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Volume 37 Number 3 September 2016

Diseases of aquaculture

The Australian Society for Microbiology

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Enquires: Christine Seers (caseers@unimelb.edu.au) or Karena Waller (klwaller@unimelb.edu.au)

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Cover image: Juvenile barramundi (Gavin Partridge); fish cleaning (Faculty of Fisheries, Nong Lam University, Vietnam); fish farming, Kimberley coast (Nicky Buller).

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Roy Robins-Browne
President of ASM

As this is my first communication with you as your new president, I should start by thanking ASM's Council, and especially the Executive Committee, for honouring me in this way and for putting their faith in me. I must also thank Jon Iredell for the wonderful job he did as ASM President for the past two years, and for the amazing support and guidance he has given me to date (and hopefully in future).

I also want to acknowledge and thank Peter Timms for his major contribution to ASM and Council. Peter is stepping down from his role as Chair of the National Examination and Qualifications Board, a position he has held for many years. He will be succeeded by Julian Rood, who as a former President of the Society will ensure that the NEQB remains in safe hands.

Times are changing for biological societies around the world, and ASM will need to embrace some changes too. Our large sibling (I hesitated to write 'Big Brother'), the American Society for Microbiology, recently coalesced its two annual flagship meetings: the Annual General Meeting and the Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) into one; and is supporting more specialised meetings as a way to compensate for the loss of one annual conference.

Our own ASM may need to do something similar. This is one of the issues that Enzo Palombo and his team will be addressing as part of the comprehensive review they are undertaking for ASM. The overarching aim of this review is to ensure that our Society remains relevant to its members. I will keep you updated on the review as things progress and will be seeking your input on all proposals and recommendations to emerge from the review in future.

I recently returned from our Annual Scientific Meeting in Perth. While there, I attended Educon (the educators' conference), all plenary sessions and several symposia. For those of you who did not attend, you missed an outstanding conference. Unlike many other scientific meetings I have been to, I felt spoiled for choice. For example, I had great difficulty choosing between concurrent symposia on 'Infections in Regional Australia', 'Viral Discovery', 'Gut Ecology', and 'Bacterial Pathogenesis', all topics that interest me greatly.

The Local Organising and Scientific Program Committees, chaired by Charlene Kahler and Megan Lloyd, respectively, deserve our thanks, admiration and warmest congratulations on an extremely valuable, interesting and enjoyable conference. The invited plenary speakers, in particular, were top class. Another excellent Annual Scientific Meeting is being planned for Hobart next year. Mark the dates 2–5 July, 2017 in your diary to ensure that you do not miss out.

Finally, please visit our website: www.theasm.org.au to see a list and photos of our award winners for 2016, as well as information regarding upcoming meetings, awards, and, for those who may be interested, our financial statements and minutes of recent meetings. Please also consider making www.theASM.org.au your Internet homepage. You may also like to follow, and contribute to ASM on Twitter, @AUSSOCMIC, to make sure you keep up with the latest news, trends and developments in microbiology in Australia and elsewhere.

Things that the ASM does to look after and promote the interests of its members

ASM is a member of Science and Technology Australia (STA)

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STA is Australia's peak body in Science and Technology. STA's mission is to bring together scientists, governments, industry and the broader community to advance the role, reputation and impact of science and technology across the nation.
The formal members of STA are some 60 Australian scientific and technological societies and organisations with total membership of 68 000 individual scientists, engineers and technologists.

STA has three formal objectives:
- encourage scientific dialogue between government, the science and technology community, and industry
- promote public understanding of science
- foster close relations between the societies

Go have a look at the STA website:

http://scienceandtechnologyaustralia.org.au

Maybe you will find something of interest and value to you.

Diseases of aquaculture

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The value of production of aquaculture in Australia is around $990 million[1] and consists of cultivation of over 40 species, most for food, but others such as pearl oysters and crocodiles are cultured for products for the fashion industry. A number of finfish are grown for food including salmon, barramundi, and silver perch, and other species include prawns, marron, abalone, oysters and mussels, whereas southern bluefin tuna are caught from the wild and farmed until they reach market size. A number of species are being investigated for aquaculture and these include octopus and sea cucumber[1].

Aquaculture is undertaken in all states of Australia plus the Northern Territory and the geographical location, environment including water source and temperature, and resources often dictates the type of aquatic animals cultured.

In New Zealand, aquaculture of oysters, mussels and salmon results in revenue of NZ$ 400 million with a range of other species being investigated for production that includes rock lobster, sea cucumbers, eels, and sea sponge[2].

Aquaculture farms may be land-based in tanks or ponds, or water-based in the ocean, river or estuary systems using cages, ponds or in the case of shellfish – cultured on racks or lines suspended in the water. By definition aquaculture is intensive farming and as such creates disease challenges related to stocking density similar to those seen in farming of terrestrial animals, or the effect of urbanisation on the epidemiology and transmission of diseases in people[3,4]. Good farming practices are crucial for disease management. A disease outbreak in any type of aquaculture system can be devastating and there are instances where a single disease outbreak resulted in the failure of that business[5].

As with all living things, the aquatic animal host is colonised by a range of microorganisms considered to be normal flora, but each host has a number of potential pathogens that includes bacteria, viruses, fungi, parasites etc.

Although not covered in this issue, a number of pathogens or saprophytes of aquatic animals or microorganisms in the aquatic environment and their biotoxins to which workers or fisherman may be exposed, are zoonotic or biohazards that can impact on human health and can cause infections or food poisoning[6]. Therefore, not only veterinary microbiology laboratories, but also medical laboratories may encounter these microorganisms.

Working in the area of aquatic animals, whether in a diagnostic laboratory or in research, presents interesting and exciting challenges, with in-depth knowledge required of a vast range of microorganisms.

Some of the challenges associated with detecting, identifying and controlling disease agents in aquaculture are presented in this issue of *Microbiology Australia*.

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Biography

Dr Nicky Buller is Senior Microbiologist in the bacteriology laboratory at Animal Health Laboratories, Department of Agriculture and Food Western Australia. She is the author of *Bacteria and Fungi from Fish and Other Aquatic Animals; a practical identification manual*, published in 2004 (first edition) and 2014 (second edition) by CABI, United Kingdom.

Antimicrobial susceptibility testing guidelines as a necessary tool to guide chemotherapeutic interventions in aquaculture

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The selection of chemotherapy in aquatic animal medicine is not as straightforward as one might believe. A multitude of factors can impact effectiveness *in situ*. Some of these factors include the pathogen(s) present and their antimicrobial susceptibility, site(s) of infection, timing of treatment, host health/disease status, dose and regimen, water salinity, and water temperature. This article will focus on the first of these factors, and how susceptibility testing of target pathogen(s) can be used to both inform therapy decisions and assist in compliance with principles of prudent and judicious use.

Antimicrobials have long been used to relieve pain and suffering and to control infections in food-producing animals, including fish. The safe and prudent prescription of effective antimicrobials by veterinarians to treat aquatic animals has contributed immensely to the increased food production capacity of aquaculture worldwide. However, the use of antimicrobials in aquaculture is not without risk. The American Veterinary Medical Association has published educational materials for veterinarians which describe prudent and judicious use guidelines for antimicrobials in aquaculture[1]. Antimicrobial-resistant bacteria, pathogenic to animals and humans, have been found in and near fish and shellfish farms where medicated feed has been administered[2–7]. In addition, fish have been implicated as potential reservoirs of zoonotic pathogens[8], some of which may carry resistance genes including extended-spectrum beta-lactamases[9]. Cabello suggested the unrestricted use of antimicrobials in aquaculture in any country has the potential to affect human and animal health on a global scale, and that this problem should be dealt with through unified local and global preventive strategies[10].

One mitigation step that can be used to minimise the dissemination of antimicrobial resistance is to make every attempt to ensure that chemotherapeutic intervention is necessary. Part of this decision process should be to determine the pathogen's susceptibility to antimicrobials approved for use in the target animal species. Whenever possible it is important to use internationally standardised antimicrobial susceptibility testing (AST) methods. The most widely used AST methods are those published by the Clinical and Laboratory Standards Institute (CLSI). The CLSI has published two guidelines: VET03-A – *Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated from Aquatic Animals*[11], and VET04-A2 – *Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals*[12], as well as an informational supplement, the VET03/VET04-S2 – *Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated from Aquatic Animals*[13]. These documents provide detailed standardised AST methods (and recommended non-standard modifications for fastidious pathogens), quality control parameters, and interpretive categories for aquatic animal pathogens at incubation temperatures of 18°C, 22°C, 28°C and 35°C. As global consensus guidelines, the quality control parameters included allow for harmonisation of AST data on an international scale. These standardised AST methods also permit performance monitoring within a lab, and development of lab-specific interpretive categories (see below) when limited data are available in the literature for a given pathogen and antimicrobial combination.

Antimicrobial susceptibility testing

The disk diffusion method is the most commonly used AST method in aquatic animal disease diagnostic laboratories. The diameter of zone of inhibition produced by a disk impregnated with an antimicrobial and placed on an agar plate is measured to the nearest millimeter. Another very popular AST method is the broth microdilution method. This method determines minimal inhibitory concentrations (MICs) for each antimicrobial, typically in a 96-well plate format. Based on an AST result, an interpretive category is attributed

to the antimicrobial and specific isolate tested. Different interpretive categories are used by diagnostic laboratories dependent upon whether results are needed to inform treatment, in which case clinical breakpoints should be used; if results are being used for antimicrobial resistance (genotype) surveillance, epidemiological cut-off values (ECVs) should be used (Figure 1).

Clinical breakpoint interpretive categories include susceptible (S), intermediate (I) and resistant (R). *Susceptible* is a category based on a breakpoint that implies that isolates are inhibited by usually achievable concentrations of antimicrobial when the dosage recommended to treat the site of infection is used. *Intermediate* is a category based on a breakpoint that includes isolates with corresponding MICs that approach usually attainable blood and tissue levels, and for which response rates may be lower than for susceptible isolates. *Resistant* is a category based on a breakpoint that implies that isolates are not inhibited by usually achievable concentrations of the antimicrobial with normal dosage schedules, and/or that demonstrate MICs that fall in the range in which specific microbial resistance mechanisms are likely, and clinical effectiveness of the antimicrobial against the isolate has not been reliably shown in treatment studies. When detection of an emerging genotype is the primary goal, an ECV which is a zone diameter or MIC that separates microbial populations into those with and without acquired and/or mutational resistance genes is used. Wild type (WT) is an interpretive category based on an ECV that describes isolates with no mechanisms of acquired resistance for the antimicrobial. Non-wild type (NWT) is a category based on an ECV that describes isolates with presumed or known mechanisms of acquired resistance and/or decreased susceptibility for the antimicrobial[14].

Incorporation of antimicrobial susceptibility testing into the diagnostic process

The OIE's *Aquatic Animal Health Code* emphasised the importance of clinical assessment, pathogen identification, and susceptibility testing before initiation of therapy[15]. Too often, particularly in developing countries, antimicrobials are obtained over-the-counter by fish farmers before a proper diagnosis can be made. In many countries where a veterinarian–client–patient relationship does exist, a veterinarian may choose to prescribe an antimicrobial without a complete knowledge of the pathogen(s) present and their susceptibility (or lack thereof) to the prescribed antimicrobial. It is understood that the pressure placed on veterinarians to intervene quickly is critical, particularly in aquaculture where losses can be sudden and devastating. However, ideally, to be in full compliance with the prudent and judicious use principles outlined by the AVMA and the OIE, additional steps should be taken prior to prescribing a medicated feed to an entire animal population (Figure 2). These include isolation and identification of the pathogen, assessment of susceptibility to antimicrobials of interest, and prescription of an approved antimicrobial at a dose appropriate for a given situation. Alternatively, improved husbandry practices may supplant the need for intervention with a medicated feed. To illustrate this approach and demonstrate how AST can be used as

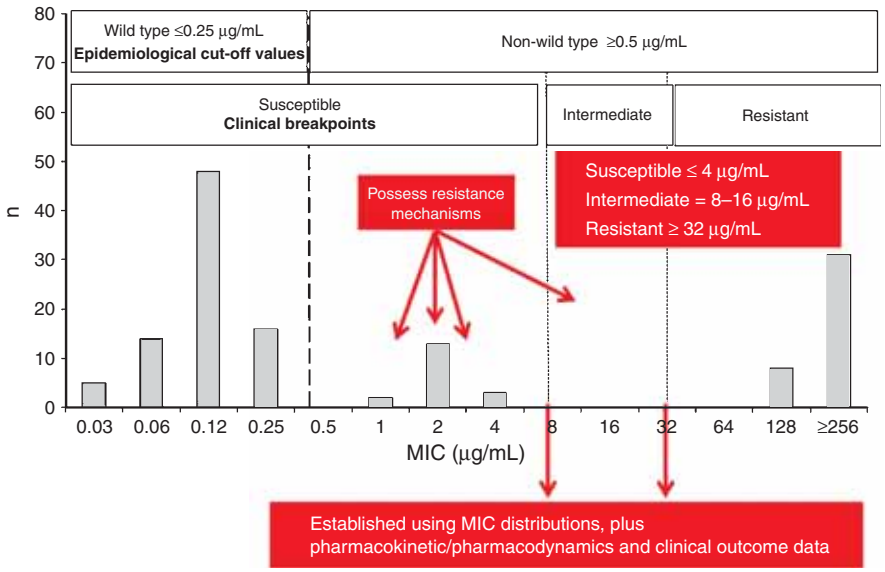

Figure 1. Epidemiological cut-off values vs clinical breakpoints. MIC, minimal inhibitory concentrations.

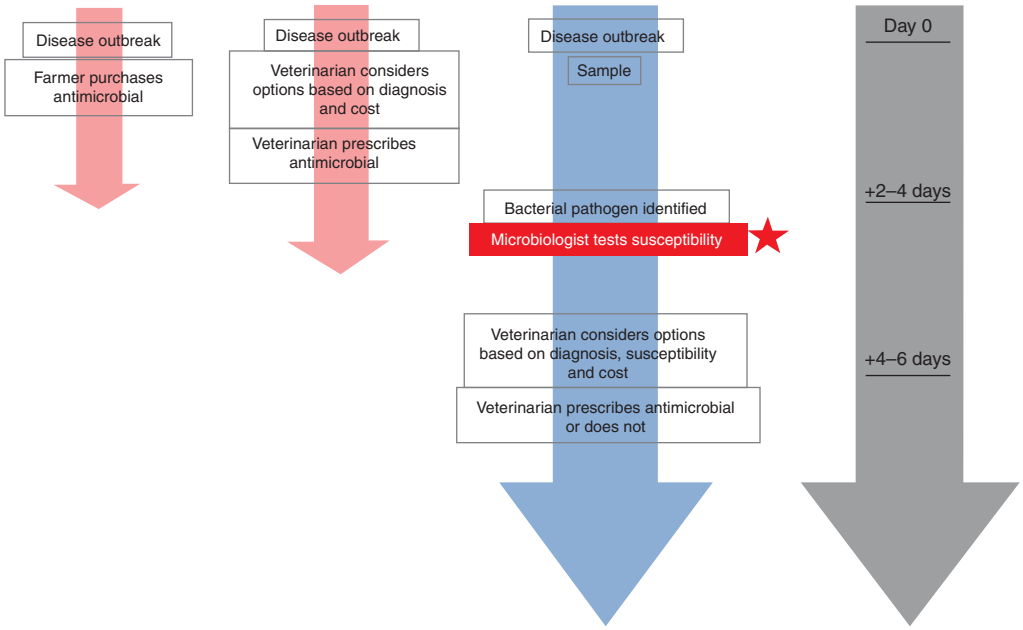

Figure 2. Common practices in veterinary medicine.

a helpful tool in intervention decisions, two scenarios are provided below.

A rainbow trout case from a large farm in Europe is found through culture and identification, to have succumbed to furunculosis caused by *Aeromonas salmonicida*. One scenario may include a need to quickly find the best course of action to prevent further losses on the farm. In the country of origin, oxolinic acid is approved by the regulatory body to treat furunculosis in trout. Upon completion of AST testing in accordance with CLSI guidelines VET03-A[11] and VET04-A2[12], the isolate has an oxolinic acid zone diameter of 30 mm and MIC of 0.12 µg/mL. What does this mean? After consulting the clinical breakpoint table (table 12) in CLSI's VET03/VET04-S2 supplement, we find this isolate is considered susceptible to oxolinic acid. The veterinarian should consider administration of oxolinic acid in feed to the affected population. Additional improved husbandry interventions may also be warranted.

Consider a second scenario where an Atlantic salmon is determined through culture and identification to have died from enteric redmouth disease caused by *Yersinia ruckeri*. After conducting standardised AST the isolate is found to have a florfenicol zone diameter of 29 mm, and an MIC of 4 µg/mL. The VET03/VET04-S2 informational supplement[13] does not provide clinical breakpoints or ECVs for *Y. ruckeri*. The veterinarian is forced to consult available literature on *Y. ruckeri* susceptibility to florfenicol, and prior experience to determine the best course of action.

In the latter scenario, there is an opportunity to develop laboratory-specific interpretive categories that may be reliable as long as the AST methods used do not change and quality control parameters are maintained. However, when laboratory-independent interpretive categories are available[13] these should be used whenever appropriate.

Conclusions

Understanding how AST can be used as a tool to guide clinical decision making in aquatic animal medicine is an emerging issue. Knowledge of and use of available standardised AST methods and interpretive categories will improve data quality, international harmonisation, and encourage prudent prescribing practices. Application of a suitable interpretive category (i.e. clinical breakpoint or ECV) for a given situation (i.e. clinical intervention or antimicrobial resistance surveillance) is also critical to minimise antimicrobial resistance development and maintain therapeutic effectiveness now and in the future.

