since long time as a major fish pathogen unable to grow at 37°C. However, some cases of human infection by putative mesophilic *A. salmonicida* have been reported. The goal of the present study is to examine two clinical cases of human infection by *A. salmonicida* in Spain and to investigate the pathogenicity in mammals of selected mesophilic *A. salmonicida* strains. An evaluation of the pathogenicity in a mouse model of clinical and environmental *A. salmonicida* strains was performed. The genomes of the strains were sequenced and analyzed in order to find the virulence determinants of these strains. The experimental infection in mice showed a gradient in the virulence of these strains and that some of them can cause necrotizing fasciitis and tissue damage in the liver. In addition to demonstrating significant genomic diversity among the strains studied, bioinformatics analyses permitted also to shed light on crucial elements for the virulence of the strains, like the presence of a type III secretion system in the one that caused the highest mortality in the experimental infection. Clinicians and microbiologists should consider these results for the inclusion of *A. salmonicida* in diagnosis tests since it is now clear that some mesophilic strains are also pathogens for humans.

Keywords

Aeromonas salmonicida; Infection; Necrotizing fasciitis; Pathogenicity; Type III Secretion Systems; Whole Genome Sequencing

1. Introduction

The Gram-negative bacterium *Aeromonas salmonicida* has been well known for decades to be a fish pathogen (Austin and Austin, 2016). Officially, *A. salmonicida* has five subspecies (Martin-Carnahan and Joseph, 2005): *salmonicida*, *smithia*, *achromogenes*, *masoucida* and *pectinolytica*. Although the taxonomy of *A. salmonicida* has always been subject to debate (Austin, 2011), it was only in 2000, with the publication of the discovery of the subspecies *pectinolytica* (Pavan et al., 2000) that the diversity of this bacterium was truly revealed. While the other defined *A. salmonicida* subspecies grow only at temperatures below 25°C, *pectinolytica* strains can grow efficiently at 37°C and are thus considered to be mesophilic (Pavan et al., 2000). This dichotomy in the maximum growth temperatures of *A. salmonicida* was reported before the official publication of the subspecies *pectinolytica* (Altwegg et al., 1990; Guérin-Faublée et al., 1997; Janda et al., 1996; Rouf and Rigney, 1971). However, at that time, the intra-species delineation of *A. salmonicida* into subspecies was not systematically used and genome sequences were not available, making conclusions difficult. Moreover, classification of *A. salmonicida* based on biochemical characteristics or 16S rRNA gene sequence has been extremely difficult and many times impossible (Beaz-Hidalgo et al., 2010).

Recently, four mesophilic *A. salmonicida* strains isolated from food in India were sequenced and characterized to shed light on genomic signatures that could explain why some evolutionarily close subspecies have such large differences in their maximum growth temperatures (Vincent et al., 2017, 2016). In accordance with previous experimental evidence based on the *salmonicida* subspecies (Tanaka et al., 2012), investigation of these genomes revealed that insertion sequences could be one of the major genomic determinants between the mesophilic and the psychrophilic strains (Vincent et al., 2017, 2016).

Although our knowledge about *A. salmonicida* has increased significantly during recent years, the infectious potential of mesophilic strains remained unknown. While psychrophilic *A. salmonicida* subspecies are known to infect various fish species (Austin and Austin, 2016), no host is certainly known for mesophilic strains. Early studies found that mesophilic *A. salmonicida* strains (known as hybridization group 3 [HG3]) could be isolated from human and animal hosts (Abbott et al., 1992; Altwegg et al., 1990; Aravena-Román et al., 2011; Janda et al., 1996; Janda and Abbott, 2010). Although rigorous, these studies were made before the democratization of DNA sequencing and the recent advances in the taxonomy of *A. salmonicida* based on core genome sequence analysis. In addition, no clinical background was available for the isolates mentioned above, letting difficult to draw conclusions on the medical importance of *A. salmonicida* for humans.

In 2008, a first case of human infection by *A. salmonicida* with clear clinical background was reported (Yang et al., 2008). More precisely, a 68-year-old diabetic woman on continuous ambulatory peritoneal dialysis was diagnosed as infected by *A. salmonicida* after having been admitted for abdominal pain and cloudy peritoneal fluid. Unfortunately, there is no indication on how the strain was identified as *A. salmonicida*. Recently, in India, *A. salmonicida* was reported to have been recovered from: (i) the blood of a 34-year-old female patient (Tewari et al., 2014), (ii) a skin infection of a 67-year-old immunocompetent male (Kamble, 2015) and (iii) the right eye of 55-year-old female who had recovered from a cataract surgery (Varshney et al., 2017). However, although interesting for clinical backgrounds, the taxonomic identification of these strains is putative given the inherent complexity of *A. salmonicida*.

In 2017, a study reported the isolation of a multidrug-resistant strain, ASG1, from a 15-year-old boy who had recovered from a finger surgery (Ruppé et al., 2017). This time, the strain was clearly identified as belonging to *A. salmonicida* species. Although it demonstrated once for all that mesophilic *A. salmonicida* could infect humans, the pathogenicity of these isolates and specific mechanisms that allow such infections are still unknown.

Here, we investigate two mesophilic *A. salmonicida* strains isolated from human patients in Spain, one that suffered from an acute gastroenteritis and the other that had a cellulitis in a foot after a trauma. These two clinical strains, in addition to four environmental mesophilic *A. salmonicida* strains, were tested for pathogenicity in an immunosuppressed rodent model. The complete genomes of the strains were also investigated to figure out the putative determinants implicated in the virulence of the strains.

2. Materials and Methods

2.1. Isolation of the clinical strains

The strain AJ83 and 947C were isolated at a hospital in Guadalajara (Spain) (Table 1). The strain AJ83 was recovered from a cellulitis in the right foot of a 49-year-old man that also suffered of fasciitis due to trauma. The strain 947C came from the faeces of an 8-year-old girl that had an acute gastroenteritis. Both strains were first identified at the hospital as *Aeromonas hydrophila* using MicroScan W/A identification system (Dade MicroScan, Inc., Sacramento, Calif). Using the same equipment and based on the Clinical and Laboratory Standards Institute guidelines of 2015, the resistance to various antibiotics was assessed for each strain. Both strains were re-identified more thoroughly as *A. salmonicida* by sequencing the *rpoD* gene using primers and condition used in another study (Beaz-Hidalgo et al., 2010).

2.2. *In vivo* experiments

All *A. salmonicida* strains included in this study (Table 1) were grown on tryptic soy agar (TSA) plates and incubated at 30°C for 24 h. The colonies were then scraped off with a sterile loop and were suspended in sterile phosphate-buffered saline (PBS) solution. For each strain, the concentration of bacterial cells was determined by plating 10-fold dilutions onto TSA plates and then by counting the number of CFU after 24 h.

Four-week-old male OF1 mice weighing approximately 30 g each (Charles River, Criffa S.A., Barcelona, Spain) were used to perform the experiments. All animals were maintained under standard conditions. The designed experiments and care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Mice were immunosuppressed 2 days prior to infection by intraperitoneal injection of 200 mg/kg body weight of cyclophosphamide (Genoxal$^{®}$; Laboratories Funk S.A., Barcelona, Spain) and thereafter the same procedure was performed once every 5 days (Sanchis et al., 2016).