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Biography

Dr Ron Miller is a regulatory review microbiologist in the U.S. FDA – Center for Veterinary Medicine's Office of New Animal Drug Evaluation. He is the Chair of the Clinical and Laboratory Standards Institute (CLSI) – Veterinary Antimicrobial Susceptibility Testing Subcommittee's Working Group on Aquatic Animals. His interests include susceptibility testing method development, antimicrobial resistance surveillance tools, and aquatic microbiology.

Antibiotic resistance associated with aquaculture in Vietnam



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The fishery sector is an important source of income, employment and food supply in Vietnam. In 2014, Vietnam was ranked the world's fourth largest exporter and the third largest producer of farmed food fish. Vietnam seafood export has attained the value of over US$6.0 billion since 2011 and reached a peak of US$7.9 billion in 2014. However, many problems and diseases confront sustainable development of the fishery sector and overuse of antibiotics is considered a major challenge. Antibiotics are used in aquaculture for both therapeutic and prophylactic reasons. Various antimicrobials used in human medicine are also used for food animals even for non-therapeutic use. The use of antibiotics in health management of aquaculture farming is of great concern due to possible residues in aquatic products and in the development of antibiotic resistance. In 2005 the Vietnamese government first promulgated a list of medicines, chemicals and antibiotics that are banned or limited for use in aquaculture and this is regularly updated and amended to tackle the growing problem of antibiotic resistance.

The fishery sector in Vietnam

Fisheries is a key national economic sector that produces 4–5% GDP and 5–6% of the total national turnover of Vietnam. Aquaculture in Vietnam has changed from a self-sufficient sector in the early 1960s into a concentrated commodity production and this model has become the most important contributor in the fishery sector since 2007. In Vietnam, the Mekong River Delta in the south and the Red River Delta in the north have been the major areas for wild caught fishing as well as intensive aquaculture farming for decades. Aquaculture production in the Mekong Delta has contributed more than 70% of the country's total production, in which catfish (fresh water) and black tiger and white leg shrimp (brackish water) are the three most important commercial species grown (Figures 1, 2). Since the 1990s, fishery products have been the third biggest export commodity of the country. Fishery exports earned over US$1 billion in 2000, US$3 billion in 2006, US$6 billion in 2011 and reached a peak of US$7.9 billion in 2014. Currently, Vietnamese fishery products are exported to more than 140 countries and territories in five continents. According to the Food and Agriculture Organisation of the United Nations (FAO), nearly 10% of the population in Vietnam obtains their main income from Fisheries. More than four million workers including a large proportion of women are employed in aquaculture and fishery activities (Figure 3).

Antibiotic resistance in aquaculture in Vietnam

In Vietnam, only limited studies on antibiotic uses and resistance in aquaculture were conducted and/or published before 2005. A study on antibiotic residues and resistance against Norfloxacin (NOR), Oxolinic acid (OXO), Trimethoprim (TRI) and Sulfamethoxazole

Figure 1. Seabass or Barramundi (*Lates calcarifer*) farming in Vietnam.

Figure 2. Tra catfish feeding in a farm in the Mekong Delta, Vietnam.

(SUL) has been conducted in four shrimp farming locations (Thai Binh, Nam Dinh, Can Gio-Ho Chi Minh City and Ca Mau provinces) in mangrove areas in Vietnam in 2003[1]. The results showed the presence of antibiotic residues and a high incidence of bacterial resistance to the four antibiotics observed in these shrimp ponds in different mangrove areas in Vietnam. Another study on antibiotic resistance of 123 bacterial isolates (from water, sediment and different fish farms (catfish, tilapia, common carp and gourami) in five provinces in the Mekong Delta) found that 90% of the isolates were resistant to Tetracycline (TET), 76% to Ampicillin (AMP), 100% to Chloramphenicol (CHL), 65% to Nitrofurantoin (NIT) and 89% to Trimethoprim-Sulphamethoxazole (SXT).

A number of extensive research projects on antibiotic resistance of commensal bacteria from several aquaculture species have been conducted by our groups. In 2003, a study on the resistance of 101 isolates (belonging to the family Enterobacteriaceae) from frozen catfish of the Mekong Delta showed high frequencies of resistance (80–100%) to Oxytetracycline (OTE), CHL, Streptomycin (STR), SXT and AMP (unpublished data). The levels of multiple resistance were also high, of which 100% of isolates were resistant to at least four antibiotics. A research study published in 2005 reported the resistance of 92 isolates (including Enterobacteriaceae (49.1%), Pseudomonas spp. (35.2%) and Vibrionaceae (15.7%) families) from three catfish farms in the Mekong Delta[2]. Rather high incidences

Figure 3. Tra catfish filleting in a seafood processing company in the Mekong Delta, Vietnam.

(20–60%) of resistance to OTE, CHL, SXT, NIT, Nalidixic acid (NAL) and AMP were observed. The multiple antibiotic resistance (MAR) index values indicated that the three farms corresponded to high-risk exposed-antibiotic sources. In a recent publication, a larger collection of samples (catfish, water and sediment) was collected from 50 catfish culture ponds (belonging to 15 catfish intensive farms) in the Mekong Delta, Vietnam[3]. All 116 *Pseudomonas* spp. and 92 *Aeromonas* spp. isolates recovered from these samples were tested for their resistance to 13 antimicrobial agents belonging to eight families. High levels of resistance to AMP, SXT, and NAL were observed. Lower frequencies of resistance occurred against Ciprofloxacin (CIP), NOR, TET, Doxycycline (DOX), Gentamycin (GEN), Neomycin (NEO), STR, and Kanamycin (KAN). The percentages of multiple resistance (resistance to three or more antimicrobial classes) was 96.6% for *Pseudomonas* and 61.9% for *Aeromonas* spp. The MAR indices showed that *Pseudomonas* and *Aeromonas* isolates were high-risk sources of contamination where antibiotics were commonly used. The level of resistance to CHL and NIT was still high despite these antibiotics being banned for use in aquaculture in Vietnam since 2005, and this resistance is possibly due to the co-transfer of resistance genes. Our recent study on the resistance of 167 *Vibrio* spp. isolated from blood cockles (16 farms of the six provinces in the Mekong Delta) showed a low incidence of resistance to nine tested antibiotics (TET, NAL, NOR, CIP, SXT, NIT, CHL, STR, KAN), with a high level of resistance to AMP only. Unfortunately, very high levels of resistance of bacteria in aquaculture environments or products have also been reported in many countries which have intensive aquaculture activities such as China[4], USA[5], Thailand[6], India[7], Indonesia[8], Australia[9], Italy[10] and Chile[11] implying the heavy use of antibiotics in aquaculture worldwide and this provides a warning on the effects of antibiotic usage in aquatic ecosystems, and the world wide emergence of antibiotic resistance in aquatic bacteria.

Integrons and the transferability of antibiotic resistant genes

In bacteria, horizontal gene transfer is the principle mechanism responsible for the spread of antibiotic resistance genes[12]. Horizontal gene transfer is facilitated by mobile genetic elements such as conjugative plasmids, transposons, and phages[13]. Integrons are not themselves mobile elements but are associated with mobile genetic elements (transposons or conjugative plasmids) enabling efficient intra- or interspecies transmission[14]. In Vietnam, studies on integrons and resistance gene transfer mechanisms have been conducted in clinical isolates[1,15–19]. However, very limited studies have been published on non-human isolates such as food-borne pathogens[20], animal pathogens[19] and catfish pathogens[21]. Recently we have done an extensive study on integrons and the transferability of antibiotic resistant genes of commensal bacteria in catfish aquaculture in Vietnam[3]. We found that the commensal isolates of catfish harbour a pool of mobile genetic elements such as plasmids and integrons, which contain various antibiotic resistance gene cassettes. Conjugation and transformation experiments demonstrated the successful transfer of all or part of the resistance phenotypes of isolates to the recipient strains of different genera and sources. Class 1 integrons associated with plasmids have facilitated the emergence and dissemination of antibiotic resistance in aquaculture environments. The transformation and conjugation experiments also indicated that CHL resistance phenotype was co-transferred in association with SXT, AMP, TET resistance phenotypes. In addition, the CHL resistance genes (*catB*) have often been associated with class 1 integrons, therefore, the co-selection is important for CHL resistance dissemination. This suggests that, in the absence of chloramphenicol selection pressure, CHL resistance is co-transferred and maintained due to gene linkage to genes encoding resistance to antimicrobials that are widely used in food animals or aquaculture[22]. The other studies worldwide also indicate that aquatic environments or products are reservoirs of antibiotic resistance and class 1 integrons have facilitated the dissemination of antibiotic resistant genes such as in the USA[23], China[24], Thailand[25], Northern Europe and North America[26], Europe, Japan and the USA[27], and Japan[28].

Conclusion

The high frequencies of resistance observed in aquaculture bacterial isolates in Vietnam, and in worldwide aquatic environmental and product isolates, reflect the global spread of resistance due to extensive use of antibiotics in aquaculture. Aquaculture resident bacteria could serve as a reservoir of resistant genes if they harbour a pool of mobile genetic elements that can readily be transferred intra- and interspecies.

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Francisellosis in fish: an emerging challenge

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Francisellosis is a bacterial disease with increasing economic impacts in the culture of tilapia and Atlantic cod since emerging in 1992. Two main strains – *Francisella noatunensis* subsp. *orientalis* (*Fno*) and *F. noatunensis* subsp. *noatunensis* (*Fnn*), have been identified, causing both acute and chronic granulomatous systemic disease. The piscine host range is increasing and *Francisella* culture should be included in routine diagnosis. Differentiation from the major zoonotic *F. tularensis* and opportunistic zoonotic *F. philomiragia* when dealing with environmental soil and water samples from fish farms is important. Diagnosis can be challenging but presentation of granulomatous pathology in fish should require use of cysteine supplemented selective media, culture at 15–28°C or culture in fish cell lines and specific PCR to exclude piscine *Fno* or *Fnn*. Control of infections in fish rely on appropriate antibiotic selection although in the long term an effective commercial vaccine that includes the pathogenic species of *Francisella* is required.

Tilapia (*Oreochromis niloticus*) production has increased from 2.6 million tons in 2005 to an estimated 4.5 million tons in 2014[1], being only second to carp in global aquaculture production[2]. Atlantic cod (*Gadus morhua*) production peaked at 22.7 tons in 2009 and dropped to 4.3 tons in 2013[3]. Francisellosis has been reported in farmed tilapia from Taiwan (1992), United States (2003), Costa Rica (2009), Indonesia (2009), United Kingdom (2010), and Brazil (2012)[4] with mortalities of 30–75%[5,6] and up to 95%[7]. During 2004–2005, outbreaks in farmed Atlantic cod in Norway resulted in approximately 40% losses, presenting a major impediment to the expansion of cod aquaculture[7]. Initially thought to be a *Rickettsia*-like organism[5,6,8] or *Piscirickettsia*-like organism[8,9], the pathogen was later confirmed as a γ-Proteobacteria in the family Francisellaceae, order Thiotrichales[7]. *Francisella noatunensis* subsp. *orientalis* (*Fno*) causes francisellosis in tilapia (a fresh and warm water fish species) and *F. noatunensis* subsp. *noatunensis* (*Fnn*) in Atlantic cod[7,8] (a marine and cold water fish species). *F. philomiragia* subsp. *noatunensis* subsp. nov. and *F. piscicida* were two different names proposed for the organisms isolated from Atlantic cod in Norwegian disease outbreaks, however it has been resolved to be synonymous with *Fnn*[7,10]. Infections associated with *F. philomiragia*/*Fnn* in Atlantic salmon (*Salmo salar*), *Francisella* spp. in three-line grunt (*Parapristipoma trilineatum*) and ornamental cichlid species are reported[7]. *Fno* infected hybrid striped bass (*Morone chrysops* × *M. saxatilis*)[8] and *Francisella halioticida*[11] infected the giant abalone (*Haliotis gigantea*)[7]. Recently, disease in marine ornamental fish species (wrasses and damselfish) was associated with *Fno*[12].

The gross pathology is typified by visceral granulomatosis causing splenomegaly and renomegaly due to multiple whitish-tan nodules with similar lesions in liver, gills or muscle. The degree and range of organ involvement differ between species. In Atlantic cod, emaciation, haemorrhagic skin and heart nodules also occur while in tilapia, gills can have the nodules in addition to exopthalmia and skin haemorrhages and scale loss[7,8]. Histopathology in affected organs feature granulomas consisting of vacuolated macrophages with the *Francisella* organisms, associated central necrosis and fibrous encapsulation[5,7–9,12,13], in the sub-acute (7 days post challenge[5]) to chronic disease. Acute disease has been experimentally replicated causing 100% mortality by 72 h post intraperitoneal inoculation of approximately 10^7 colony-forming unit (cfu) per fish where bloody ascites, increased melanomacrophage centres but no granulomas were observed[6]. For tilapia, epizootics typically occur in cooler, winter water temperatures with higher mortalities at 15°C than 30°C[7] or no mortalities at 26.5–29.2°C[8]. Francisellosis causes more mortalities as water temperatures increase towards 20°C in summer for Atlantic cod[7]. Epidemiologically, piscine Francisellae cause disease in both fresh and marine waters and morbidity can be extremely elevated for Atlantic cod and tilapia[8]. Fish pathogenic *Francisella* can enter a viable but non-culturable state in cold water after 30 days at 8°C and 16 days at 12°C[8], meaning that they are non-virulent. A reservoir of the *F. philomiragia* in the aquatic protozoan *Acanthamoeba castellanii* and the aquatic biofilm has been reported[14] with implications of transmission to fish.

Francisella are 0.1–1.5 µm, strictly aerobic, facultatively intracellular, non-motile, Gram-negative coccobacilli[7] to pleomorphic

spherical[6,9,15,16], halophilic[15] or freshwater[6] organisms. Culture of *Fno* and *Fnn* from kidney, spleen, blood or granulomatous lesions is made on enriched blood agar plates supplemented with 0.1% cysteine and 1% glucose, cysteine heart agar with 1% haemoglobin (CHAH) or cysteine heart agar with 5% sheep blood (CHAB) or Thayer-Martin media[6–9]. The organism fails to grow on trypticase soy agar (TSA) supplemented with 5% sheep blood[6] and can be easily overgrown with or inhibited by contaminant or secondary bacteria[6,8,9]. Polymixin B (100 U/mL) and/or ampicillin (50 µg/mL) maybe added to reduce these bacteria[6]. Incubation temperature is 15–20°C for *Fnn* and 25°C for *Fno*[9] with *Fnn* growing poorly at 30°C and *Fno* preferring 28°C[4,8]. Colonies develop slowly, taking up to 30 days but may appear as smooth, white to greyish within 3–6 days[9]. Differentiation can be made from the zoonotic *F. tularensis* and *F. philomiragia* in that these organisms can grow at 35–37°C while *Fnn* and *Fno* do not[8]. Further, *F. philomiragia* does not have an essential requirement for cysteine to grow[7,17]. Biochemical reactions for *Fno* and *Fnn* are the same, with negative reactions for cytochrome oxidase activity, acid production from sucrose, β-galactosidase and no enzymatic activity for O-nitrophenyl N-acetyl-β-D-glucosamide (ONAG), P-nitrophenyl-β-D-galactopyranoside (PNPG), leucine arylamidase, and N-acetyl-β-glucosaminidase[7]. However, *Fnn* metabolises D-glucose but does not have indoxyl phosphate (IDP)[7,18] activity, while *Fno* is the reverse for these tests[7]. Molecular testing based on the G1,L1 primers targeting the internal transcribed sequence (ITS) with Eub A and Eub B primers targeting 16S rRNA, followed by sequence homology analysis is able to differentiate *Fno* (in tilapia and three-line grunt) from *Fnn* (Altlantic cod and Atlantic salmon)[7,18]. Of note, *Fnn* shows 99.3% and *Fno* shows 98.6% 16S rRNA similarity to *F. philomiragia*, but they are more genetically dissimilar to *F. tularensis*[7,8,16]. Cell culture isolation has been demonstrated for *Fno* using chinook salmon embryo (CHSE-214[5]) and tilapia ovary cells (TO)[9]. Similarly *Fnn* can be grown using salmon head kidney (SKK-1) and Atlantic salmon kidney (ASK)[8]. Serological testing using antiserum raised against *Fnn* detects *Fno* as well, with *F. philomiragia* agglutinating slightly to the *Fnn* antiserum[7] but there was no cross reaction with monoclonal antibody against *F. tularensis*.

In terms of zoonotic risk, *F. tularensis* is a major environmental and tick or insect vector-borne human pathogen causing pneumonic tularemia[6,19], with *F. tularensis* subsp. *tularensis* being the most virulent strain and of biological weapon concern[6,7]. Recently, tularemia in Turkey has been associated with beaver, muskrat and voles which infect surface waters suggesting that the aquatic environment is an important risk factor in its epidemiology[20,21]. *F. philomiragia* is a rare disease and is associated with immune-compromised patients as in chronic granulomatous disease (CGD) and in near drowning events causing pneumonia or fever-bacteraemia[13,15,17]. *F. philomiragia* has been isolated from brackish water in an area where repeated tularemia cases occurred[19]. Therefore, it may be prudent to consider that zoonotic species of *Francisella* could be transmitted through the aquatic environment when dealing with aquatic environmental samples, including those from fish farms. To date, *Fno* and *Fnn* are considered to have negligible zoonotic risk as they cannot grow at 37°C and for *Fno* in tilapia, there has been no documented case of human infection despite it being a major aquaculture product processed for human consumption[7,8].

Control of clinical infections of francisellosis in tilapia has been reported with 30–50 mg/kg oxytetracycline over a 10–14 day treatment, but the high infectivity, a low infective concentration, high morbidity and inappetance in severely infected fish may render sustainable management ineffective[8]. Isolates may be resistant to trimethoprim-sulfamethoxazole, penicillin, ampicillin, cefuroxime and erythromycin, gentamicin and ciprofloxacin[7]. Florfenicol at 15 mg/kg has been demonstrated experimentally to improve survival to challenge with *Fno*, and it is suggested that this antibiotic could penetrate intracellularly to clear the organism[7]. To date there is no commercial vaccine for piscine francisellosis although development work based on attenuation of *Fno* by mutation of the *iglC*[*] gene provided effective protection in tilapia[7,8]. Formalin-killed *Fno* bacterin with a mineral oil adjuvant provided a relative percentage survival (RPS) value in tilapia of 100% at day 27 post intraperitoneal challenge, with a specific antibody response at 15, 30 and 45 days post vaccination[1].

There are a number of key issues with piscine francisellosis:

- improving the efficiency of definitive diagnosis to mitigate the inadvertent dissemination of infected carrier fish hosts. This will require veterinary pathologists and microbiologists to be up-to-date regarding the case presentation of the disease. As a standard approach, fish with granulomatous disease should be subject to *Francisella* sp. exclusion, as part of the differential diagnoses.
- research into the epidemiology (in particular the diversity of reservoir host species) and virulent factors or genes of *Fno* and *Fnn* as part of the process for development of commercial vaccine products. This is important as warm water and cold water francisellosis are likely to present different scenarios in terms of disease management.
- finally, regarding the zoonotic risk of *F. tularensis* and *F. philomiragia* with these being isolated also from aquatic environments[15,19], bacteriological culture conditions to exclude these zoonotic Francisellae from fish samples is an important exercise. This will avoid inadvertent human infection from aquatic or aquaculture environments.

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Providencia rettgeri septicaemia in farmed crocodiles

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Bacterial septicaemia is a major cause of morbidity and mortality in farmed saltwater crocodiles (*Crocodylus porosus*) in the Northern Territory. *Providencia rettgeri* is the most common aetiological agent. Efficacy of antibiotic treatment is dubious and there are high levels of resistance to antibiotics commonly used by farms, underlining the need for exploration of new approaches to managing the disease.

Saltwater crocodile farming is a growing industry in Australia, with an annual gross value of over $50 million, the main product being high quality skins for the luxury leather market. In the Northern Territory, there are several farms, the largest having approximately 40 000 crocodiles. Berrimah Veterinary Laboratories (BVL) is situated within 30 km of the four largest farms, facilitating a close collaborative relationship. Each year, BVL receives from 50–100 farmed crocodile

diagnostic submissions, the vast majority concerning hatchling to juvenile crocodiles (2–8 months of age). In this age group, a very common cause of death is bacterial septicaemia, with *Providencia rettgeri* predominating (Figure 1).

Grossly, affected crocodiles may exhibit regional subcutaneous and serosal vascular congestion and oedema (Figure 2*a*, *b*). Despite young crocodiles being offered food every 2–3 days, septicaemic crocodiles invariably have no stomach content at necropsy, indicating that affected crocodiles are inappetent. Histological findings include fibrinous pyogranulomatous cellulitis and polyserositis, intravascular coagula of fibrin, macrophages and degranulating heterophils, and acute multifocal splenic necrosis and heterophil infiltration (Figure 2*c*).

Definitive diagnosis in cases of suspected septicaemia is achieved by bacterial culture of at least two, aseptically sampled, blood filtering organs (lung, liver, spleen or lung). Tissues are homogenised aseptically in physiological saline and a swab soaked in homogenised samples is inoculated on tryptic soy agar with sheep's blood and MacConkey agar for aerobic culture. The plates are incubated at 35°C and are examined for bacterial growth after overnight and 48 hours incubation. The predominant colony type is then selected for biochemical testing. In cases of *P. rettgeri* septicaemia, the bacterium is typically present as a moderate to heavy growth in pure culture.

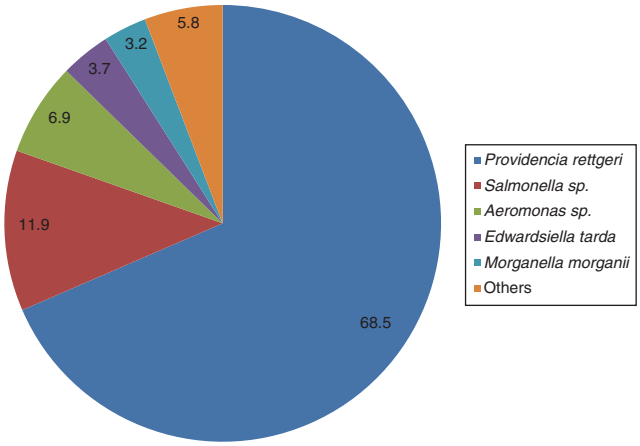

Figure 1. Predominant isolates in farmed crocodiles with bacterial septicaemia. Numbers are percentages of a total of 220 isolates from 159 cases that occurred during 2010–2015. *P. rettgeri* was the cause of septicaemia in significantly more cases than other bacterial species ($P < 0.0001$, Chi-squared = 427, df = 5).

Providencia rettgeri appears non-haemolytic on sheep's blood agar and is an aerobic, Gram-negative bacillus, oxidase negative and catalase positive (Figure 3). A commercial kit, Microbact 24E (Oxoid Ltd), is routinely used for biochemical identification and the test results after overnight incubation at 35°C are as follows: negative reactions for lysine decarboxylase, ornithine decarboxylase, H_2S production, ONPG, acetoin production, gelatin liquefaction, malonate inhibition, arginine dihydrolase and fermentation of xylose, sorbitol, sucrose, lactose, arabinose and raffinose; positive reactions

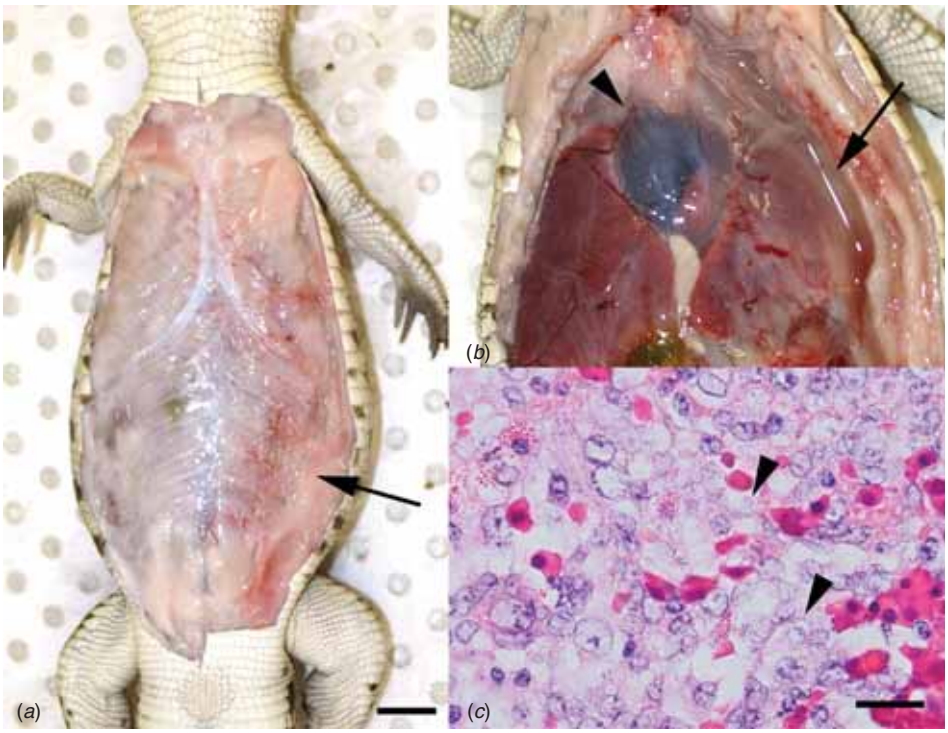

Figure 2. Pathology of *P. rettgeri* septicaemia in a hatchling. (*a*) Crocodile in dorsal recumbency with ventral skin removed to show subcutaneous erythema and oedema (arrow). Bar = 1 cm. (*b*) Thoracic cavity of same crocodile opened revealing severe serosal oedema with accumulation of watery, slightly cloudy fluid (arrow) and injection of serosal vessels overlying heart (arrowhead). (*c*) Histological image of spleen showing deposition of pink material (fibrin), infiltration with degranulating heterophils, and macrophages containing phagocytised bacterial rods (arrowheads). Haematoxylin and eosin stain; bar = 10 µm.

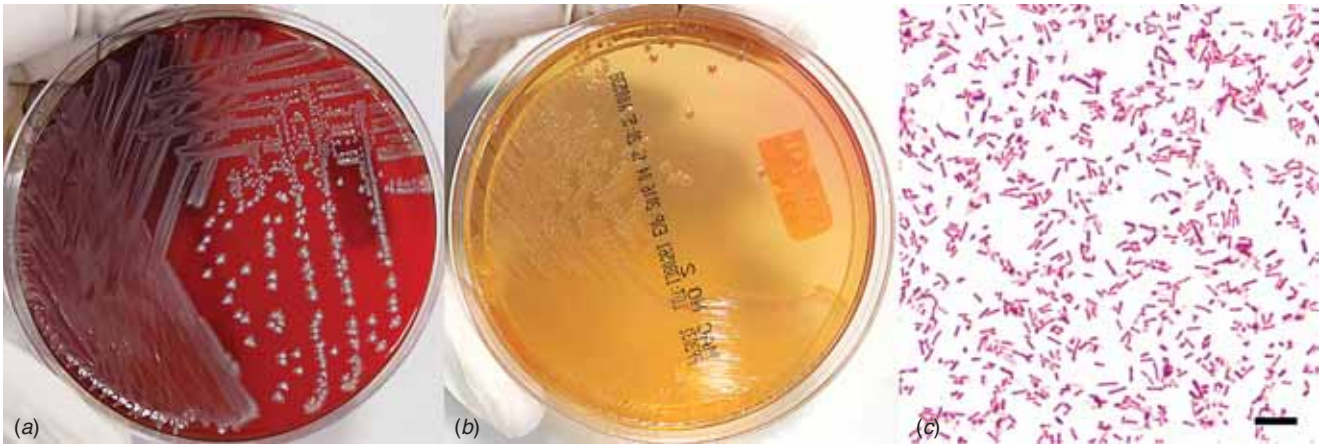

Figure 3. Bacteriological features of *P. rettgeri*. Colony morphology on sheep's blood (*a*) and MacConkey (*b*) agars after 24 h incubation. (*c*) Gram's stain of *P. rettgeri* from culture medium after overnight incubation. Bar = 5 µm.

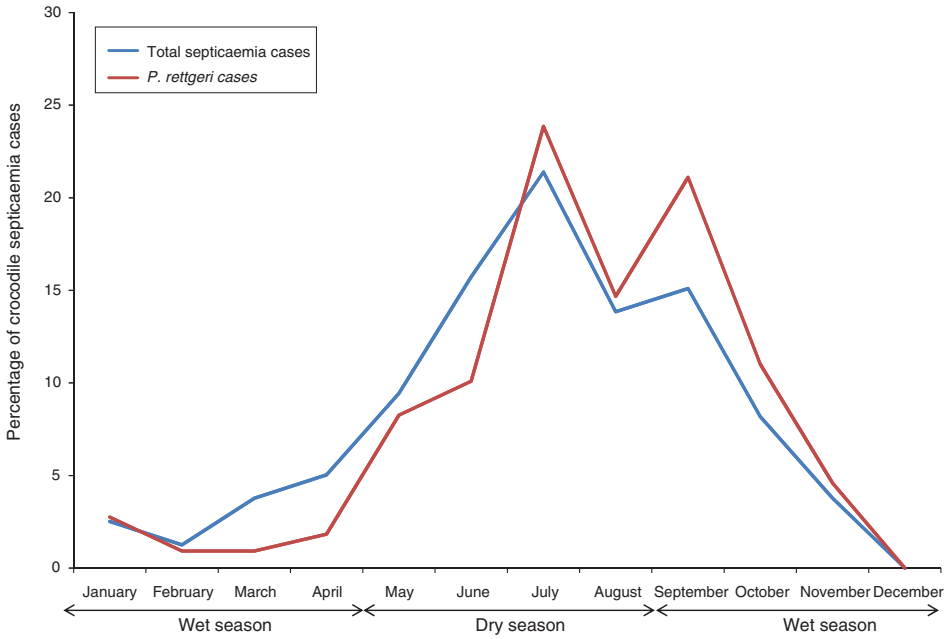

Figure 4. Seasonal occurrence of bacterial septicaemia in farmed crocodiles in the Northern Territory of Australia, presented as monthly percentages of a total of 159 cases from 2010–2015. There are significantly more cases of septicaemia in the 'dry' season compared to the 'wet' season ($P < 0.0001$, Chi-squared = 136, df = 1). There is no significant seasonal difference in the occurrence of septicaemia due to *P. rettgeri* versus all cases combined.

for indole, urease, citrate utilisation, TDA and fermentation of glucose, mannitol, adonitol and inositol; mostly positive but occasional negative reactions for fermentation of rhamnose and salicin (octal codes: 06331212, 06331012, 06331210 or 06331010).

The *in vitro* antimicrobial susceptibility testing on *P. rettgeri* is performed by disc diffusion method using the Clinical and Laboratory Standards Institute guidelines[1,2]. The three antibiotic treatments, namely sulphafurazole, tetracycline and sulphamethoxazole with trimethoprim, are routinely tested at BVL on crocodilian bacterial isolates as requested by the local crocodile farmers, since these are the antibiotics added to food for treatment. A total of 139 *P. rettgeri* isolates were tested between 2010 and 2015, of which 44% were sensitive to all three antibiotic treatments mentioned above, 21% were resistant to all three antibiotic treatments, 21% were resistant only to tetracycline and 14% were resistant only to sulphonamides. Antimicrobial resistance in crocodilian bacterial isolates may be due to development either of resistance in response to antibiotics used at the farm, or selective pressure for innately resistant bacteria in the environment. The high level of antibiotic resistance, and the fact that antibiotics are being used in food to treat septicaemic crocodiles that are likely not eating, are clear indications of the need for an alternative approach to the use of antibiotics to overcome this problem.

Bacteria belonging to the genus *Providencia*, family *Enterobacteriaceae*, are opportunistic pathogens that have been isolated from a range of environments and hosts including humans[3]. *P. rettgeri*

has been associated with a variety of infections in humans including, travellers' diarrhoea[4], urinary tract infection, especially in certain types of immunocompromised patients[5], hospital -acquired and community-acquired neuroinfection[6], ocular infections[7] and peritonitis[8]. Although *P. rettgeri* is a relatively uncommon isolate in septicaemic reptiles[9], it has been found to be a frequent isolate from some reptilian environments, either as a part of their normal flora or in opportunistic infections[10]. The bacterium has also been noted to cause granulomatous pneumonia and hepatitis in a crocodile monitor lizard[11], respiratory tract infection in a ball python[12], septicaemia and meningitis in American alligators[13], and meningitis in hatchling saltwater crocodiles[14].

The occurrence of bacterial septicaemia in farmed crocodiles in the Northern Territory seems to be influenced by age and climate. The infection is rare in crocodiles older than one year of age, suggesting that adaptive immunity likely plays a role in resistance to infection. In young crocodiles, after hatching in the late wet season, the majority of infections do not occur until the onset of the dry season, in which there are relatively low atmospheric and/or water temperatures (average daily maximum temperature is 33°C during both seasons, but minimum temperature averages 18°C during the dry season compared to 24°C during the wet season) (Figure 4). The bactericidal function of crocodilian complement, an important component of the innate immune system, significantly decreases at temperatures below 15°C and above 30°C[15,16]. The experimental bacterial killing assay on crocodile plasma revealed that juvenile crocodiles have a more established innate ability to neutralise *Escherichia coli* compared with *P. rettgeri*[17]. Avenues for further investigation include characterisation of *P. rettgeri* virulence factors, determination of environmental factors on crocodile farms that may be promoting its presence and ability to cause infection, and efficacy of alternative methods to antibiotics to decrease the environmental and intestinal load and impact of *P. rettgeri* in crocodiles, such as alternative pen cleaning and water treatment methods and/or use of probiotics or vaccination.

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Vaccination against streptococcal infections in farmed fish

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Aquaculture produces more than 50% of fish for human consumption and, in spite of major improvements since the adoption of injectable vaccines in the 1990s, bacterial diseases still account for considerable losses, particularly in tropical and warm temperate species. Streptococcosis, caused predominantly by *Streptococcus iniae* and *S. agalactiae*, manifests as a generalised septicaemia and meningitis followed by rapid mortality. Vaccination against streptococcal infections is difficult as a result of multiple, poorly defined serotypes and consequent vaccine escape (reinfection of previously vaccinated animals). However, genomics applied to reverse vaccinology is providing novel insights into diversity among these aquatic pathogens and is identifying cross-serotype targets that may be exploited for new generation streptococcal vaccines for aquaculture.