Groups of 8 animals were infected intravenously at the tail with 0.2 mL of sterile PBS containing 1×10^7 or 1×10^9 CFU/mouse of the respective *A. salmonicida* strains. Parameters were selected based on previous experiments of mouse infections with *Aeromonas* (Romero et al., 2016). In all experiments, a control group of 8 mice injected with only 0.2 mL of PBS was used. At the end of the experiment, mice were euthanized by anoxia in a CO_2 chamber, followed by cervical dislocation.

The Kaplan-Meier function was used through the R package survival to verify if the survival curves were significantly different from each other. The p-values from the log-rank test were adjusted with the Bonferroni method ($\alpha = 0.05$).

2.3. Bacteria quantification from the different organs and histopathological analysis.

The liver and kidney from the mice infected at both concentrations were directly aseptically collected when the animal died on day 10 post-infection. Each organ was divided in two parts: one part was directly frozen at –80°C and was used for bacterial DNA quantification by real time PCR (qPCR), and the other half was directly fixed in 10% buffered formalin for histopathological studies.

The DNA was extracted using the Easy-DNA^{TM} Kit (Invitrogen, CA), according to the manufacturer's instructions. Real-time PCR was performed on the purified DNA using the kit DNA TargetSpecies dtec-qPCR Test for *Aeromonas* sp. (Genetic PCR solutions, SP) and the StepOnePlus™ Real-Time PCR System (Applied Biosystems) equipment. The number of copies was calculated on the basis of the standard curve and the corresponding amplification cycle threshold (Ct). At the time of collection of the liver and kidney, the organs were examined to detect any macroscopic lesions. After fixation, the tissues were embedded in paraffin and sectioned before staining with hematoxylin, eosin, and Giemsa. The sections were evaluated with microscopy (CX 33, Olympus).

2.4. DNA extraction, sequencing and analysis

The strains AJ83, 947C and A308 were grown on TSA at 30ºC for 24 h and the genomic DNA extracted using Easy-DNA^{TM} Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The DNA of strain A308 (=Popoff C316), was also sequenced since this environmental strain is considered to be a reference of the mesophilic *A. salmonicida* (known as hybridization group 3 [HG3]) by several studies (Abbott et al., 1992; Altwegg et al., 1990; Martínez-Murcia et al., 2005).

The purified DNA was used to prepare sequencing libraries using a KAPA Hyper Prep kit. The resulting libraries were sequenced using Illumina MiSeq technology (IBIS, Université Laval). The final reads were *de novo* assembled using A5-miseq version 20160825 (Coil et al., 2015). The resulting sequences were annotated using the Prokaryotic Genome Annotation Pipeline (PGAP) of the NCBI and were deposited in GenBank (Table 1).

All the genome sequences of mesophilic *A. salmonicida* strains (including AJ83, 947C and A308 that are from the present study), the ones of selected psychrophilic *A. salmonicida* strains and finally the ones of 30 other *Aeromonas* were annotated using Prokka version 1.12 (Seemann, 2014) (see Supplementary Table S1). Homologous links between the translated coding sequences were defined using GET_HOMOLOGUES version 20180103 (Contreras-Moreira and Vinuesa, 2013) with two algorithms: COG and OMCL (see Supplementary Fig. S1). The 2026 gene sequences (excluding paralogs) corresponding to the softcore, defined as the sequences present in more than 95% of the genomes, were recovered and aligned by codons using TranslatorX version 1.1 (Abascal et al., 2010). The resulting alignments were filtered using BMGE version 1.12 (Criscuolo and Gribaldo, 2010) and concatenated in a partitioned supermatrix using AMAS (Borowiec, 2016). The best-fit model of each partition was determined using ModelFinder (Kalyaanamoorthy et al., 2017) through IQ-TREE version 1.6.1 (Nguyen et al., 2015). The maximum-likelihood phylogeny was itself done using IQ-TREE by performing 10,000 ultrafast bootstraps (Hoang et al., 2017). The Average Nucleotide Identity (ANI) values were computed for genome sequences of *A. salmonicida* using pyani (https://github.com/widdowquinn/pyani).

The antibiotic resistance genes were predicted using ABRicate version 0.8.7 (https://github.com/tseemann/abricate) and the CARD database (Jia et al., 2016). A gene sequence had to have a minimum of 80% identity on at least 70% of the length in order to annotate it as an antibiotic resistance gene. Annotation of the genes was then manually curated.

3. Results

3.1. Clinical pictures

In 2007, a 49-year-old man was hospitalized at the Guadalajara University Hospital for cellulitis and fasciitis in the right foot after trauma. The clinical background of the patient includes diabetes mellitus and Reiter syndrome, being treated with prednisone. The patient was treated by piperacillin/tazobactam and surgical debridement. The patient healed without complications. A microbial investigation at the wound exudate revealed a polymicrobial infection of *Aeromonas hydrophila*, *Staphylococcus aureus and Klebsiella oxytoca*, after MicroScan identification. No stool or blood culture was performed, since the patient did not have a fever. The *rpoD* sequence of the *Aeromonas* strain, named AJ83, revealed that this strain does not belong to the *hydrophila* species, but surprisingly to the *salmonicida* species (data not shown). This strain is resistant to three antibiotics: cefazolin, ampicillin and ticarcillin, while sensitive to piperacillin/tazobactam (see Supplementary Table S2).

One year later, at the same hospital, an 8-year-old girl without a clinical background was hospitalized for an acute gastroenteritis. The patient had bloody stools with mucus. The stool culture revealed the presence of *Campylobacter jejuni* in addition to *Aeromonas hydrophila*. A blood culture was not performed since the young girl had no fever. She had a treatment with hydration and no antibiotic was administered. Like strain AJ83, the *rpoD* sequence of the *Aeromonas* strain (named here 947C) revealed that it belongs to the species *salmonicida*. Strain 947C was shown to be resistant to cefazolin, ampicillin and cotrimoxazole (see Supplementary Table S2).

3.2. Taxonomic validation of the strains

The genome of the clinical strains AJ83 and 947C was sequenced, *de novo* assembled and used to perform a robust molecular phylogeny based on 2026 gene sequences (Fig. 1). Without any doubt, the clinical strains AJ83 and 947C are belonging to the *salmonicida* species as they clustered with the type strain of the subspecies *pectinolytica* (34mel^T) and with the other already known *A. salmonicida* mesophilic strains. Moreover, they cluster along other mesophilic strains, as strain A308. Interestingly, strain 947C cluster with strain A308, which is environmental. On its side, strain AJ83 form a group with Y567 and Y47, two strains isolated from food in Mumbai (India) and for which no host is known (Fig. 1). The ANI values revealed that available genomes of mesophilic strains are distant in terms of nucleotide sequences, although they come from strains of the same species (ANI ≥ 0.96) (Fig. 1). Only strains AJ83 and Y567 were more closely related comparatively to other strains (ANI value of 99%).

3.3. Pathogenicity of strains

The pathogenicity of six mesophilic *A. salmonicida* strains was evaluated by infecting mice (Fig. 2). Two doses were tested, 1×10^7 and 1×10^9 CFU/mouse. At 1×10^7, a clear dichotomy in the survival rate of mice can be observed between strains (Fig. 2-A). The most virulent strain is the clinical one 947C followed by strain A308, which has been isolated from fresh water. There is no statistical significant difference in the mortality caused by both strains (see Supplementary Table S3), which are in the same phylogenetic cluster (Fig. 1). The less virulent strains include A527, Y47, AJ83 and 34mel^T (subspecies *pectinolytica*). Here again, there is no significant statistical difference in the mortality caused by those strains.