Aquaculture reached a significant milestone in 2013 as global production for food use overtook beef production for the first time and now accounts for more than 50% of the global seafood supply[1]. Aquaculture has wrestled with social license throughout its rapid growth in developed economies, including Australia, through the 1980s and 90s[2,3]. Objections have been raised around environmental issues such as eutrophication of marine sediments, escape of domesticated fish into wild stocks and pressure on wild fisheries for fishmeal for aquaculture diets. Disease transmission between farmed and wild stock and high antibiotic use for controlling bacterial infections in farmed fish have also attracted attention. Granted, antibiotic use was high in salmonid aquaculture in the 1980s and early 90s, with aquaculture outstripping both human and terrestrial animal use in Norway[4]. However, the widespread adoption of oil-adjuvanted injectable vaccines in salmonid aquaculture during the mid-1990s all but eliminated antibiotic use from salmonid production[4,5]. Nevertheless, most current and future expansion of finfish aquaculture is occurring in warm temperate and tropical regions where farmed species and the diseases from which they are at risk are not yet adequately controlled by vaccination.

Streptococcal infections occur in warm-temperate and tropical waters wherever fish are farmed and occasionally cause wild fish kills[6]. While there are a number of streptococcal species that cause disease in fish, the most prevalent and damaging are *S. iniae* and *S. agalactiae*. Infection of fish by either pathogen results in rapid onset of generalised septicaemia, meningitis often associated with bilateral exophthalmia (Figure 1*a–d*) and death with mortalities often exceeding more than 70% within a few days of infection in experimental models.

Streptococcus iniae was first isolated in 1972 from an abscess on a captive Amazon freshwater dolphin *Inia geoffrensis* from which it derives its name[7]. In aquaculture, the major species affected by *S. iniae* are rainbow trout in Israel[8], grouper in Taiwan[9], tilapia, catfish and hybrid bass in the USA[10] and, in Australia, barramundi[11]. Vaccination against *S. iniae* is accomplished using formalin inactivated bacterins by intraperitoneal injection of fish under general anaesthetic in tilapia[12], trout[13], grouper[9] and barramundi (Figure 1*e,f*)[14]. But such vaccines are serotype-specific[14–16] and in the absence of a robust serotyping scheme or typing antisera, vaccine failures may occur[13,14] where the prevalent strain does not match the serotype of the vaccine used. Serotype is defined by the polysaccharide capsule in *S. iniae*[8,14,17,18] and antigenic changes result from non-synonymous mutations in a limited repertoire of the genes in the capsular operon that alter monomer composition,

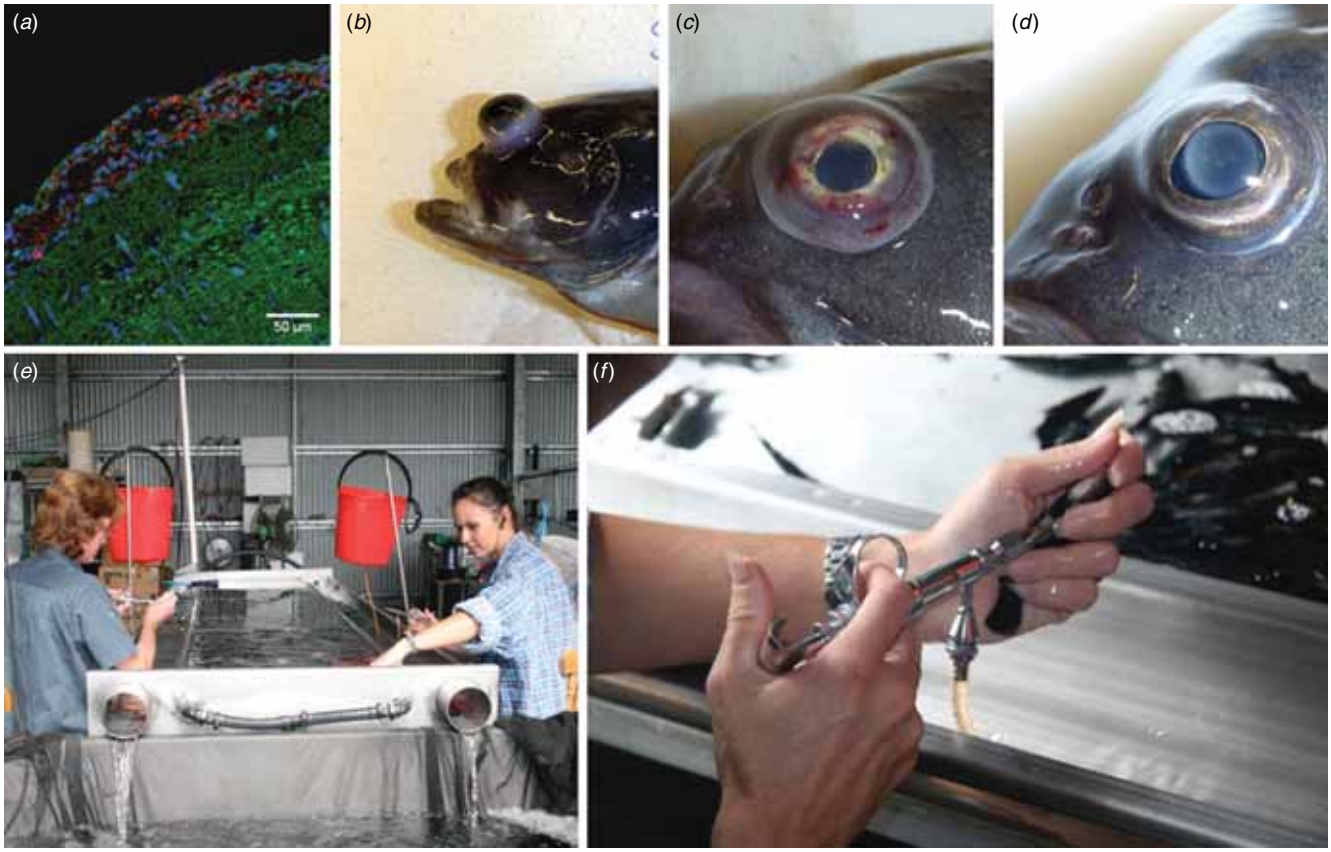

Figure 1. (*a*) Infection of the meninges shown by immunohistochemistry of infected brain section. Blue: DAPI stained tissue; red: rabbit anti-GBS polysaccharide capsule of *S. agalactiae*. (*b*) Exophthalmia and associated meningitis. (*c*) Corneal haemorrhage and (*d*) corneal opacity. Photographs *a–d* from acute infection of giant grouper *Epinephelus lanceolatus* with *S. agalactiae* ST261 serotype 1b (Photos: Dr Jerome Delamare-Debouteville). (*e*, *f*) Vaccination of barramundi against *S. iniae* by intraperitoneal injection. (*e*) Vaccination table with central anaesthetic pool and flowing, oxygenated water along the side channels to a recovery tank. Vaccines are contained in sterile blood-bags on ice in the buckets. (*f*) Vaccines are delivered directly into the peritoneal cavity of the fish under anaesthetic using a self-refilling syringe. Needles must be changed frequently and regularly de-scaled to prevent injury of the fish, which can lead to infection of the injection pore. (Photos: Andy Barnes).

polymer chain length and quantity of the capsule[14]. When a vaccine is deployed, new serotypes periodically arise through mutations in variable capsular genes. These serotypes may be already present in the pool of extant strains co-existing on the farm (serotype replacement, adaptation through standing variation) or originate under immune pressure (adaptation through *de novo* variation). Whole genome sequencing coupled with fluctuation analysis (a statistical method of measuring mutation rate in bacteria based on frequency with which resistance to antibiotic occurs in highly replicated laboratory experiments) suggests both are likely to occur, with a role for hypermutators (variants with greatly elevated mutation rates) facilitating adaptation to the immune host (Figure 2). To overcome the inherent plasticity in the capsular structure, several proteins have been proposed as potential vaccine immunogens based on analysis of the first available genome sequences[21], but these have not been uniformly effective[22–24]. With the falling cost of whole genome sequencing, using informatics to identify surface associated and secreted proteins that are conserved across different capsular serotypes has become a fast and cost-effective route to new experimental vaccines that may be cross-protective across multiple serotypes.

Streptococcus agalactiae is a Lancefield group B *Streptococcus* (GBS). Most fish-pathogenic isolates fall into either (multilocus sequence type) ST260 or ST261 with capsular serotypes Ia and Ib. There have also been reports of fish disease caused by the broad host range ST7 but these have been associated with environmental contamination from terrestrial sources[25,26]. The 'true' fish pathogens are quite distinct from their terrestrial con-specifics, with substantially reduced genomes, depleted virulence factor repertoire and reduction of carbohydrate metabolic pathway genes[26]. Indeed, fish pathogenic GBS was classified as a separate species, *S.* '*difficilis*', until these isolates were later assigned to the species *S. agalactiae* based on whole cell protein analysis in the late 1990s[27] and confirmed by DNA : DNA hybridisation in 2005[28]. Infection and mortality caused by GBS is one of the most significant issues facing tilapia culture globally. Injectable vaccines are effective but type-specific[29]. Once again the potential for exploiting the falling costs of whole genome sequencing for design of cross-serotype protective

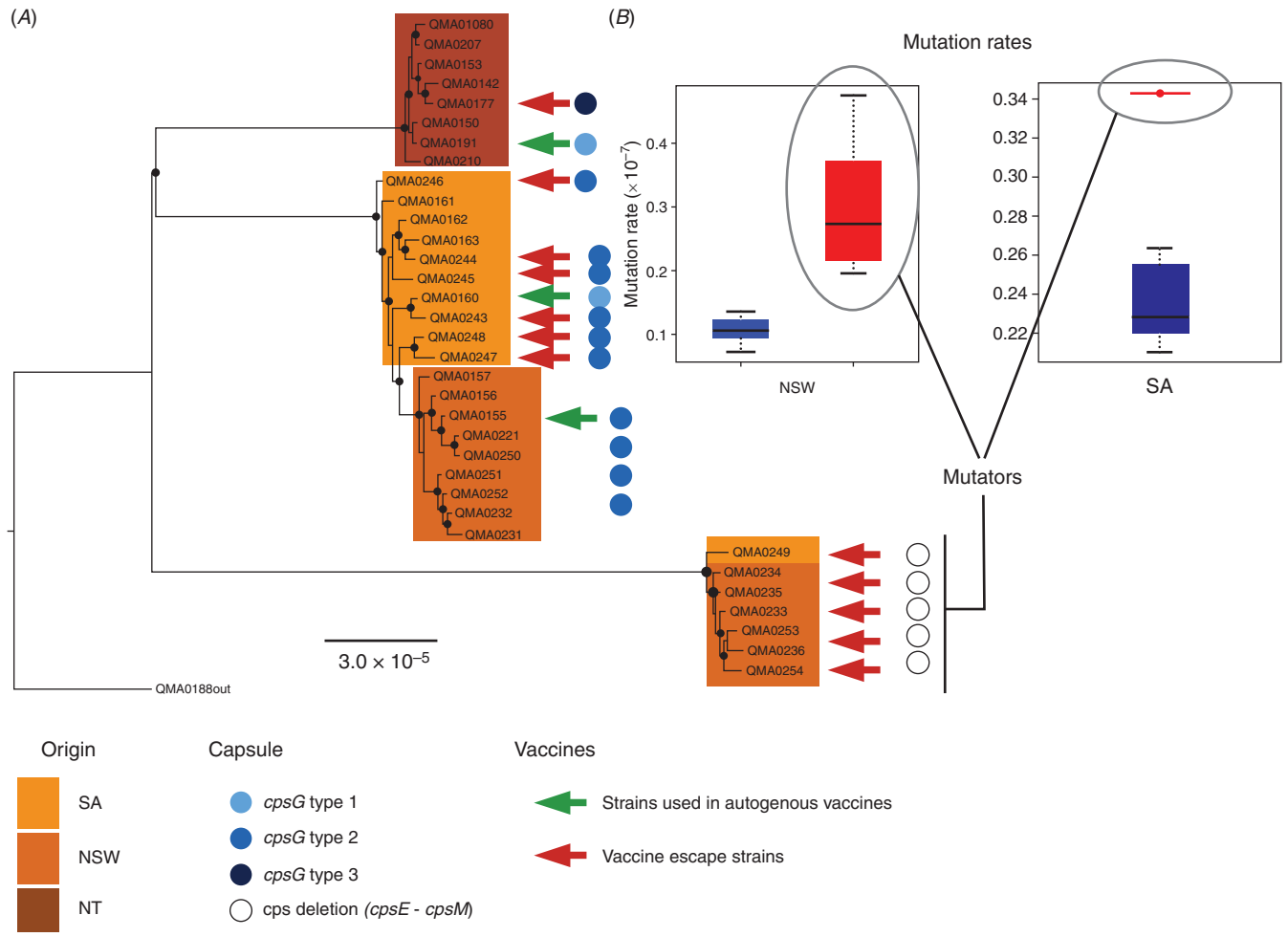

Figure 2. Evidence for role of *S. iniae* mutators in reinfection of vaccinated barramundi in Australia. A) Rooted maximum likelihood tree (RAxML v 8.1.3; GTR+GAMMA model) of *S. iniae* isolates from vaccination cases in Australia based on alignment of core genome single nucleotide polymorphisms (SNPs), filtered for regions of recombination (using Gubbins[19]) and corrected for ascertainment bias[20]. Green arrows indicate strains used to vaccinate the fish on the different farms whilst red arrows indicate strains subsequently isolated from vaccinated fish in which disease had reoccurred. The capsular serotype, defined in most of these cases by mutations in *cpsG*, which controls glucose : galactose ratio in the surface polysaccharide, is indicated by blue circles. The extended branch length supporting the cps defective isolates (white circles) from vaccinated fish in NSW and SA is indicative of a much faster nucleotide substitution rate in these strains and evidence that they are likely mutators. This is supported in (B), which shows experimentally determined mutation rates for these isolates (red) and other isolates from the same farm (blue). Taken together this is supportive of adaptation by both standing and de novo variation with a role for mutators in the latter.

vaccines is substantial and has been used to great effect in human medicine[30]. A similar approach is ongoing for aquatic isolates, and surface expressed proteins unique to aquatic isolates but conserved across the ST260 and ST261 sequence types have been identified and tested for efficacy in preliminary trials in tilapia.

Whilst large-scale whole genome sequencing is identifying antigens conserved across the most important serotypes, there are a number of further problems that must be resolved for viable streptococcal vaccines for aquaculture. First, fish are a low value commodity and even in salmon, which fetch a relatively high wholesale price, margin per dose of vaccine is low relative to other animal vaccines, except poultry. Consider that the farm gate price of most warm water species is substantially lower than salmon, with tilapia valued at less than one third of the lowest salmon price, and one can envisage that the cost per dose of vaccine has to be very low indeed. Whilst there is some margin in simple formalin-killed bacterins, recombinant protein vaccines are not economically viable in this market. Therefore, maximising expression of conserved antigens, identified through genomics, in culture for improvement of killed bacterins makes more commercial sense. The second problem relates to adjuvants. The success of vaccination in cool water salmonid aquaculture was founded upon oil emulsions that enable a single vaccination to protect for the complete farm lifecycle. This duration of immunity in excess of two years necessitates very slow antigen release from the emulsion. This works against warm water species that are farmed for maybe 9–12 months in the case of tilapias, particularly for streptococcal vaccines, where achieving an effective antigen dose against non-carbohydrate antigens is already challenging due to very low growth densities of aquatic streptococci. This will necessitate clever formulation of vaccines to enable initial fast antigen release, but also sustained protection for several months,

and all at a price of a few cents per dose. This represents a substantial challenge for the industry and the science.

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Biographies

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Testing the efficacy of probiotics for disease control in aquaculture

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Infectious diseases have been estimated to cost the global aquaculture industry billions of dollars annually[1,2]. With concerns over emerging resistance and residues of antibiotics in food[3] many such chemicals are now being banned and environmentally friendly alternatives are being sought. Probiotics influence the composition of the gut microbiota and confer health benefits to their host[4,5] and are one of several alternative approaches gaining significant popularity in aquaculture. Whilst primarily used to manage bacterial disease, there is also some evidence that probiotics can provide protection against parasites[4] and viruses[6]. Probiotics can inhibit the growth of pathogens in the gut through the excretion of antagonistic substances including bacterocins[6,7]; prevent pathogen adhesion in the gut through competition of space and nutrients[3] and by modulating the immune system[5]. Some probiotics have been reported to improve growth and feed utilisation efficiency[5] and others can also improve water quality[2,8,9], which confers indirect benefits to host health[6].

It has been estimated that 50 000 tonnes of probiotics are used annually in the aquaculture industry[10] yet analysis of the literature reveals a great deal of equivocal data on their efficacy. This is likely due, at least in part, to the wide diversity of both hosts and probiotic species within this industry and the fact that probiotic efficacy against particular pathogens is often both host specific and probiotic strain specific[5]. These factors demonstrate the need for testing to ensure probiotics are fit for purpose. Aquaculture species span many phyla and their probionts are far more diverse than the typical lactic acid bacteria (LABS) used in terrestrial animals[6] and which do not dominate in the normal gut flora of aquatic animals[2].

The diversity of probiotics used in aquaculture is highlighted by Newaj-Fyzul *et al*. who reviewed 18 genera of Gram-negative and 19 genera of Gram-positive bacteria that have been used in aquaculture[4]. This diversity reflects that of the aquatic habits where aquaculture species live and closely interact with these microbes[3,7].

Despite this diversity, *Bacillus subtilis* and *B. licheniformis* remain the most commonly used commercial probiotics in aquaculture[5], due to their spore forming nature and subsequent proclivity for long term storage and stability in formulated feeds[6,11]. Evidence exists, however, that probiotics isolated from host gut or their environment are more likely to outperform commercial products because they are more likely to colonise the host gut and effectively compete with pathogens[6,12]. The first stage of screening potential probiotics usually occurs *in vitro* and seeks to measure antagonism against pathogens of interest using methods including well diffusion, cross streaking and disc diffusion and the co-culture method[3,13]. These methods can also be used to test antagonism of putative probiotics against pathogens of interest. Whilst such methods allow cost effective, simultaneous screening of many probiotic candidates, several authors have pointed out the limitations of relying on *in-vitro* tests[3,7]. Given the many different modes of action of probiotics, a lack of *in vitro* antagonism does not necessarily exclude the bacteria as a probiotic. Conversely, it has also been demonstrated that the expression of antagonism *in vitro* does not guarantee that a candidate will perform effectively *in vivo*[2,6].

Administration of the probiotic to the host and measuring *in vivo* performance is therefore critical and indeed Kesarcodi-Watson *et al.* advocates for the use of *in vivo* testing in the preliminary screening phase to prevent exclusion of those probiotics which do not exhibit antagonism but which may still be effective based on other modes of action[3]. Whilst probiotic administration for such *in vivo* testing is usually achieved in dry feed, in hatchery applications probiotics can be delivered using live feeds as delivery vectors to the larval host or via the culture water. Application via culture water can also be effective in ponds[8].

Measurement of effectiveness *in vivo* can be achieved in several ways. As effective probiotics should colonise and thrive in the host gut[5,7], the first step can therefore simply involve confirming such colonisation and indeed not all commercial strains of probiotics originally developed for terrestrial animals thrive in the gut

of aquatic species[5]. Gatesoupe, however, points out that colonisation is not essential and that transient bacteria may also make effective probiotics if they can be provided continuously at high doses[6].

Challenging the host with a live pathogen of interest following probiotic administration is the most effective method of determining a probiotic's efficacy. Whilst such tests do not elucidate the mode of action of the probiotics, they are the most relevant in proving efficacy against pathogen-induced mortality. Appropriate pathogen dosages must be selected to ensure the probiotic effect can be detected without completely overwhelming the host. Preliminary testing should therefore be conducted to determine an appropriate LD_{50} and an appropriate route of administration for the pathogen of interest (for example bath immersion versus injection). Care must also be taken to ensure the virulence factors of the pathogen are not lost in culture prior to administration. When biosecurity or animal ethical considerations prevent challenge trials using live pathogens, administration of inactivated pathogens or pathogen-associated molecular patterns (PAMPS) may elicit measurable immune responses that indicate probiotic efficacy. Conversely, live pathogens can be used *in vitro* to measure immune response in certain host tissues. Many options exist for measuring the immune response in the host following probiotic application. These range from simple assessments of basic haematological parameters[11] to the measurement of factors such as lysozyme and serum bactericidal activity, immunoglobulin levels, phagocytosis and respiratory burst activity to more complex methods such as quantifying gene expression profiles for inflammatory markers such as cytokines[5].

Determining host-specific and strain-specific optimum probiotic dose and treatment duration are also key elements that requires *in vivo* testing to ensure optimum efficacy[12,14]. Effective in-feed dose rates generally range from 10^{6-10} CFU/gram of feed and in water treatments from 10^4 to 10^5 CFU/mL, with excessive doses shown to cause deleterious effects in the host[5,8]. For most species for which probiotics have proven effective, improved immunity has been detected 1 to 10 weeks following commencement of treatment, however, extended use can also be detrimental[3].

Finally, testing probiotics for safety, both for the host and the human consumer, is also very important. This is particularly relevant in the field of aquaculture where many probiotics being considered for use have closely related strains that are known pathogens. Testing for safety should therefore include confirmation of non-transmission of antimicrobial resistance genes or virulence plasmids, as well as using molecular techniques to confirm with certainty the identity of the species under investigation[15].

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Biography

Dr Gavin Partridge is the Principal Research Scientist at the Australian Centre for Applied Aquaculture Research and an Adjunct Associate Professor at the School of Veterinary and Life Sciences at Murdoch University. He conducts applied research to aid the development of a marine fish aquaculture industry in Western Australia and has a research interest in the role of nutrition in improving health and immune function in fish.

Aquaculture: exotic diseases and surveillance

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Aquaculture is a rapidly growing global industry. Half of all seafood is sourced from aquaculture and Australia is part of the trend. A major emerging threat to this industry is disease.

Australian aquaculture production in 2012–13 was valued at >$1 billion with farmed salmonids alone contributing $497 million[1]. However, although only six species (pearl oysters (*Pinctada maxima*), Atlantic salmon (*Salmo salar*), Pacific oysters (*Crassostrea gigas*), prawns (*Penaeus* spp.) and southern Bluefin tuna (*Thunnus maccoyii*)) account for 90% of the production, there are some 40 species under cultivation. A characteristic of Australian aquaculture is that, with a few exceptions, all of the species under cultivation are Australian native animals, the main exceptions being salmonids, introduced from Europe in the 1860s[2,3] and Pacific oysters (*Crassostrea gigas*) introduced[4] between 1947 and 1970. Farming native species provides two unique challenges. First, for most of the species under cultivation there is no previous aquaculture experience, and second, as culture intensifies, diseases that are unique to Australia are emerging as a threat to production. Adding to the mix are those disease agents that have either been accidentally introduced and are emerging as a threat to native species or those diseases still offshore that pose a threat to Australian flora and fauna. Because of the intensification of both aquaculture and global trade, diseases are now spreading at a faster rate than regulatory process can respond. This spread has been exacerbated by inadequate biosecurity measures on many farms, though that is changing slowly[5,6].

An example of the slow regulatory response is provided by koi herpesvirus. This highly contagious virus affects only common carp (*Cyprinus carpio*) and carp hybrids. Affected fish die between 24 and 48 hours after the initial onset of gill lesions and mortality may exceed 90%[7]. Survivors can act as carriers. Common carp are raised as food in many countries and koi carp are a component of the ornamental fish trade. First identified in fish farms in Israel in 1998, the disease spread globally for about 8 years before the World Organisation for Animal Health added koi herpesvirus to the list of internationally notifiable diseases. Australia has remained free of this disease due to the prohibition on importing carp, but research is underway to release the virus in an attempt to control invasive feral carp[8].

The detection of potentially exotic diseases in Australian aquaculture farms is facilitated by surveillance, of which there are two types: passive surveillance, which relies on detection of disease signs on farm and a prompt robust system to acquire a diagnosis; and targeted surveillance. Targeted surveillance is intelligence-led and risk based – looking for specific diseases of concern which may establish in specified high risk areas. An Australian example that illustrates both types of sampling is provided by White Spot Syndrome Virus of crustaceans. The disease is exotic to Australia but was detected by passive surveillance (through investigating mortalities) at a hatchery in Darwin. The hatchery was destocked and a nationwide targeted surveillance program was instituted, sampling aquaria and hatcheries where imported frozen prawns (the source of the infection) might have been fed to crustacean brood stock or wild populations. The survey results were negative, allowing Australia to retain its free status[9].

A more complex example is provided by studies of the molluscan parasites informally grouped as 'microcells' because of their small size (about 2 microns). The genera *Microcytos* and *Bonamia* are relatively easy to detect by histology but species determination is much more problematic. It was by histology and transmission electron microscopy that *Bonamia exitiosa* with cells of 2–5 µm was found in New Zealand Foveaux Strait oysters (*Ostrea chilensis*) in 1986. A related parasite, *Mikrocytos roughleyi* later renamed *Bonamia roughleyi* was described from *Saccostrea glomerata* in southeastern Australia in 1988[10]. Unlike *B. exitiosa* it causes lesions in the host and has smaller cells of 1–3 µm. Subsequently *Bonamia* sp. a molluscan parasite of Australian flat oysters (Ostreiidae) was reported from Australia in 1991[11]. It has cells of the same size (2–5 µm) as the New Zealand *B. exitiosa* but there are minor differences in morphology, ultrastructure and histopathology between the New Zealand and Australian microcells[12]. However, DNA sequencing has shown that, despite the differences, *Bonamia* sp. and *Bonamia exitiosa* are both members of a *B. exitiosa* clade,

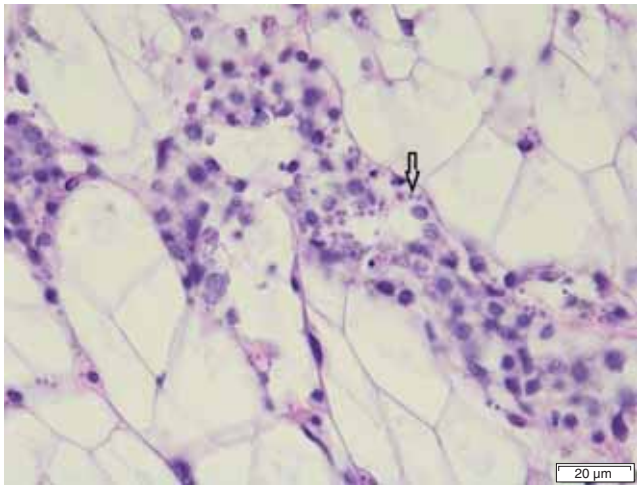

Figure 1. Heavy infection of oyster *Ostrea chilensis* with *Bonamia exitiosa* microcells (arrow), found in haemocytes and free in haemolymph spaces.

and that *B. roughleyi* is a *nomen dubium*[13]. Thus, *Bonamia exitiosa* is no longer regarded as an exotic disease in Australia (Figure 1).

Our understanding of pathogens themselves is also changing. Now that the DNA of disease agents can be sequenced it is much easier to not only detect incursions but also the genes that confer virulence. This adds a new layer of complexity on surveillance, since it's not just the organism that must be detected, but the arrival of more virulent forms of a disease perhaps already well established but tolerated. Herpesvirus-like viruses, associated with mortalities in bivalve hatcheries and detected by histology and transmission electron microscopy in bivalve shellfish, had been recorded in New Zealand, USA, Europe, and in Western Australia in the 1990s[14]. Subsequently, a micro-variant strain of a herpesvirus (named OSHV-1) emerged in France in 2008 that caused high mortalities only in *Crassostrea gigas*[15]. The relationship between the micro-variant strain now detectable by PCR and the herpesvirus-like virus seen in earlier studies by TEM has never been established. The micro-variant form of OSHV-1 spread through European oyster farms, appeared in New Zealand in 2010 and then a few months later in the Georges River, NSW in November 2010[16]. Identification of the herpesvirus by molecular methods, and identification of the characteristic deletions in the genome, confirmed that it was the microvariant strain that was causing the deaths[16]. A strict imposition of biosecurity controls by the NSW government appeared to limit the geographical spread of infection, but in 2016 the microvariant was also detected in Tasmania following mortalities.

In response to the growing threats posed by emerging diseases, attention is moving from a reliance on country border protection to a much greater emphasis on farm biosecurity. This is accompanied by a greatly increased awareness of the need for both passive and active surveillance, not only at the state level, but also at the level of the individual farm.

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Pacific oyster mortality syndrome: a marine herpesvirus active in Australia






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Genotypes of *Ostreid herpesvirus 1* (OsHV-1) known as microvariants cause the disease Pacific oyster mortality syndrome (POMS). Since its appearance in NSW in 2010, OsHV-1 microvariant has prevented the farming of Pacific oysters (*Crassostrea gigas)* in the affected estuaries near Sydney, following the initial massive outbreaks[1,2]. The arrival of the disease in southeast Tasmania in January 2016 has put the entire $53M industry in Australia in jeopardy[3]. The virus is a member of the Family *Malacoherpesviridae*[4], which includes several invertebrate herpesviruses. The OsHV-1 genome consists of 207 439 base pairs, with organisation similar to that of mammalian herpesviruses. However, OsHV-1 contains two invertible unique regions (U_L, 167.8 kbp; U_S, 3.4 kbp) each flanked by inverted repeats (TR_L/IR_L, 7.6 kbp; TR_S/IR_S, 9.8 kbp), with an additional unique sequence (X, 1.5 kbp) between IR_L and IR_S[4]. Unlike many herpesviruses which are host specific, OsHV-1 strains have been transmitted between marine bivalve species[5] and the virus is transmitted indirectly. The virus may have relatively prolonged survival in the environment, has extremely high infection and case fatality rates, and latency is unproven. Along with pilchard herpesvirus[6–8] and abalone ganglioneuritis virus[9,10], it is part of a dawning reality that marine herpesviruses are among the most virulent of pathogens. Finding solutions for industry requires more than laboratory-based research.

In 2008, the microvariant genotype OsHV-1 µVar[11] emerged as a dominant isolate against a background of prior endemic OsHV-1 strains in France, and has devastated the *C. gigas* industry there. Similar microvariant genotypes of OsHV-1 have since appeared throughout Europe, in New Zealand, and in Australia[1], with similar devastating impact (Figure 1). In 2010–11 when an emergency response was required in Australia, almost no information existed on the epidemiology of OsHV-1 infection, and the only management responses internationally that could be drawn from were to develop resistant oysters through selective breeding approaches. In Australia, a breeding program commenced immediately but it was complemented by research to identify mechanisms of viral transmission and environmental triggers, in order to develop husbandry recommendations to mitigate losses.

Figure 1. Since its appearance in NSW in 2010, OsHV-1 microvariant has prevented the farming of Pacific oysters (*Crassostrea gigas*) in the affected estuaries near Sydney. Dead oysters in a tray after an OSHV-1 mortality event.

Figure 2. Oyster farmer Len Drake with a tray of experimental oysters to be placed in the Georges River to study the transmission of the disease.

Oysters are often grown in the intertidal zone. Replicated experiments were conducted in the Georges River estuary near Sydney in summer 2011–12 and 2012–13, to determine whether immersion time in seawater influenced viral exposure and mortality in different age classes of oyster (Figure 2). The findings were remarkably consistent. Reduction in immersion time by about 2 hours per tide cycle led to 50% reduction in mortality in valuable adult oysters[12,13]. Furthermore, assumptions about uniform seawater exposure to virus were shattered by observations of highly clustered infection and mortality patterns at scales of 1 km to a few cm. This was consistent with distribution patterns of estuarine plankton[14]. Accurate counts of thousands of live and dead research oysters placed in multiple locations in successive summers led to a hypothesis: that OsHV-1 may be carried in plankton[15].

Most of the Pacific oysters grown in Australia are produced in Tasmania in large commercial hatcheries, certified free from diseases, and then shipped to farmers in New South Wales, South Australia and Tasmania to be grown and marketed. For this reason

Figure 3. Olivia Evans collecting water samples to test for OsHV-1.

the occurrence of OsHV-1 in Tasmania was predicted to be a massive risk to the entire industry. Based on the idea that OsHV-1 was probably carried on organic or inorganic particles with the plankton, experiments were conducted to prevent mortality by filtering seawater or aging it by sedimentation to remove the causative agent. Filtration would not have been an obvious solution for a viral aetiology were it not for the epidemiological observations, and evidence that testing a pellet derived from low speed centrifugation of seawater improved detection of the virus[16] (Figure 3). Controlled experiments on these water treatments were successful[17] and the results were taken up by the hatcheries. In January 2016, when POMS emerged in Tasmania, one hatchery had appropriate water treatment in place and did not experience mortality in its stock while a second had not completed its water treatment program and was severely affected by the disease.

The state jurisdictions implemented quarantine controls over affected estuaries in both NSW and TAS and this has been very effective in slowing the spread of the disease. This has been accompanied by research to confirm appropriate disinfection protocols. Surprisingly, OsHV-1 remained viable for at least a week within dried oyster tissues, such as those that might adhere to plastic farm trays and baskets in which oysters are grown. Disinfection of such material with high concentrations of chlorine was ineffective. However, quarternary ammonium compounds were effective[18]. Biosecurity controls apply only to oysters and oyster farming equipment, leaving many potential pathways for spread of the virus. Pleasure boats, commercial shipping, and oceanic currents may all play a role in international and regional spread of aquatic pathogens.

Pathogen distribution and disease expression may be favoured by environmental conditions. POMS occurs in Sydney estuaries when water temperature is ~22–26°C[1,2]; this is ~5°C higher than in France[19,20]. An experimental infection model has been

developed and used in Australia to confirm these field observations on water temperature; it revealed an important interaction between water temperature and infectious dose[21,22]. As part of FRDC funded research we have established water temperature monitoring on oyster leases in all major *C. gigas* farming areas in Australia to provide insight into a very dynamic thermal environment. The aim is to develop a risk management and early warning system.

Over time a combination of biosecurity, husbandry and genetic approaches will need to be applied to continue farming Pacific oysters in Australia.

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Biographies

Richard Whittington, PhD, FASM, is Professor of Farm Animal Health. **Paul Hick**, PhD, is Senior Lecturer in Veterinary Virology. **Olivia Evans**, BAnVetBioSc, is a final year PhD student. **Ana Rubio**, PhD, is a marine scientist. **Navneet Dhand**, PhD, is Associate Professor in Biostatistics and Epidemiology. **Ika Paul-Pont**, PhD, is Research Fellow in Environmental Immunology. Their studies combine the disciplines of virology, epidemiology, pathobiology and marine science to discover weaknesses in disease biology that can be exploited for disease control and prevention in aquaculture and fisheries.