Three groups of strains based on virulence can be observed at the dose of 1×10^9 CFU/mouse (Fig. 2-B). The pathogenicity of 947C is striking, with all mice being death after only three days post-infection. As seen in the test at the dose of 1 × 10^7 CFU/mouse, strains A527, Y47 and 34mel^T are the less virulent and without significant difference in the mortality caused by them (see Supplementary Table S4). At this dose, the environmental strain A308 showed to have an intermediate virulence, along with strain AJ83. Although they are less virulent than strain 947C, both strains A308 and AJ83 killed all mice before the end of the experiment.

Interestingly, both clinical strains 947C and AJ83 and the Indian strain Y47 produced lesions on the mouse tails, at the injection site (Fig. 2-C). This cutaneous infection that is typical of necrotizing fasciitis was only seen at the lowest dose (1 × 10^7 CFU/mouse) for 947C, likely because mice died too quickly at the dose of 1 × 10^9 CFU/mouse. Strains A308, A527 and 34mel^T did not produce visible cutaneous infections.

3.4. Bacteria quantification from the different organs and histopathological studies

The presence of bacterial DNA in the liver and kidney of mice was quantified by qPCR (Fig 3). Significantly higher amounts of DNA (p<0.05) were found in both organs for the clinical strains (947C and AJ83), than for the environmental strains. A higher amount of *Aeromonas* DNA was detected in liver than in kidney. It is interesting to note that the DNA of the environmental strain A308 was present in larger quantities than other environmental strains, in both organs. In liver and at a higher dose, more DNA of strain A308 was detected than clinical strain AJ83 (Fig. 3A). The results obtained with the clinical strains showed a significantly greater amount of DNA of strain 947C, the most pathogenic one, at a lower dose (1 × 10^7) than for strain AJ83 at both doses.

Histopathological examination with hematoxylin and eosin or Giemsa staining showed no damage in the kidney (see Supplementary Fig. S2). However, the liver revealed various levels of multifocal and diffuse necrotic changes and infiltration of polymorphonuclear cells (PMNs), with inflammatory response as shown in Fig. 4. Specifically, tissues collected from animals infected with strain 947C at dose 1 x 10^7 showed more PMN infiltration and necrotic cells (Fig. 4A), than for strain AJ83 at dose 1 x 10^9 (Fig 4B). In addition, the Giemsa staining confrimed the observation of PMN cells and the inflammatory response (Fig 4C).

3.5. Genomic investigation

When checking the genome of strain 947C, the most virulent one, several genes involved in a type III secretion system (T3SS) were found (see Supplementary Table S5). However, it is unclear what make strains AJ83 and A308 virulent. A high number of genes that encode for hypothetical proteins were predicted to be encoded in their genomes and we cannot rule out that some of them are implied in virulence.

When looking for the presence of CDSs that encode known virulence factors (Rasmussen-Ivey et al., 2016), the gene *ast* (cytotonic enterotoxin) was found exclusively in the genomes of clinical strains 947C and AJ83 (see Supplementary Table S6). Finally, the mouse infections clearly demonstrated that strains 947C, AJ83 and Y47 can cause necrotizing fasciitis (Fig. 2). Only five orthologous genes, not yet associated with virulence in *Aeromonas salmonicida*, were found to be present in the genomes of these three strains and absent from those of strains 34mel^T, A308 and A527 (Table 2). Interestingly, four of these five genes were already listed in the literature as virulence factors in human pathogens such as *A. hydrophila*, *Helicobacter pylori*, *Leptospira* sp. and *Salmonella enterica* (Table 2).

It was also interesting to investigate the genes that could be involved in antibiotic resistance for the mesophilic strains of *A. salmonicida* (see Supplementary Table S7). All strains have genes predicted to be involved in antibiotic resistance (from 2 to 12 genes). Two genes were predicted to be encoded in the genome of all strains: *OXA-12* (resistance to cephalosporin and penam) and *cphA5* (resistance to carbapenem) genes. The two strains with the most antibiotic resistance genes are ASG1 (12 genes), isolated from a human patient, and ECFood+05 (10 genes) for which little information is available. The most virulent strain in the mouse model (Fig. 2), 947C, is predicted to have genes involved in resistance to several compounds: aminoglycoside, cephalosporin, penam and carbapenem. The second strain isolated from a human patient for the present study, AJ83, presents almost the same antibiotic resistance pattern as 947C, only differing by the absence of the gene involved in resistance to aminoglycoside compounds.

4. Discussion

Earlier studies on human cases of *A. salmonicida* infections lack clinical metadata or are taxonomically uncertain (Abbott et al., 1992; Altwegg et al., 1990; Aravena-Román et al., 2011; Janda et al., 1996; Kamble, 2015; Ruppé et al., 2017; Tewari et al., 2014; Varshney et al., 2017) compared to what can be done now with core genome phylogeny (Vincent et al., 2016). Recently, the strain ASG1, clearly identified as *A. salmonicida*, was isolated from a 15-year-old boy that recovered from a finger surgery (Ruppé et al., 2017). Unfortunately, another pathogen, *Stenotrophomonas maltophilia*, was co-isolated with strain ASG1, making it impossible to draw firm conclusions on clinical aspects of the ASG1 strain. The present study clearly demonstrated for the first time by combining experimental infection essays and whole genome analyses that some mesophilic *A. salmonicida* strains are able to infect mammals.

It is not surprising that T3SS seems to be a major virulence factor, as shown by the striking mortality caused by strain 947C. T3SS is known to be an important virulence factor in several Gram-negative bacteria, including the human pathogens *A. hydrophila* and *Aeromonas veronii* (Chacón et al., 2004; Vilches et al., 2004) and the fish pathogen *A. salmonicida* subsp. *salmonicida* (Frey and Origgi, 2016).

Interestingly, some *A. salmonicida* strains have the ability to cause cutaneous infections that look like necrotizing fasciitis. In addition to the pathogenicity tests done in the present study, ASG1 was isolated from a finger that recovered from surgery (Ruppé et al., 2017) and AJ83 isolated from the right foot of 49-year-old man that suffered of fasciitis due to trauma. Investigation of the genomes revealed five genes that are candidates to explain why only three strains cause necrotizing fasciitis (Table 2). In addition to these genes, which may help explain the ability of some strains to cause necrotizing fasciitis, it was observed that even a low level of virulence can cause this type of infection. Strain 947C, which is the most virulent, causes a necrotizing fasciitis only at the lowest dose (Fig. 2). The other two strains that can cause this skin infection, AJ83 and Y47, cause a low or intermediate mortality level. It is possible to postulate some similarity with the subspecies *salmonicida*, which causes two forms of furunculosis in salmonids (Austin and Austin, 2016). The chronic form of the disease causes a low mortality rate and is often characterized by a cutaneous appearance known as furuncles, hence the name of the disease. The acute form of the disease causes a high mortality rate (2 to 3 days) due to septicaemia and does not manifest cutaneously.

A significant amount of *Aeromonas* DNA was found in the livers of fish (more than in their kidneys) by qPCR (Fig. 3). Similar results were described with *A. hydrophila* in channel catfish, where the bacterium was detected only in the liver more than 48 hours post-infection and was eliminated from the other organs, including the kidney, of the fish (Zhang et al., 2016). The quantification obtained from the clinical strains correlated with the results of the histopathological examination, which showed important pathological changes in the liver while no damage was observed in the kidney (Fig .4 and Supplementary Fig. S2). The fact that bacterial

DNA was detected in the kidney at relatively low levels could be related to the process of their elimination with the urine.