Identification of bacteria from aquatic animals

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A wide range of aquatic animal species are cultured for human consumption, the fashion industry, research purposes or re-stocking natural populations. Each host species may be colonised by bacterial saprophytes or infected with pathogens that have specific growth requirements encompassing temperature, salinity, trace elements or ions. To ensure successful culture and identification of potential pathogens, the microbiologist must have in-depth knowledge of these growth requirements and access to the appropriate resources. Identification techniques include traditional culture and biochemical identification methods modified to take into account any growth requirements, identification using mass spectrometry, detection of nucleic acids, sequencing 16S rRNA or specific genes, and whole genome sequencing.

More than 70 aquatic host species ranging from finfish, crustaceans, bivalves, amphibians, algae and corals are grown throughout the world for either human consumption, the fashion industry, food for aquacultured hosts, research or re-stocking of natural populations. Each host species has saprophytic and pathogenic bacteria that may have specific growth requirements, and the microbiologist must have the appropriate knowledge and access to resources to enable their successful culture and identification.

Standard bacteriological procedures similar to those performed for the isolation of bacterial pathogens from human and terrestrial animals are used with modifications that take into account growth requirements for temperature, salinity, seawater salts/ions, nutrients or growth factors. As a general rule samples from freshwater are cultured to blood agar plates, whereas those from marine sources are cultured to blood agar containing 2% NaCl final concentration[1]. The majority of bacterial pathogens from aquaculture samples are incubated at 23–25°C.

Many bacteria have specific growth requirements. For example, *Tenacibaculum* (*Flavobacterium*) *maritimum*[2], has an absolute requirement for ions present in seawater together with low nutrients and must be cultured on a minimal nutrient agar such as Anacker-Ordal[3] containing seawater salts (Sigma). *Flavobacterium psychrophilum* has an optimum temperature range of 15–20°C, variable growth at 25°C and no growth at 30°C[4]. Samples from brackish waterways such as coastal rivers and estuaries may contain a mix of freshwater and marine bacteria, and therefore, should be cultured to media with and without NaCl. Likewise, samples from marine mammals may also require the use of culture media with and without NaCl, as these animals typically harbour members of the *Enterobacteriaceae* as well as bacteria of marine origin. It may be prudent to incubate duplicate sets of media at 25°C and 37°C.

Microbiologists must be aware of pathogens exotic to Australia and New Zealand, as some of these such as *Renibacterium salmoninarum*[5], a slow growing Gram-positive rod that has an absolute requirement for cysteine and a temperature range of 15–17°C, will not be detected using 'general culture' conditions.

Phenotypic identification of a majority of bacteria from aquatic animals is achieved using conventional biochemical test methods that include carbohydrate fermentation, enzyme hydrolysis or carbon utilisation[6], and comparing the results to designated Type strains and well-characterised strains[1,7]. A number of bacteria from the marine environment generally will grow in physiological conditions, produce virulence factors and cause disease, however may not fully express all enzymes or biochemical reactions normally associated with their identification profile. These bacteria must be grown in biochemical identification media at their optimal conditions for temperature and salinity[1,7]. A number of bacteria including *Vibrio* species, *Photobacterium damselae* and *Yersinia ruckeri* produce different biochemical reactions, especially enzyme

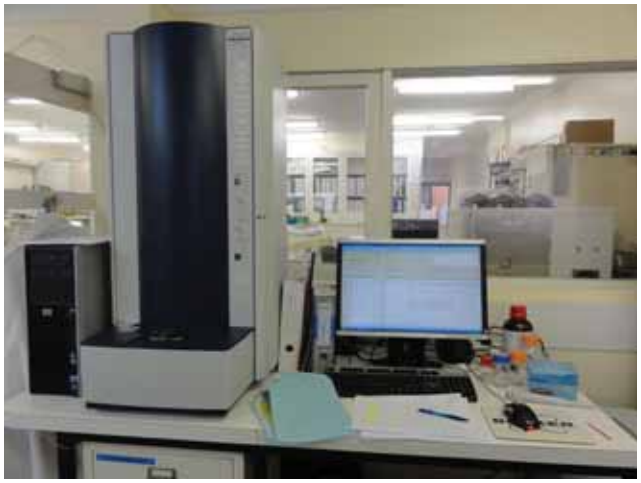

Figure 1. MALDI-TOF (Bruker Daltonics).

reactions at 25°C compared to 37°C and at 0.85% NaCl compared to 2% NaCl[8,9].

Some bacteria infecting aquacultured species are also zoonotic and both medical and veterinary laboratories must be aware of these bacteria that can infect wounds in people handling aquatic animals or cause food poisoning by their consumption[10]. Misidentification can result if these bacteria are not grown at their optimal salinity and temperature[8,9].

Many laboratories do not have their own media preparation laboratories, but rely on commercially available media and identification systems such as the API kits (Biomerieux). The kits are designed for bacteria from medical sources and their interpretation for bacteria from aquatic animals must be used with caution and results interpreted using a reliable database[1]. Other bacteria such as some of the genera within the *Flavobacteriaceae* family are difficult to identify by phenotypic means and the specialised media required for many bacteria means that some laboratories may lack the resources to culture such organisms.

Advances in phenotypic identification have seen the advent of matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometry (Figure 1) and to some extent this technology overcomes the constraints of the traditional methods of identifying bacteria based on the biochemical pathways of the cell. Mass spectrometry still requires the organism to be cultured, however, the identification can be performed on individual colonies growing on the primary plate. A bacterial colony is smeared within a well on a target plate and the cellular proteins are released using formic acid and a matrix solution of saturated alpha-cyano-4-hydroxycinnamic acid containing acetonitrile and trifluoroacetic acid. The high abundance ribosomal proteins between 2–20 kiloDaltons are analysed within 1–2 minutes. Identification is achieved by pattern matching of the generated protein peaks against a stored library containing spectra for 6000 species. The intensity is correlated and used for ranking results. Thus, an assigned score of >2.0 identifies the bacterium to species level, whereas a score of 1.7–2.0 identifies to genus level only (Bruker MALDI biotyper instrument, Bruker Daltonics). Although mass spectrometry has been available for protein analysis for over 50 years, it is the bioinformatics that has enabled the technology to be applied to the identification of bacteria, yeast and fungi. Only true fungi are present in the database. Oomycete fungi such as *Aphanomyces* and *Saprolegnia* that are pathogens or saprophytes in aquatic animals are not in the commercial databases at present. MALDI-TOF technology has been used for the separation of species and subspecies, strain typing, and the detection of antibiotic resistant genotypes[11,12].

The MALDI-TOF database contains 51 of the 121 described *Vibrio* species, 21 of the 45 described *Aeromonas* species, and many other genera and species in the family *Enterobacteriaceae* including the exotic fish pathogen *Edwardsiella ictaluri*. The database also contains aquatic species from the genera *Flavobacterium* and *Tenacibaculum* (*T. discolor* and *T. ovolyticum*) but not the pathogenic species *T. maritiumum*, *Flavobacterium columnare* or *F. psychrophilum*. *Carnobacterium maltaromaticum*, a cause of pseudokidney disease in salmonids. *Vagococcus fluvialis* and *V. lutrae* are present but not the pathogens *V. salmoninarum* or *Renibacterium salmoninarum*. The database of 69 *Streptococcus* species does not include the human and fish pathogen *Streptococcus iniae*. Although MALDI-TOF identification of some bacteria is very robust, it can be unreliable for certain genera such as *Vibrio* and *Aeromonas*, and like all aspects of microbiology, the microbiologist must be aware of the limitations. The recommendation is for all unknown isolates to be tested in duplicate on the target plate. For many of the *Vibrio* and *Aeromonas* species, the same score can be obtained for duplicate spots (unpublished data) and biochemical testing or specific PCR must be done. The database tends to be more robust for human pathogens or where there are multiple strains in the database[13]; however, this will improve as more strains are added.

Molecular identification of many aquatic pathogens can be problematic with published PCRs for specific pathogens often cross reacting with closely related species (unpublished data). In the case of *Vibrio* and *Aeromonas* species, clonal groups or clades within these genera are so similar that at least seven housekeeping genes must be sequenced and concatenated to determine phylogenetic differences[14,15]. The advent of next generation sequencing technology is likely to overcome some identification problems, but at present costs are high, data storage is problematic, and the volume

of data generated requires significant time to process and analyse; however, like all technology, these drawbacks will be minimised as the technology advances.

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Biographies

The biography for **Dr Nicky Buller** is on page 103.

Sam Hair is a Microbiologist and Research Officer in the Bacteriology and Molecular Biology laboratories at Animal Health Laboratories, DAFWA. Sam is currently undertaking a PhD investigating the role of bacteria in the nutrition of post-larval abalone. Sam and Nicky work closely with pathologists and scientists at the Fish Health Unit, Department of Fisheries WA, which is co-located at DAFWA.

Common pathogens found in yellowtail kingfish *Seriola lalandi* during aquaculture in Australia

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Yellowtail kingfish aquaculture in sea-cages is an emerging industry in Australia. Monogenean, myxozoan and bacterial pathogens sometimes cause health issues that require diagnosis or monitoring.

Yellowtail kingfish *Seriola lalandi* aquaculture in Australia is in its infancy with just one commercial farm in South Australia at the present time. Currently there is also interest in sea cage aquaculture of this species in Western Australia and New South Wales. The natural range of this species is from Queensland right around the south of the mainland and as far as the mid-west of Western Australia. The fish is able to spawn and grow in captivity, is very fast growing and is a firm white fleshed fish which is especially popular for sashimi.

There have been several mortality events during aquaculture of this species. The fish are generally quite robust when held in tanks on land but once they are exposed to the extra stressors associated with sea cage culture they are more likely to have periods of reduced growth and spikes of mortality. The cause of these events can be difficult to identify and are often suspected of being multifactorial in nature with various stress factors being identified and opportunistic pathogens in some but not all of the fish. Bacteria such as *Vibrio harveyi*, *V. alginolyticus*, *Photobacterium damselae* subspecies *damselae* or a *P. damselae* subspecies *piscicida*-like bacterium are sometimes isolated from the spleen and kidney of moribund fish. At times the bacteria appear to have caused a chronic hepatitis, cholangitis and pancreatitis from an ascending infection from the intestine. At other times the pseudotubercular lesions, reported in *P. damselae* subspecies *piscicida* infections overseas[1], can be seen grossly in yellowtail kingfish in Australia. A deficiency in taurine due to feeding of incompletely formulated, manufactured fish pellets is one factor shown to predispose fish to ill thrift and subsequent infections resulting in mortalities. Other possible stress factors which may lead to immunosuppression in the fish could include low dissolved oxygen events, the presence of predators such as sharks, birds or seals around the cage and extremes of water temperatures outside the normal expected range for the species.

Monogenean parasites can have a severe detrimental impact on yellowtail kingfish in sea cages and continual monitoring of the fish to determine the intensity of infestation with these parasites is required. The main skin parasites of concern are the capsalid monopisthocotyleans *Neobenedenia* sp. (pers. comm., ID Whittington, South Australian Museum) (Figure 1) and *Benedenia seriolae*[2]. These are both tissue grazing parasites that are extremely irritating to the fish and fish become inappetant when parasite numbers are high. The blood feeding polyopisthocotylean parasite *Zeuxapta seriolae*[2] (Figure 2) occurs on gills and can cause severe anaemia and death if infestations are not managed appropriately.

Fish in sea cages become infected from wild fish in the waters around the sea cage. Most of the monogenean parasites infecting yellowtail kingfish are hermaphrodites that have a direct life-cycle and tanned eggs that are resistant to chemical treatments. The eggs may have

Figure 1. *Neobenedenia* sp. in a wet preparation. The parasite is very irritating to the fish and attaches to the fish by paired hooks on the haptor. Bar = 500 µm.

Figure 2. Wet preparation of *Zeuxapta seriole* demonstrating the clamps on the haptor that attach to the gill. Developing eggs can be seen in the parasite on the right. Bar = 500 µm.

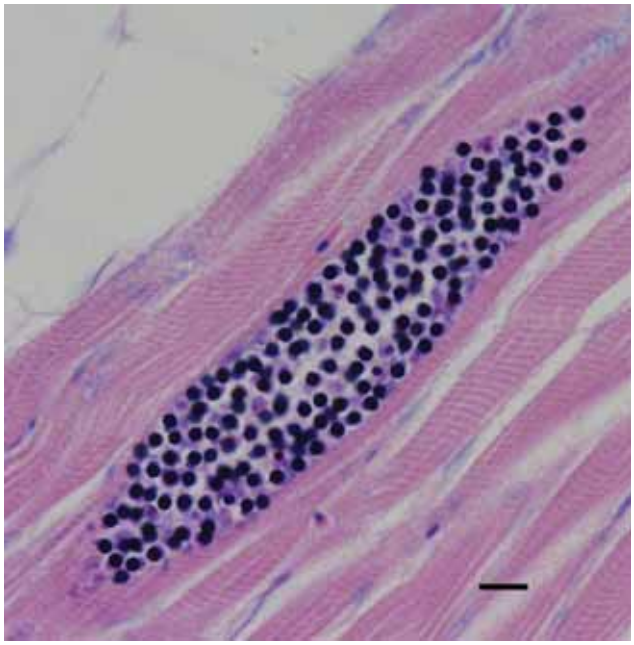

Figure 3. *Unicapsula seriolae* spores in skeletal muscle. Each spore has a single polar capsule. Giemsa. Bar = 10 µm.

a filament that becomes entangled on structures around the cage such as the net and moorings. Individual parasites can produce large numbers of eggs daily when water temperatures are suitable. *Zeuxapta seriolae* is particularly fecund[2]. One management strategy is for nets to be regularly cleaned or changed to reduce the number of monogenean eggs present in the sea cage environment. This also removes fouling organisms that reduce water flow through the cage. Parasite infection intensity is monitored by sampling live fish from the sea cage and placing the fish in a diluted praziquantal treatment bath. The parasites detach from the fish and can be counted before the fish is returned to the cage. Chemotherapy using hydrogen peroxide or paraziquantal baths is labour intensive as the sea cage must first be surrounded by an impervious tarpaulin. Then the oxygen in the bath water must be monitored and the static water aerated to ensure the fish have access to sufficient oxygen. The treatment dose must be calculated accurately and then well dispersed through the bath water. Timing is critical in these bathing processes and must be aligned with the lifecycle of the monogenean being treated.

A number of tissue dwelling myxozoan parasites have been observed infecting yellowtail kingfish that are confined in sea cages. Of these, *Unicapsula seriolae* (Figure 3) has the greatest potential to impact the industry due to the parasites' ability to cause unacceptably soft or liquefied flesh when high numbers of spores are present in the skeletal muscle[3]. It was first identified in wild fish in Queensland and also occurs in Western Australia[4]. It can infect yellowtail kingfish in sea cages that are in relatively shallow water close to the probable habitat of its intermediate host[5]. At the present time it does not appear to be a major impediment to the aquaculture of yellowtail kingfish. *Unicapsula seriolae* has not been identified as a problem in South Australia[6].

Several other myxozoan parasites have been seen in aquaculture operations but their presence has not been definitively linked with major morbidity or production loss in Australia. In one locality in Western Australia *Kudoa neurophila* was highly prevalent in the brains of yellowtail kingfish[5,7]. These fish had been spawned and reared in a nearby hatchery that used water pumped from the adjacent harbour where their likely invertebrate intermediate hosts (e.g. polychaete worms) were abundant. It is suspected that the fish became infected in the hatchery but the presence of infection did not present with pseudocysts in the brain until after the fish were stocked into sea cages. This parasite has not been seen in other hatcheries or yellowtail kingfish in aquaculture in Western Australia but did occur in striped trumpeter in a hatchery in Tasmania. The incoming water at that hatchery was infected with the infective stage of the parasite[8].

Other potential pathogens include myxozoans in the epicardium and in the lumen of kidney tubules in some fish. The *Kudoa* species in the heart is similar to *K. pericardialis* that occurs in Japan[9]. Uncharacterised Apicomplexan parasites have also been seen in the intestines of fish with ill thrift. Blood flukes of the Aporocotylidae were identified as a potential risk factor for yellowtail kingfish aquaculture in Australia[6] and have occurred in sea cage aquaculture in South Australia.

In summary the Monogenea have been the most troublesome pathogens of yellowtail kingfish culture in Australia. Their

management requires regular monitoring of the intensity of infestation together with time consuming and logistically demanding therapeutic bathing at strategic times. Other pathogens cause sporadic problems that require management on a case by case basis.

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Biography

Fran Stephens is a Fish Pathologist at the Fish Health Unit of the Department of Fisheries in Western Australia. She has an interest in diseases of aquatic animals and the management of aquaculture production units.

A simple summary of ASM Finances

The audited financial statements are available on the website, but, as required, they amalgamate all the financial activities of the society. This brief summary is provided to give members more insight into the individual areas that make up ASM finances.

1. The annual scientific meeting
The meeting held in Canberra in 2015 was a scientific, social and financial success, resulting in a final profit of $50 000, 10% of which is retained by the NSW branch.

2. State branches
Most states have financial holdings representing five years of capitation payments, therefore enabling them to organise most events without needing to charge members or obtain outside sponsorship. In total the branches hold a total of $250 000.

3. ASM
(i) Day-to-day operations:
99% of our income of $262 000 is derived from membership fees.
Capitation payments to the states of $52 000 represent 20% of this amount.
Expenditure of $190 000 is due mainly to payment for membership services provided by ASN ($96 000) and the production of *Microbiology Australia* ($45 000). Sponsorship of other meetings and membership of STA account for $9000 and $14 000 respectively.