Although preliminary, the degree of pathogenicity does not seem to be associated with strains of a specific phylogenetic group. However, the study of the pathogenicity of mesophilic *A. salmonicida* is still in its infancy and strains from various hosts will be needed to clarify the evolutionary links between these strains. The fact that the genomes of only two out of ten mesophilic *A. salmonicida* strains are similar at the nucleotide level demonstrates a great diversity in the mesophilic strains of this bacterium (Fig. 1). One of these two strains, AJ83, has a clinical origin while the second, Y567, was isolated from food.

The psychrophilic strains of *A. salmonicida* are officially divided into different subspecies: *salmonicida*, *smithia*, *achromogenes* and *masoucida*, whereas there is only one official mesophilic subspecies, *pectinolytica*. However, according to the molecular phylogeny and ANI values, the mesophilic strains of *A. salmonicida characterized* so far have greater genetic diversity than the psychrophilic strains of the same species. This fact rises, as mentioned before (Vincent et al., 2017), a certain taxonomic dilemma. It is obvious that it will be necessary to review the taxonomy of *A. salmonicida* in order to unify in a cohesive manner the mesophilic and psychrophilic strains of this species. A first scenario could be to classify mesophilic strains into different subspecies. A second scenario would be to make two subspecies, one comprising all the mesophilic strains and the other all the psychrophilic strains. In any case, before considering one of these scenarios, it will be necessary to continue to isolate new mesophilic and psychrophilic strains of *A. salmonicida* in order to obtain a broader view of the different genetic and phenotypic characteristics, thus making it possible to establish a robust and representative taxonomy of this bacterium. Also, it is crucial to take into account that the mesophilic

A. salmonicida strains can be easily misidentified as *A. hydrophila* and that the use of molecular methods such as the sequence of the *rpoD* gene are required to correctly assign the taxonomy of these strains (Beaz-Hidalgo et al., 2010).

The two clinical strains investigated in the present study were shown to be resistant to some antibiotics (see Supplementary Table S2) and also to harbor genes known to be involved in resistance (see Supplementary Table S7). In fact, the resistance gene repertoires of strains 947C and AJ83 differ only in that 947C has a gene that causes resistance to aminoglycoside antibiotics. It is surprising that strain AJ83 is resistant to ticarcillin, belonging to the penem drug class, while strain 947C is sensitive to this antibiotic (see Supplementary Table S2). Similarly, strain 947C is resistant to cotrimoxazole, belonging to sulphonamide/diaminopyrimidinedrug class, while strain AJ83 is sensitive. It is still unclear why strains 947C and AJ83 differ in their resistance to these antibiotics. Other species of the genus *Aeromonas* are known to harbor genes involved in antibiotic resistance (Piotrowska and Popowska, 2015). This is the case, for example, of the fish pathogen *A. salmonicida* subsp. *salmonicida*, for which several strains are multi-resistant to all antibiotic approved in aquaculture in Canada (Trudel et al., 2016; Vincent et al., 2014). A similar pattern of multiple resistance seems to be apparent in mesophilic strains of the *salmonicida* species where some strains, such as ASG1 and ECFood+05, were predicted to harbor more than 10 genes involved in resistance to antibiotic compounds. This is even more interesting given the context that these two strains cluster together in the phylogenetic tree (Fig. 1), suggesting that some mesophilic *A. salmonicida* strains that arise from a particular common ancestor could be more prone to having antibiotic resistance genes. Given that both ASG1 and ECFood+05 only share four genes (*OXA-12*, *cphA5*, *aadA* and *tet*(E)), it is reasonable to believe that the other genes could have been acquired by horizontal gene transfers. Moreover, the multiple resistance to antibiotics of ASG1 strain was confirmed experimentally (Ruppé et al., 2017). Closely monitoring mesophilic *A. salmonicida* will be essential to effectively treat cases of infection by strains of this bacterium.

4.1. Concluding remarks

In this study, it was possible to demonstrate robustly that the mesophilic strains of *A. salmonicida* can infect mammals, with varying levels of pathogenicity between strains. It will be essential in the future to isolate new mesophilic *A. salmonicida* strains and to verify their geographical distribution. The clinical strains AJ83 and 947C investigated in the present study come from Spain. However, several clinical studies have documented cases in India of infections in humans from putatively mesophilic *A. salmonicida* strains (Kamble, 2015; Tewari et al., 2014; Varshney et al., 2017). Moreover, environmental strains from India have clearly been identified as mesophilic *A. salmonicida* (Vincent et al., 2017, 2016).

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Conflicts of interest

The authors have no conflicts of interest to declare.

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References

Abascal, F., Zardoya, R., Telford, M.J., 2010. TranslatorX: Multiple alignment of nucleotide sequences guided by amino acid translations. Nucleic Acids Res. 38, W7-13. doi:10.1093/nar/gkq291

Abbott, S.L., Cheung, W.K.W., Kroske-Bystrom, S., Malekzadeh, T., Janda, J.M., 1992. Identification of *Aeromonas* strains to the genospecies level in the clinical laboratory. J. Clin. Microbiol. 30, 1262–1266.

Altwegg, M., Steigerwalt, A.G., Altwegg-Bissig, R., Luthy-Hottenstein, J., Brenner, D.J., 1990. Biochemical identification of *Aeromonas* genospecies isolated from humans. J. Clin. Microbiol. 28, 258–264.

Aravena-Román, M., Harnett, G.B., Riley, T. V., Inglis, T.J.J., Chang, B.J., 2011. *Aeromonas aquariorum* is widely distributed in clinical and environmental specimens and can be misidentified as *Aeromonas hydrophila*. J. Clin. Microbiol. 49, 3006–3008. doi:10.1128/JCM.00472-11

Austin, B., 2011. Taxonomy of bacterial fish pathogens. Vet. Res. 42, 20. doi:10.1186/1297-9716-42-20

Austin, B., Austin, D.A., 2016. Aeromonadaceae Representative (*Aeromonas salmonicida*), in: Austin, B., Austin, D.A. (Eds.), Bacterial Fish Pathogens: Disease of Farmed and Wild Fish. Springer International Publishing, Cham, pp. 215–321. doi:10.1007/978-3-319-32674-0_5

Beaz-Hidalgo, R., Alperi, A., Buján, N., Romalde, J.L., Figueras, M.J., 2010. Comparison of phenotypical and genetic identification of *Aeromonas* strains isolated from diseased fish. Syst. Appl. Microbiol. 33, 149–153. doi:10.1016/j.syapm.2010.02.002

Borowiec, M.L., 2016. AMAS: a fast tool for alignment manipulation and computing of summary statistics. PeerJ 4, e1660. doi:10.7717/peerj.1660

Chacón, M.R., Soler, L., Groisman, E.A., Guarro, J., Figueras, M.J., 2004. Type III secretion system genes in clinical *Aeromonas* isolates. J. Clin. Microbiol. 42, 1285–1287. doi:10.1128/JCM.42.3.1285-1287.2004

Coil, D., Jospin, G., Darling, A.E., 2015. A5-miseq : an updated pipeline to assemble microbial genomes from Illumina MiSeq data. Bioinformatics 31, 587–589. doi:10.1093/bioinformatics/btu661