(ii) Share portfolio:
The society has a share portfolio with a value of approximately $500 000, comprising seven holdings of comparatively equal value in the following companies: AGL, ANZ, BHP, CBA, CSL NAB and Westpac. The annual yield of approximately $24 000 is reinvested to maintain the value of the portfolio.

4. Research trust
The Research trust holds funds to the value of $1 028 000. These are held in capital notes and preference shares in CBA (50% of holding), ANZ, Macquarie, NAB (10% each) and the residual 20% in Bendigo Bank, BOQ and Westpac. These holdings yield an annual income of $47 500, which is used to pay all the society's awards. Any residual funds are reinvested.

The society's finances are responsibly and competently managed by Bree Knights and Kerrie Harris-Spencer at ASN, Tammy Currie at FAME, Rollo Morgan at Morgan's financial services and Eric Townsend (Auditor).

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Pseudomonas anguilliseptica infection as a threat to wild and farmed fish in the Baltic Sea

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The transport of live fishes related to the growth of the fish farming industry worldwide may increase the transfer of previously known bacterial pathogens into new geographic areas and new host species, but also facilitate the introduction of completely new bacterial pathogens. Species belonging to the genera *Vibrio* and *Aeromonas* are well known in many countries, infecting a large number of fish species. Other bacterial fish pathogens like *Pseudomonas anguilliseptica* species, up to now considered less harmful, may constitute a potential threat to a developing fish farming industry, especially of European whitefish.

Pseudomonas anguilliseptica is a fish pathogenic bacterium infecting mainly farmed fish in brackish and marine environments. The pathogen was initially reported from diseased farmed Japanese eel (*Anguilla japonica*) in Japan in 1971, and named 'sekiten-byo' or red spot disease[1]. Since 1981, *P. anguilliseptica* has also been isolated from cultured European eel (*Anguilla anguilla*) in different European countries[2–5]. Although *P. anguilliseptica* was initially considered a pathogen closely associated with eel culture it appeared that this pathogen infects a number of different fish species in different water areas. It has been isolated from farmed fish species, like ayu (*Plecoglossus altivelis*)[6], cod (*Gadus morhua*)[7], gilthead seabream (*Sparus aurata*)[8], sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*)[9], striped beakperch (*Oplegnathus fasciatus*)[10] and recently from lumpsucker (*Cyclopterus lumpus*)[11].

The disease signs associated with *P. anguilliseptica* infections in fish are characterised by petechial haemorrhages in the peritoneum and in the skin on the ventral side of the fish (Figure 1). Sometimes haemorrhages are also present in the liver and adipose tissue of visceral organs. Occasionally spleen and kidney are soft in consistency and enlarged[1,12,13]. *P. anguilliseptica* has in some occasions caused significant mortalities in farmed eel[3,14] and Atlantic salmon[13] suggesting this pathogen has the potential to be a serious problem for farmed fish especially if left untreated.

P. anguilliseptica is an aerobic, motile, Gram-negative rod, producing slow-growing colonies on agar plates. The bacterium is cytochrome oxidase-positive, catalase positive and does not produce acid from glucose and has a low metabolic reactivity for many different carbohydrates. The inclusion of this pathogen into the genus *Pseudomonas* has been questioned, and it has been suggested that it could be classified into *Alcaligenes* or *Deleya* or even to a newly described genus[15].

In the Baltic Sea area in northern Europe, in the middle of the 1980s *P. anguilliseptica* has been almost simultaneously isolated from farmed European eel in Denmark[16], in Sweden (*Eva Jansson, pers. comm.*) and from different farmed salmonids in Finland (Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta* m. *trutta*), European whitefish (*Coregonus lavaretus*) and rainbow trout (*Oncorhynchus mykiss*))[13]. Subsequently, occasional disease outbreaks associated with *P. anguilliseptica* have been reported from farmed eel in Sweden (*Eva Jansson, pers. comm.*), although the number of eel farms in the Baltic Sea area were rather low during the past 20–30 years. Although in the northern Baltic Sea, initially *P. anguilliseptica* was isolated from several farmed salmonid species, it is today mainly associated with disease in European whitefish and to lesser extent with diseased rainbow trout. Both species are farmed in net pens in brackish water (salinity = 4–6‰). During 1986–1991, 2 to 17 disease outbreaks associated with *P. anguilliseptica* were recorded per year[17]. Lately, 2–5 disease outbreaks both in European whitefish and in rainbow trout have been recorded per year (T. Wiklund, unpubl. data)[18]. Initially *P. anguilliseptica* was often co-isolated with other bacterial fish pathogens like *Vibrio anguillarum*, and *Aeromonas salmonicida* subsp. *salmonicida*[17], suggesting a compromised immune system of the fish facilitating the infection with several bacterial species. Now the majority of farmed European whitefish and rainbow trout in Finland are vaccinated against vibriosis and furunculosis, but *P. anguilliseptica* is still causing disease outbreaks, mainly in European whitefish.

So far, Finland seems to be one of the few countries where *P. anguilliseptica* is causing disease outbreaks in farmed salmonids

Figure 1. Farmed European whitefish (*Coregonus lavaretus*) with *Pseudomonas anguilliseptica* infection showing haemorrhages in the skin and loss of scales.

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from marine and brackish water. In Sweden *P. anguilliseptica* was recently reported from diseased rainbow trout in freshwater (*Eva Jansson, pers. comm.*). Disease outbreaks in fish from freshwater associated with *P. anguilliseptica* are uncommon. *P. anguilliseptica* has previously been isolated from tilapia farmed in fresh water[19] and the bacterium seems to be present also in fresh water environment as reported from a river in India in rather high concentrations[20].

Reports of *P. anguilliseptica* in wild fish are rather rare in literature. The pathogen has been isolated from wild European eel[17] and from wild Atlantic salmon, sea trout and Baltic herring (*Clupea harengus membras*) with eye lesions in the Baltic Sea[21]. The Baltic herring suffered from haemorrhages in the eyes and in some specimens the cornea was punctured. Additionally haemorrhages in the fins and head and blood containing ascites were present. The isolates from Baltic herring were, however, of low pathogenicity for rainbow trout. The role of *P. anguilliseptica* as the etiological agent of the observed eye lesions in Baltic herring remained unsolved, and the authors concluded that the bacterium might have been a secondary invader[21].

Although *P. anguilliseptica* has been associated with disease outbreaks in different fish species in the Baltic Sea, the most significant impact today is on European whitefish. Infections with *P. anguilliseptica* have been treated with trimethoprim/sulfamethoxazole or florfenicol. Both antibiotics are efficient if the treatment is applied immediately in the onset of a disease outbreak. In contrast, oxytetracycline has been reported to be of limited effect against this pathogen[13]. In Japan and Scotland *P. anguilliseptica* infections in eels have been controlled by raising the water temperature temporary to above 27°C[3,22]. However, this procedure to treat the infection is not possible for salmonids.

It can be concluded that *P. anguilliseptica* seems to be present in different areas of the Baltic Sea. The pathogen has the potential to negatively impact future large scale farming of European whitefish and European eel in brackish water.

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Biography

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Disease threats to wild and cultured abalone in Australia

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Abalone species are important for recreational and commercial fisheries and aquaculture in many jurisdictions in Australia. Clinical infections with viral, bacterial and parasitic pathogens can cause significant losses of wild and cultured stock, and subclinical infections may result in decreased productivity and growth. Infections with abalone herpesviruses (AbHV), *Vibrio* spp. and parasites of the genus *Perkinsus* are of particular concern to Australian fisheries. Here we provide a brief overview of these three major pathogen groups and their diagnoses from an Australian perspective.

Perkinsus olseni

The protistan parasite *Perkinsus olseni*, was first described as a parasite of the abalone, *Haliotis rubra*, in the south of Australia[1]. *P. olseni* belongs to the order Perkinsida and is the causative agent of perkinsosis, a disease associated with extensive mortalities of molluscs worldwide[2–5]. *P. olseni* is included on the list of reportable disease of the World Organisation for Animal Health (OIE) because infections cause mass mortalities in oysters and clams and significant economic losses (http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2016/). *P. olseni* is also listed on the Network of Aquaculture Centres in Asia-Pacific (NACA). This parasite induces lesions that can impede the respiration, and other physiological processes such as growth and reproduction, sometimes leading to death, impacting fishery and aquaculture productivity[6–8].

P. olseni has three main life stages. The trophozoite stage occurs in the tissues of the live host and proliferate by undergoing successive bipartitionning (schizogony) that yields up to 32 daughter cells (Figure 1)[9,10]. The rupture of the wall allows the liberation of immature trophozoites that will enlarge[9,10]. In the dying host, trophozoites gradually enlarge and become mature trophozoite or prezoosporangia. When released in the water column and under favourable environmental conditions, the prezoosporangia divide internally into hundreds of biflagellated ellipsoidal zoospores that are formed within the original cell wall and leave the zoosporangium via a discharge tube. The motile zoospores can then infect a new host. It is not yet well understood which stage is the most effective or principal stage for transmitting the disease in the natural environment[11].

In the 1970s, soft white-yellow abscesses were observed in the flesh of the blacklip abalone *Haliotis rubra* collected in South Australia[10]. When clusters of *Perkinsus* cells are found near the surface of the abalone, they appear as a soft white nodule or microabscess[10]. Microabscesses develop to form brown spherical abscess or pustules up to 8 mm or more in diameter[10]. These abscesses are observed in the foot and muscle[10]. Identical lesions were observed in *H. laevigata* but lesions are absent in infected *H. scalaris* and *H. cyclobates*[10]. This parasite was associated with severe mortalities in *H. laevigata* wild populations in 1980s, leading to local extinction on the western shore of Gulf St Vincent, South Australia[10,12]. Outbreaks also occurred during the same period in *H. laevigata* aquaculture facilities in South Australia, when 40% of the stock died.

Mass mortalities of blacklip abalone (*H. rubra*) occurred from 1992 to 2002 along approximately 500 km of the NSW coastline between Port Stephens and Jervis Bay[13]. Histological examination of moribund abalone since 1992 and a survey of infection prevalence in abalone using Ray's test in 2002, confirmed infections by a variant strain of *P. olseni*, suggesting that this parasite contributed to the mortalities observed[13]. Indeed, substantial tissue and organ damage occurred in abalone with high intensity of infection. Disruption of the gut epithelium and infarction in the gills suggested impairment

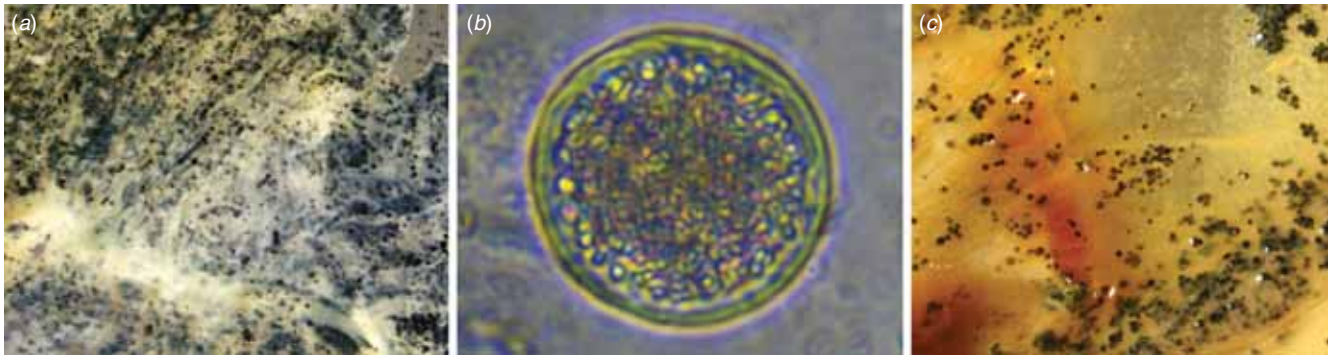

Figure 1. *In situ* iodine stained trophozoites of *Perkinsus* sp. (black dots) in the gills (*a*) and mantle (*c*) of heavily-infected Manila clams (*Ruditapes philippinarum*). Prezoosporangium containing hundreds of zoospores isolated from the gills of greenlip abalone (*H. laevigata*) in Western Australia (*b*).

to normal nutrient absorption and respiration[14]. There is some indication that stress such as that from high temperatures exacerbate the disease but the conditions under which the disease progresses are not well understood.

Perkinsus olseni was formally identified and reported in Western Australia in 2015 from wild greenlip abalone. Surveys of wild and cultured abalone stocks in WA are currently ongoing to evaluate the prevalence of this parasite and monitor any potential negative impacts.

Abalone herpesvirus infections

Infections with herpes-like viruses resulting in the disease Abalone Viral Ganglioneuritis (AVG), were first identified and characterised in Australia around 2005 from Victorian land-based greenlip abalone (*H. laevigata*) culture facilities[15]. The disease is listed as reportable by the OIE and NACA and is characterised by marked inflammation and necrosis of nervous tissues (cerebral, pleuropedal and buccal ganglia, branches of the pedal nerve and peripheral nerves) in infected abalone[15–17]. Since their initial detection, abalone herpesvirus (AbHV) infections have been implicated in causing mass mortalities in wild abalone stocks in Victoria and in culture facilities in Tasmania, resulting in strict stock movement restrictions and enhanced biosecurity practices being enforced by jurisdictions[15,16,18,19]. Five genotypic variants of AbHV have now been identified from Australian *Haliotis conicopora*, *H. laevigata* and *H. rubra* populations, and experiments have confirmed that all five variants may cause disease and subsequent mortalities in these abalone species[18,20,21].

Diagnosis of AbHV infections in abalone typically involves histopathological examination of neural tissues, electron microscopy and nucleic acid sequencing[17,18,21,22]. Rapid quantitative real-time PCR assays targeting the five Australian AbHV variants have been developed by the Fish Disease Laboratory at the Australian Animal Health Laboratory to aid in quickly assessing presence or absence of virus in abalone stocks for biosecurity and translocation protocols by relevant jurisdictions[21,23].

Vibrio spp. infections

Infections with *Vibrio* spp. have been implicated in causing severe mass mortalities in cultured abalone in numerous localities worldwide, including Australia[24]. These pathogens are generally considered opportunistic, causing acute infection and mortality in physically or environmentally stressed individuals[25–27]. The condition 'Summer Mortality Syndrome' observed in Australian greenlip (*H. laevigata*) and blacklip (*H. rubra*) abalone and their hybrids, refers to an increase in mortalities in association with increased water temperatures which promote infection with *Vibrio* species such as *V. harveyi*[28].

Confirmative diagnosis for *Vibrio* spp. infection in abalone includes observing bacteria within affected tissues on histopathological examination of moribund abalone, isolation and culture of bacteria followed by matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF) or DNA sequencing[29].

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Amoebic gill disease: a growing threat



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The risk of disease outbreaks is predicted to increase due to climate change. For farmed fish an example is amoebic gill disease (AGD). While initially reported only in farmed salmonids in Washington State, USA, and Tasmania, Australia, it has now become an issue for Atlantic salmon farming worldwide and affects a range of other farmed marine fish species. Local high temperature anomalies and a lack of rainfall have been associated with the outbreaks of AGD. This worldwide presence is at least partly due to the cosmopolitan nature of the parasite and its low host-specificity. The disease can be treated using freshwater or hydrogen peroxide baths, but the treatments increase the cost of salmon production. Management of AGD contributes 20% to production costs of Atlantic salmon in Tasmania.

AGD, caused through infection of fish gills by the facultative parasite *Neoparamoeba perurans*, was first documented in sea-caged salmonids in 1985[1]. Since its initial observation AGD has become a primary health concern globally for the marine salmonid industry, resulting in mortalities as great as 80% when left untreated[2]. Clinical signs of AGD include respiratory distress, lethargy and inappetence, which are associated with grossly visible gill lesions[3]. Histologically, gill lesions are characterised by epithelial hyperplasia, interlamellar vesicles with associated amoebae and lamellar fusion[4] (Figure 1).

Because *N. perurans* was only recently identified[5] and shown to cause AGD[6], minimal information is available on its biology and ecology. Amoebae of the genus *Neoparamoeba* (Amoebozoa, Dactylopodida) are ubiquitous in the marine environment[7], and *N. perurans* specifically have been detected throughout the water column on and near Atlantic salmon (*Salmo salar* L.) farms[8,9]. All species from the genus *Neoparamoeba* harbour at least one intracellular endosymbiont known as a *Perkinsela amoebae*-like organism (PLO)[10]. The details of the symbiotic relationship between the PLOs and *Neoparamoeba* are unknown; however, the strict phylogenetic congruence of PLOs and their *Neoparamoeba* hosts suggests that PLOs are vertically transmitted from parent to daughter cells during mitotic division[10]. Species of *Neoparamoeba* all

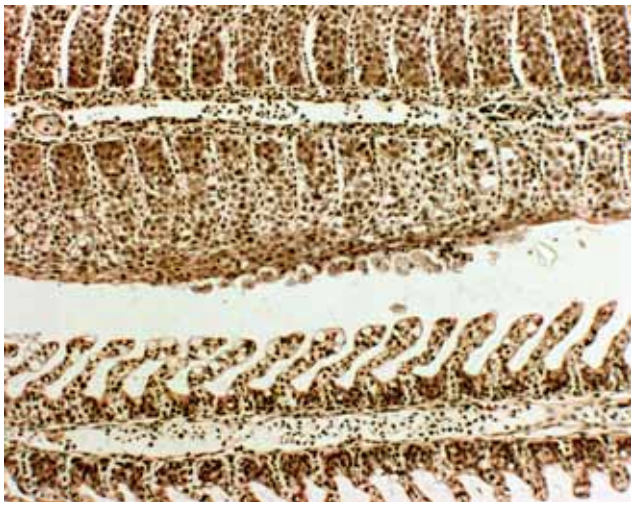

Figure 1. Histological section showing two filaments from gills of infected Atlantic salmon, the top filament is affected and the bottom one is normal. Epithelial hyperplasia and lamellar fusion associated with presence of numerous amoebae can be seen in the top filament.

share the same general ultrastructural characteristics and cannot be differentiated morphologically[11].