Contreras-Moreira, B., Vinuesa, P., 2013. GET_HOMOLOGUES, a versatile software package for scalable and robust microbial pangenome analysis. Appl. Environ. Microbiol. 79, 7696–7701. doi:10.1128/AEM.02411-13

Criscuolo, A., Gribaldo, S., 2010. BMGE (Block Mapping and Gathering with Entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments. BMC Evol. Biol. 10, 210. doi:10.1186/1471-2148-10-210

Eshghi, A., Lourdault, K., Murray, G.L., Bartpho, T., Sermswan, R.W., Picardeau, M., Adler, B., Snarr, B., Zuerner, R.L., Cameron, C.E., 2012. *Leptospira interrogans* catalase is required for resistance to H2O2 and for virulence. Infect. Immun. 80, 3892–3899. doi:10.1128/IAI.00466-12

Frey, J., Origgi, F.C., 2016. Type III secretion system of *Aeromonas salmonicida* undermining the host's immune response. Front. Mar. Sci. 3, 130. doi:10.3389/FMARS.2016.00130

Gilbreath, J.J., Dodds, J.C., Rick, P.D., Soloski, M.J., Merrell, D.S., Metcalf, E.S., 2012. Enterobacterial common antigen mutants of *Salmonella enterica* serovar *typhimurium* establish a persistent infection and provide protection against subsequent lethal challenge. Infect. Immun. 80, 441–450. doi:10.1128/IAI.05559-11

Guérin-Faublée, V., Charles, S., Chomarat, M., Flandrois, J.P., 1997. Reappraisal of the effect of temperature on the growth kinetics of *Aeromonas salmonicida*. Lett. Appl. Microbiol. 25, 363–366.

Hoang, D.T., Chernomor, O., von Haeseler, A., Minh, B.Q., Le, S.V., 2017. UFBoot2: Improving the ultrafast bootstrap approximation. Mol. Biol. Evol. 35, 518–522. doi:10.1093/molbev/msx281

Janda, J.M., Abbott, S.L., 2010. The Genus *Aeromonas*: Taxonomy, Pathogenicity, and Infection. Clin. Microbiol. Rev. 23, 35–73. doi:10.1128/CMR.00039-09

Janda, J.M., Abbott, S.L., Khashe, S., Kellogg, G.H., Shimada, T., 1996. Further studies on biochemical characteristics and serologic properties of the genus *Aeromonas*. J. Clin. Microbiol. 34, 1930–1933.

Jia, B., Raphenya, A.R., Alcock, B., Waglechner, N., Guo, P., Tsang, K.K., Lago, B.A., Dave, B.M., Pereira, S., Sharma, A.N., Doshi, S., Courtot, M., Lo, R., Williams, L.E., Frye, J.G., Elsayegh, T., Sardar, D., Westman, E.L., Pawlowski, A.C., Johnson, T.A., Brinkman, F.S.L., Wright, G.D., McArthur, A.G., 2017. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res. 45, D566-D573. doi:10.1093/nar/gkw1004

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., Von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: Fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589. doi:10.1038/nmeth.4285

Kamble, R., 2015. *Aeromonas salmonicida* furunculosis in an adult male. Int.J.Curr.Microbiol.App.Sci. 4, 59–63.

Martin-Carnahan, A., Joseph, S., 2005. *Aeromonadales* ord. nov., in: Brenner, D., Krieg, N., Staley, J., Garrity, G., Boone, D., De Vos, P., Goodfellow, M., Rainey, F., Schleifer, K.-H. (Eds.), Bergey's Manual® of Systematic Bacteriology SE - 12. Springer US, pp. 556–587. doi:10.1007/0-387-28022-7_12

Martínez-Murcia, A.J., Soler, L., Saavedra, M.J., Chacón, M.R., Guarro, J., Stackebrandt, E., Figueras, M.J., 2005. Phenotypic, genotypic, and phylogenetic discrepancies to differentiate *Aeromonas salmonicida* from *Aeromonas bestiarum*. Int. Microbiol. 8, 259–269. doi:10.2436/im.v8i4.9534

Nagar, V., Shashidhar, R., Bandekar, J.R., 2011. Prevalence, characterization, and antimicrobial resistance of *Aeromonas* strains from various retail food products in Mumbai, India. J. Food Sci. 76, M486-92. doi:10.1111/j.1750-3841.2011.02303.x

Nguyen, L.T., Schmidt, H.A., Von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32, 268–274. doi:10.1093/molbev/msu300

Pavan, M.E., Abbott, S.L., Zorzópulos, J., Janda, J.M., 2000. *Aeromonas salmonicida* subsp. *pectinolytica* subsp. nov., a new pectinase- positive subspecies isolated from a heavily polluted river. Int. J. Syst. Evol. Microbiol. 50, 1119–1124. doi:10.1099/00207713-50-3-1119

Piotrowska, M., Popowska, M., 2015. Insight into the mobilome of *Aeromonas* strains. Front. Microbiol. 6, 494. doi:10.3389/fmicb.2015.00494

Rasmussen-Ivey, C.R., Figueras, M.J., McGarey, D., Liles, M.R., 2016. Virulence factors of *Aeromonas hydrophila*: In the wake of reclassification. Front. Microbiol. 7, 1337. doi:10.3389/fmicb.2016.01337

Romero, A., Saraceni, P.R., Merino, S., Figueras, A., Tomás, J.M., Novoa, B., 2016. The animal model determines the results of *Aeromonas* virulence factors. Front. Microbiol. 7, 1574. doi:10.3389/fmicb.2016.01574

Rouf, M., Rigney, M., 1971. Growth temperatures and temperature characteristics of *Aeromonas*. Appl. Microbiol. 22, 503–506.

Ruppé, E., Cherkaoui, A., Wagner, N., La Scala, G.C., Beaulieu, J.-Y., Girard, M., Frey, J., Lazarevic, V., Schrenzel, J., 2017. *In vivo* selection of a multidrug-resistant *Aeromonas salmonicida* during medicinal leech therapy. New Microbes New Infect. 21, 23–27. doi:10.1016/j.nmni.2017.10.005

Sanchis, M., Guarro, J., Sutton, D.A., Fothergill, A.W., Wiederhold, N., Capilla, J., 2016. Voriconazole and posaconazole therapy for experimental *Candida lusitaniae* infection. Diagn. Microbiol. Infect. Dis. 84, 48–51. doi:10.1016/j.diagmicrobio.2015.09.010

Seemann, T., 2014. Prokka: Rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069. doi:10.1093/bioinformatics/btu153

Tanaka, K.H., Dallaire-Dufresne, S., Daher, R.K., Frenette, M., Charette, S.J., 2012. An insertion sequence-dependent plasmid rearrangement in *Aeromonas salmonicida* causes the loss of the type three secretion system. PLoS One 7, e33725. doi:10.1371/journal.pone.0033725

Tewari, R., Dudeja, M., Nandy, S., Kumar Das, A., 2014. Isolation of *Aeromonas salmonicida* from human blood sample: A case report. J. Clin. Diagnostic Res. 8, 139–140. doi:10.7860/JCDR/2014/6883.4032

Trudel, M. V., Vincent, A.T., Attéré, S.A., Labbé, M., Derome, N., Culley, A.I., Charette, S.J., 2016. Diversity of antibiotic-resistance genes in Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida*: dominance of pSN254b and discovery of pAsa8. Sci. Rep. 6, 35617. doi:10.1038/srep35617

Varshney, A., Das, M., Chaudhary, P., Kumari, R., Yadav, K., 2017. *Aeromonas salmonicida* as a causative agent for postoperative endophthalmitis. Middle East Afr. J. Ophthalmol. 24, 213–215. doi:10.4103/meajo.MEAJO_238_17

Vilches, S., Urgell, C., Merino, S., Chacón, M.R., Soler, L., Castro-Escarpulli, G., Figueras, M.J., Tomás, J.M., 2004. Complete type III secretion system of a mesophilic *Aeromonas hydrophila* strain. Appl. Environ. Microbiol. 70, 6914–6919. doi:10.1128/AEM.70.11.6914-6919.2004

Vincent, A.T., Rouleau, F.D., Moineau, S., Charette, S.J., 2017. Study of mesophilic *Aeromonas salmonicida* A527 strain sheds light on the species' lifestyles and taxonomic dilemma. FEMS Microbiol. Lett. 364, fnx239. doi:10.1093/femsle/fnx239

Vincent, A.T., Trudel, M. V, Freschi, L., Nagar, V., Gagné-Thivierge, C., Levesque, R.C., Charette, S.J., 2016. Increasing genomic diversity and evidence of constrained lifestyle evolution due to insertion sequences in *Aeromonas salmonicida*. BMC Genomics 17, 44. doi:10.1186/s12864-016-2381-3

Vincent, A.T., Trudel, M. V, Paquet, V.E., Boyle, B., Tanaka, K.H., Dallaire-Dufresne, S., Daher, R.K., Frenette, M., Derome, N., Charette, S.J., 2014. Detection of variants of the pRAS3, pAB5S9, and pSN254 plasmids in *Aeromonas salmonicida* subsp. *salmonicida*: multidrug-resistance, interspecies exchanges, and plasmid reshaping. Antimicrob. Agents Chemother. 58, 7367–7374. doi:10.1128/AAC.03730-14

Wahid, S.U.H., 2017. Structural and functional characterization of the *Helicobacter pylori* cytidine 5'-monophosphate-pseudaminic acid synthase PseF: molecular insight into substrate recognition and catalysis mechanism. Adv. Appl. Bioinform. Chem. 10, 79–88. doi:10.2147/AABC.S139773

Wattam, A.R., Davis, J.J., Assaf, R., Boisvert, S., Brettin, T., Bun, C., Conrad, N., Dietrich, E.M., Disz, T., Gabbard, J.L., Gerdes, S., Henry, C.S., Kenyon, R.W., Machi, D., Mao, C., Nordberg, E.K., Olsen, G.J., Murphy-Olson, D.E., Olson, R., Overbeek, R., Parrello, B., Pusch, G.D., Shukla, M., Vonstein, V., Warren, A., Xia, F., Yoo, H., Stevens, R.L., 2017. Improvements to PATRIC, the all-bacterial bioinformatics database and analysis resource center. Nucleic Acids Res. 45, D535–D542.

Yang, X., Yang, Q.Q., Guo, Q.Y., Yi, C.Y., Mao, H.P., Lin, J.X., Jiang, Z.P., Yu, X.Q., 2008. Aeromonas salmonicida peritonitis after eating fish in a patient undergoing CAPD. Perit. Dial. Int. 28, 316–317.

Zeng, W.B., Chen, W.B., Yan, Q.P., Lin, G.F., Qin, Y.X., 2016. Hemerythrin is required for *Aeromonas hydraphlia* to survive in the macrophages of *Anguilla japonica*. Genet. Mol. Res. 15, gmr8074. doi:10.4238/gmr.15028074

Zhang, D., Moreira, G.S.A., Shoemaker, C., Newton, J.C., Xu, D.H., 2016. Detection and quantification of virulent *Aeromonas hydrophila* in channel catfish tissues following waterborne challenge. FEMS Microbiol. Lett. 363, fnw080. doi:10.1093/femsle/fnw080

Table 1. Mesophilic strains of *A. salmonicida* used in the present study.

Strain	Source	Country	Year	Accession number	Reference
34mel[T]	River	Argentina	1988	NZ_CP022426.1	(Pavan et al., 2000)
Y47	Chicken[a]	India	2006	JZTF00000000	(Nagar et al., 2011)
A527	Giant river prawn[a]	India	2007	CP022550	(Nagar et al., 2011; Vincent et al., 2017)
A308[b]	Fresh water	France	1962	PSZJ00000000	Present study
AJ83	Human	Spain	2007	PSZI00000000	Present study
947C	Human	Spain	2008	PSZK00000000	Present study

a: Isolated in food markets in India (Nagar et al., 2011). The real hosts are considered unknown.

b: Strain A308 = Popoff C316 = CDC 0434-84 = CECT 5171 = LMG 13451. This strain is considered as a reference for mesophilic *A. salmonicida* (Abbott et al., 1992; Altwegg et al., 1990; Martínez-Murcia et al., 2005).

Table 2. CDSs present only in strains 947C, AJ83 and Y47

Protein	Virulence trait	Ref
Two pore domain potassium channel family protein	N/A[a]	N/A
Hemerythrin	*A. hydrophila* survival in host macrophages	(Zeng et al., 2016)
Pseudaminic acid cytidylyltransferase	Colonisation of *H. pylori*	(Wahid, 2017)
Catalase KatE[b]	Virulence of *Leptospira* spp. in animal models	(Eshghi et al., 2012)
UDP-N-acetylglucosamine-1-phosphate transferase[c]	Production of enterobacterial antigen in *S. enterica*	(Gilbreath et al., 2012)

a: N/A, none-applicable

b: The catalase was annotated as KatE by PATRIC (Wattam et al., 2017)

c: The CDS in strain Y47 appears to be divergent compared to those of strains 947C and AJ83

FIGURES LEGENDS

Figure 1. Phylogenetic tree of 51 strains of *Aeromonas*. The tree is based on 2026 gene sequences using the methodology described in the Materials and Methods section. For the sake of clarity, the focus is on mesophilic (red) and psychrophilic (blue) strains of the species *salmonicida*. Bootstrap values are only shown if they are less than 100. The heatmap represents the ANI values.

Figure 2. Virulence tests in a mouse model. Survival rate of mice at doses of (A) 1×10^7 and (B) 1×10^9 CFU/mouse of six mesophilic *A. salmonicida* strains. (C) Pictures showing the lesions at the infection site caused by strains 947C, AJ83 and Y47. The pictures of the mouse tail infected with A308 provides a negative control for the lesions observed with the other strains.

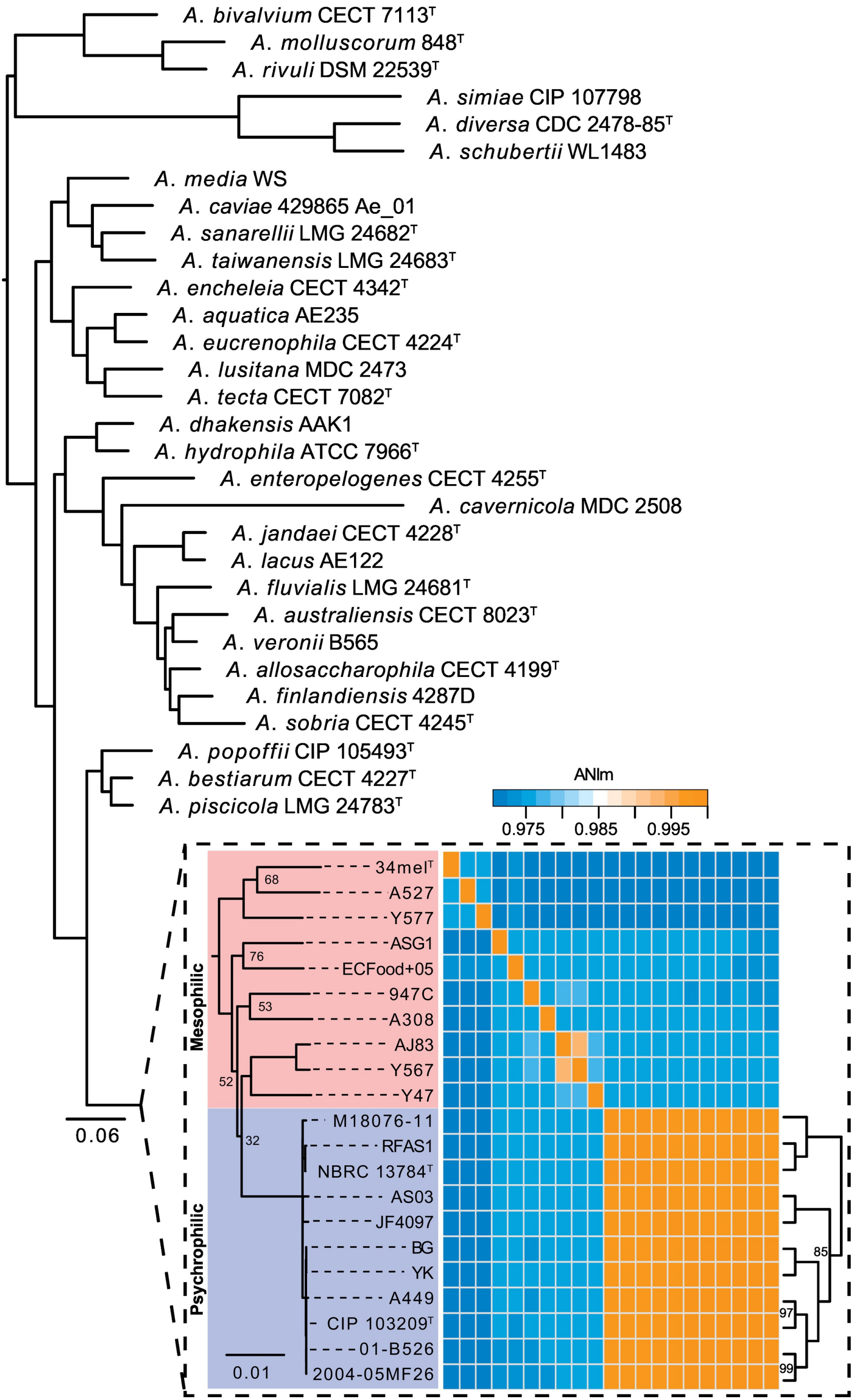
Figure 3. Concentration of *Aeromonas* DNA determined by qPCR in mice liver (A) and kidney (B) tissues 10 days after intravenous infection at doses 1×10^7 and 1×10^9. *Statistical significance ($p < 0.05$). Tissue = non-infected tissue, Water = only water without tissue; both used as negative controls.

Figure 4. Histopathological examination of mouse liver tissue 10 days after intravenous infection with two *Aeromonas* strains (AJ83 and 947C) of clinical origin. (A) Strain AJ83 at dose 1×10^9 CFU with hematoxylin/eosin staining. (B) Strain 947C at dose 1×10^7 CFU with

hematoxylin/eosin staining (C) Strain 947C at dose 1 × 10^7 CFU with Giemsa staining. Bars represent 100 µm.

Highlights

- Significant evolutionary diversity within the mesophilic *A. salmonicida* strains;
- Some *A. salmonicida* mesophilic strains can infect mammals, including humans;
- There is variability in the pathogenicity of mesophilic *A salmonicida* strains;
- Necrotizing fasciitis can be caused by mesophilic *A. salmonicida* strains;
- Some genetic determinants can explain the strains' infectivity capacity.


A. bivalvium CECT 7113T
A. molluscorum 848T
A. rivuli DSM 22539T
A. simiae CIP 107798
A. diversa CDC 2478-85T
A. schubertii WL1483
A. media WS
A. caviae 429865 Ae_01
A. sanarellii LMG 24682T
A. taiwanensis LMG 24683T
A. encheleia CECT 4342T
A. aquatica AE235
A. eucrenophila CECT 4224T
A. lusitana MDC 2473
A. tecta CECT 7082T
A. dhakensis AAK1
A. hydrophila ATCC 7966T
A. enteropelogenes CECT 4255T
A. cavernicola MDC 2508
A. jandaei CECT 4228T
A. lacus AE122
A. fluvialis LMG 24681T
A. australiensis CECT 8023T
A. veronii B565
A. allosaccharophila CECT 4199T
A. finlandiensis 4287D
A. sobria CECT 4245T
A. popoffii CIP 105493T
A. bestiarum CECT 4227T
A. piscicola LMG 24783T
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ANIm
0.975
0.985
0.995
Mesophilic
Psychrophilic
68
76
53
52
32
34melT
A527
Y577
ASG1
ECFood+05
947C
A308
AJ83
Y567
Y47
M18076-11
RFAS1
NBRC 13784T
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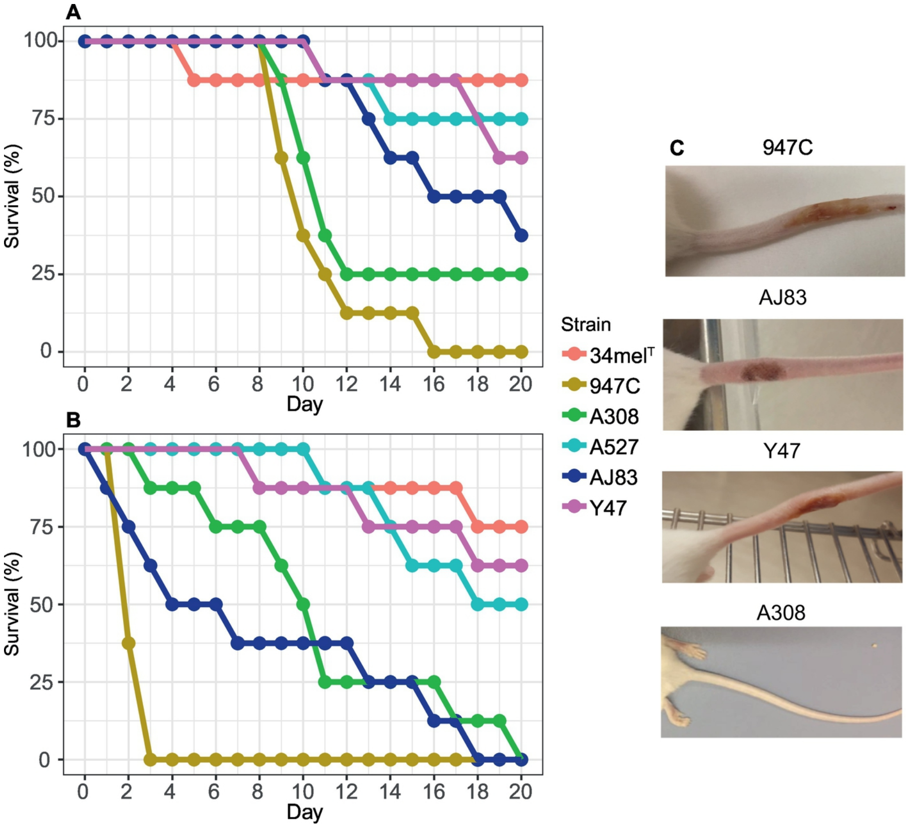
Figure 1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Survival (%)
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Figure 2

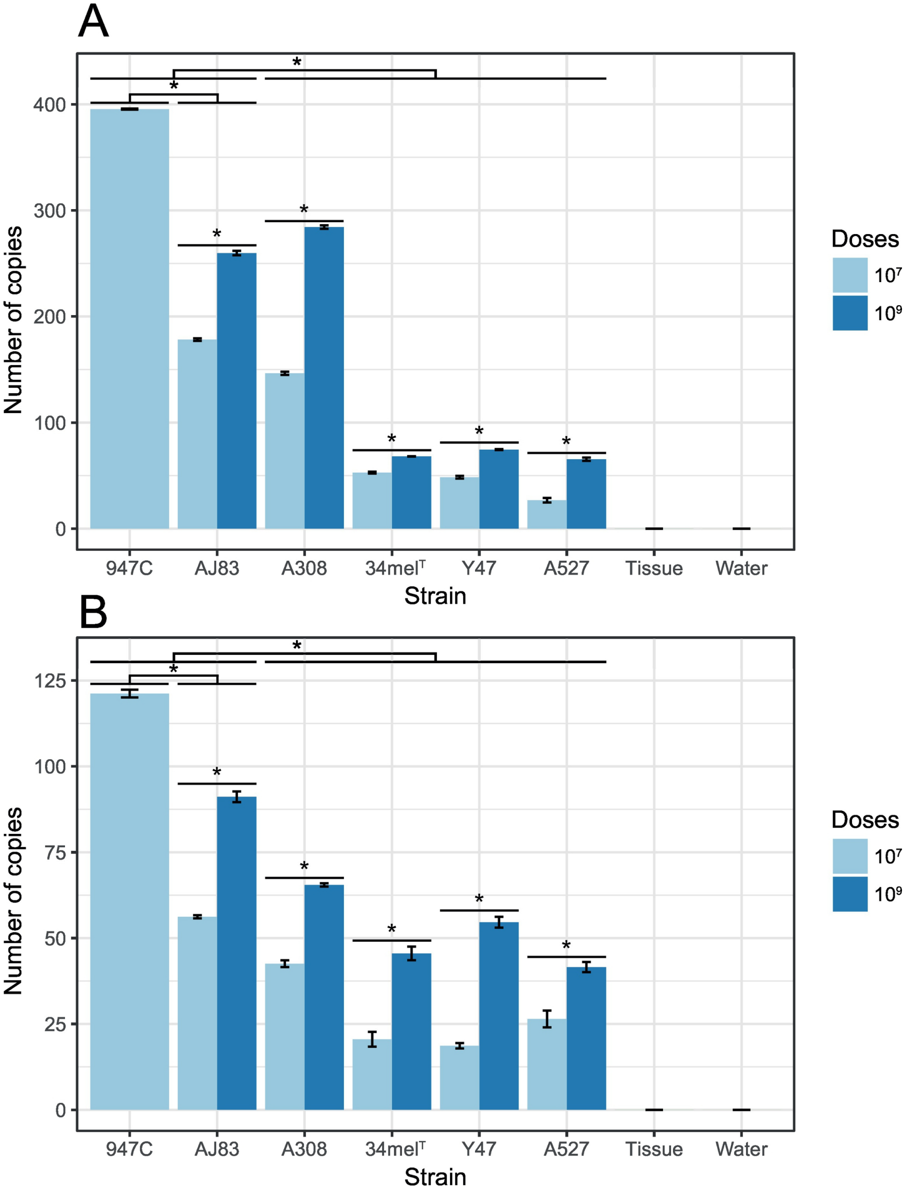
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Figure 3

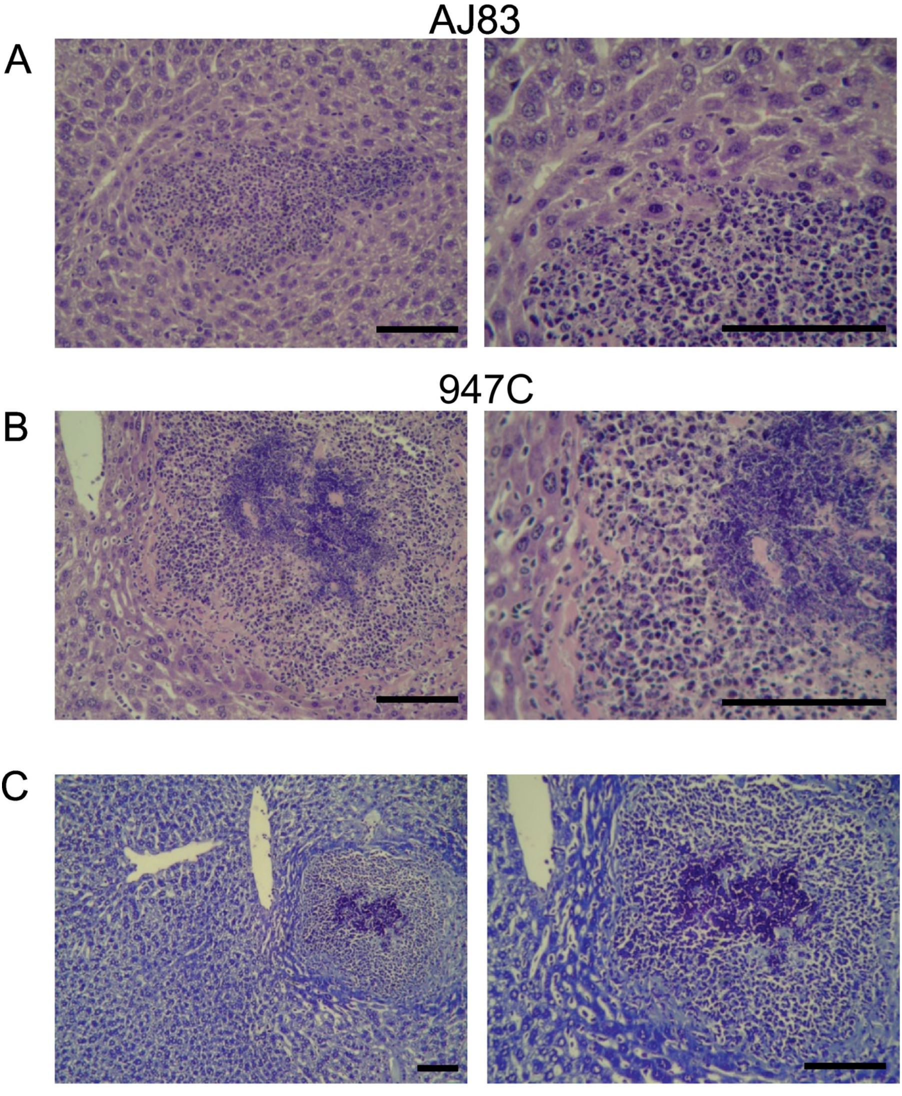

Figure 4