Despite numerous studies investigating potential reservoirs of *N. perurans*, no significant reservoir outside farmed salmon has been identified[9]. Extensive surveys of the water column[8,12] and wild fish[13–15] have detected only minimal evidence of *N. perurans*. Studies of metazoan ectoparasites, for example copepods or isopods, on farmed salmon have detected low frequencies of *N. perurans*[16,17], but no evidence of a reservoir population. Additional studies using genus specific identification methods detected *Neoparamoeba* spp. in sediment samples[18] and net biofouling[19], but as yet no species specific testing has been conducted to detect *N. perurans* in these potential reservoirs.

Along with seemingly no reservoir, *N. perurans* is also an opportunistic parasite with no apparent host specificity. The pathogen has been detected not only in the commercially important Atlantic salmon[20], but also in a variety of farmed and non-farmed finfish species around the world[9] (Figure 2). Presently AGD is a major issue for aquaculture in Tasmania, Ireland, Scotland, Norway and the United States with varying levels of impact from 10% to 82% mortality in some cases[9]. Additional outbreaks have been reported in Chile, France, Spain, South Africa, and most recently Canada and the Faroe Islands[9,21].

Beyond salmon, *N. perurans* has been found on the gills of an additional 14 finfish species including ayu in Japan[22], sea bass in the Mediterranean[23], and olive flounder in Korea[24]. There is no traceable pattern from one of these outbreaks to another making it unlikely that it is a specific sub-population that causes the disease or that amoebae are transferred from one outbreak site to another. What is known of its lifecycle suggests an asexual clonal evolutionary pattern. It has been postulated by statistical analysis that the sheer number of individuals in any given microbial species is so large that dispersal would rarely be restricted by contrived geographical barriers[25], especially in marine environments[7].

The cosmopolitan nature of *N. perurans* and lack of host specificity make discerning trends and risk factors for AGD challenging. A recent meta-analysis which considered all reports of AGD to date suggests locally high temperature anomalies, rather than absolute temperature, are related to disease outbreak[9]. Salinity also plays an important role in AGD. *N. perurans* is a marine amoeba with minimal tolerance for low salinity. Freshwater bathing for 2–4 hours is the most commonly utilised commercial treatment for AGD[26], and though many reports do not include information on rainfall, the few which have report lower than average rainfall preceding outbreaks[3,27–29]. Given the predicted increase in ocean temperatures and altered rainfall patterns associated with climate change, there is concern that AGD associated costs will continue to increase for the salmonid industry moving forward[30].

Although research into AGD has come a long way in the past 30 years there are still many knowledge gaps in key areas from basic biology

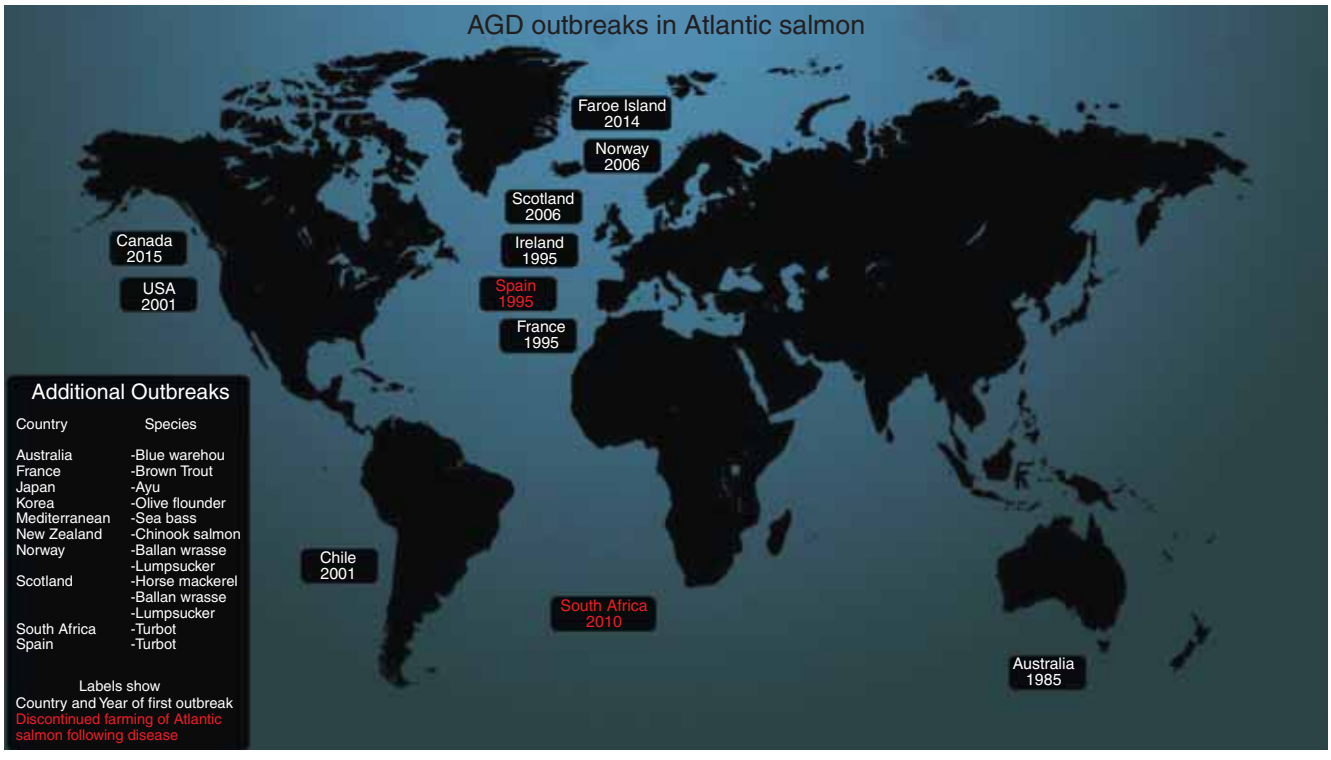

Figure 2. Map showing reported confirmed (PCR and/or ISH) cases of amoebic gill disease (AGD) in farmed Atlantic salmon.

to industry research. For instance, little is known about the parasite *N. perurans*. The mechanisms behind the successful transfer of the PLO from mother to daughter cell, and benefits of the symbiosis, are not yet known. In addition there is little information on how the amoebae cause disease and whether there is potential for vaccines or drug targets. On a more practical side, extensive and thorough testing of sediment, biofouling and other potential reservoirs would also be beneficial for predicting outbreaks and controlling this globally increasing threat.

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Biographies

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Tina Oldham is a PhD student in the Aquatic Animal Health group at the University of Tasmania. Her primary interest is in development of sustainable, resilient aquaculture.

Barbara Nowak is a Professor at Institute of Marine and Antarctic Studies at University of Tasmania. Her research focuses on aquatic animal health. She has been working on amoebic gill disease for the past 25 years.

Unprecedented toxic algal blooms impact on Tasmanian seafood industry

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While most microscopic algae provide food for filter-feeding shellfish and larvae of crustaceans and finfish, other so-called Harmful Algal Blooms (HABs) can have negative effects, causing severe economic losses to aquaculture, fisheries and tourism. Of greatest concern to human society are blooms of toxic HAB species that cause illness and death of fish, seabirds and mammals via toxins transferred through the food web. Unprecedented *Alexandrium* (Dinophyceae) blooms along the East Coast of Tasmania in 2012 and 2015, a previously low biotoxin risk area, led to major impacts on the local oyster, mussel, scallop and rock lobster industries. Four human hospitalisations also occurred from eating wild shellfish.

One of the first recorded fatal cases of human Paralytic Shellfish Poisoning (PSP) after eating shellfish contaminated with dinoflagellate toxins occurred in 1793, when Captain George Vancouver and his crew landed in British Columbia in an area now known as Poison Cove. He noted that it was taboo for local Indian tribes to eat shellfish when the sea became bioluminescent due to plankton blooms[1]. The causative organism, the dinoflagellate *Alexandrium* was formally described in 1936 and the neurotoxin it produces (saxitoxin, STX) chemically characterised in 1975[2,3] (Figure 1). Doses of 1 mg cause moderate symptoms in humans (tingling sensations around finger tips and lips) but doses of 10 mg can be lethal, resulting in death from respiratory paralysis. To prevent human poisonings, the United States Food and Drug Administration (US FDA) introduced a compulsory monitoring program in 1937 whereby seafood products are regularly tested in accredited laboratories using mouse bioassays[4]. Shellfish containing more than 0.8 mg PST/kg are deemed unsuitable for human consumption and prohibited from sale. Due to increasing concern over animal ethics, many algal toxins are now analysed using sophisticated Ultra Performance Liquid Chromatography (UPLC) and Liquid Chromatography Mass Spectrometry (LCMS) methods. These analyses are expensive ($500–$800/test) and when compounded by sample transport problems, result in frustrating delays for fishermen and regulators. In Tasmania, seafood biotoxin testing is conducted through the Tasmanian Shellfish Quality Assurance Program, using a specialist analytical laboratory in Sydney for routine testing – at an annual cost of up to $450 000.

In October 2012 a shipment of blue mussels (*Mytilus galloprovincialis*) from the East Coast of Tasmania, Australia, was tested by Japanese import authorities and found to be contaminated with unacceptably high level of Paralytic Shellfish Toxins (10 mg PST/kg)[5]. Subsequent testing showed that oysters, scallops, clams, and abalone and rock lobster viscera were also contaminated along the entire Tasmanian East Coast. This led to precautionary fishery

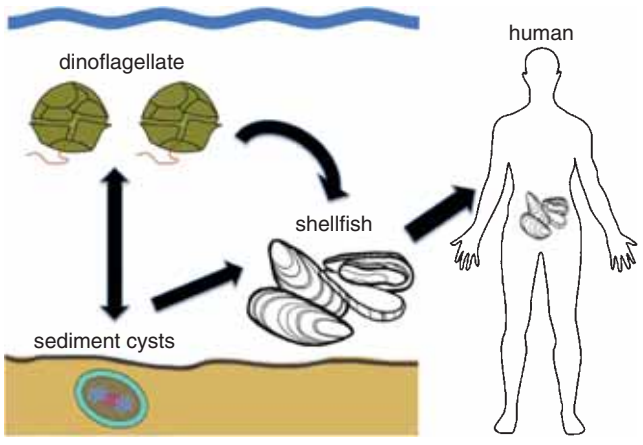

Figure 1. Transfer of dinoflagellate toxins via shellfish to humans where they can cause paralytic shellfish poisoning.

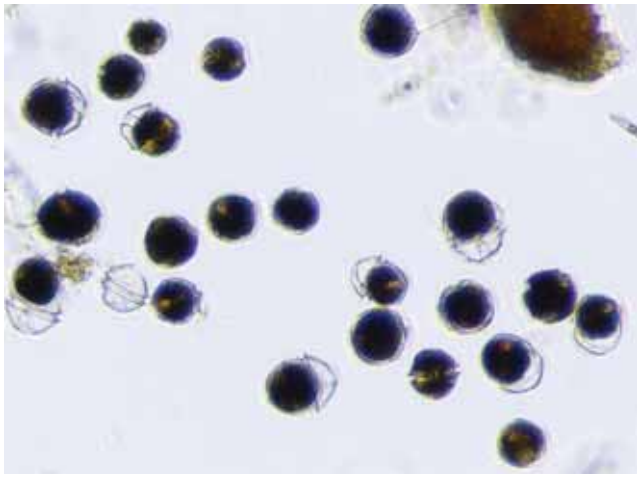

Figure 2. East Coast Tasmanian plankton bloom dominated by the toxigenic dinoflagellate *Alexandrium tamarense* (30–40 µm diameter), some cells of which are seen to escape from their diagnostic cellulose cell walls.

closures also for the high-value Southern Rock Lobster and abalone industries and an international shellfish product recall with losses of more than $23 million to the local economy. Following low toxicity events and no lengthy farm closures in 2013 and 2014, a more severe bloom event recurred during June–Oct 2015 (up to 300 000 dinoflagellate cells/L) resulting in >15 mg/kg PST in mussels, >6 mg/kg PST in oysters, and four human hospitalisations after consumption of wild shellfish by recreational collectors unaware of public health warnings. The causative dinoflagellate *Alexandrium tamarense* (Figure 2) had been previously detected in the area in very low cell concentrations over the past 10–15 years, but cultured strains had been mostly non-toxic or weakly toxic[6]. Accordingly, the area had been assigned a low biotoxin risk and monitored at low frequency, particularly in winter and autumn. Unexpectedly, all outbreaks since 2012 have been dominated by a highly toxic *A. tamarense* genotype never previously seen in bloom proportions in Australia. While we considered the possibility of ballast water introduction or climate-driven range extension[7], preliminary molecular evidence suggests the causative dinoflagellate is a previously cryptic (rare) genotype in the area, now favoured by increased water column stratification associated with southward extension of the East Australian Current. Increased seafood and plankton monitoring now include *Alexandrium* quantitative molecular detection (qPCR) that offers more sensitive early detection of the causative dinoflagellate[8] and routine immunoassay screening for toxins, in parallel with routine UPLC toxin testing[9]. The rapid immunoassay PSP test kits[10] (Figure 3) provide an on-site or on-board qualitative yes/no result within 20 minutes, allowing producers to make on-farm harvest decisions prior to product processing and transport, thus reducing their business risk. Once validated at a national and international level, these tests can also be used by regulators as a pre-screening test to reduce the cost of testing negative samples, and improve public health outcomes.

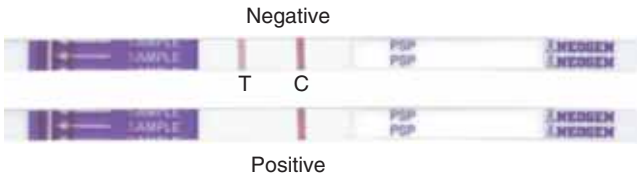

Figure 3. Pregnancy type immunological test kits used in Tasmania for rapid 20 minute screen testing whether a shellfish sample is positive (1 line) or negative (2 lines) for paralytic shellfish toxins (from http://foodsafety.neogen.com/pdf/ProdInfo/R2-PSP.pdf).

Acknowledgements

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Biographies

Gustaaf Hallegraeff is a Professor at the Institute of Marine and Antarctic Studies at the University of Tasmania. His research focuses on harmful algal blooms impacting on human health, the fish farm and shellfish industries, their stimulation by coastal eutrophication, climate change and global spreading via ship's ballast water.

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Report from ASM 2016: New Frontiers

Charlene Kahler

Chair of the local organising committee for ASM Perth 2016

We are very grateful for the support of the ASM membership and our industry partners for their attendance at the 44th Annual Scientific Meeting and Trade Exhibition held in Perth in July 2016. We are delighted to report that our 420 delegates had excellent weather with panoramic views from the conference center! Nevertheless, the exciting scientific program and the fantastic lunches encouraged our delegates to stay and network extensively with each other and our exhibitors!

Our conference began on Saturday with Educon, which was held at the School of Pathology and Laboratory Medicine, UWA. Delegates were put through their paces with demonstrations of the use of the schools E-learning suite, active learning and flipped classrooms. Delegates discussed the importance of laboratory work for prospective student employment and how industry-led student internships can enhance undergraduate access to laboratory skills. I would like to thank Megan Lloyd for excellent liaison with the EduCon organisers to make this a successful event!

Over 170 delegates attended the Sunday workshop program and were suitably impressed with the sessions from our special interest groups and our company sponsors, Thermo Fisher and Illumina. I would like to thank the workshop convener Fiona Daga and our catering convener Suzi McCarthy for their hard work in organising the day.

Our official program began with a public lecture entitled 'WHO global alerts and the traveller!' and included three eminent speakers; Dr Chris Baggoley AO, Dr Paul Effler and Professor Tania Sorrell. They provided their personal stories and professional insight into the issues facing the WHO including the challenges we face to bring health care to developing nations while maintaining the safety and security of our health care system in Australia. I would like to specifically thank Dr Allison Imrie who organised this session and acknowledge the support of the Center for Research Excellence in Infectious Diseases.

Public lecture. Dr Charlene Kahler introducing the speakers Dr Paul Effler, Dr Chris Baggoley and Dr Tania Sorrell.

Our official scientific program opened with the Bazeley oration, which is an award supported by the Commonwealth Serum Laboratories. We are very grateful for the continuation of this award by CSL over many years at our annual conference and are excited to have it placed in our opening session! This year, Professor Ulrike Holzgrabe presented an excellent overview of the challenges in modern drug design.

In keeping with the theme of 'New Frontiers', the conference covered the main thematic areas of 'Antimicrobial Resistance: Impact, mechanisms and solutions to a growing health care crisis' and 'The Good, the Bad and the Useful: Microbes in healthy ecologies, disease and industry'. I would like to thank our theme leaders: Professor Peter Hawkey, Professor Dan Andersson, Dr Brian Conlon, Associate Professor Susan Lynch, Professor Victor Nizet and Associate Professor Anna Durbin who delivered outstanding presentations that were highly praised by our delegates.

The plenary sessions were supported by a diverse array of national and international speakers who contributed to the 3-day scientific program. I would like to thank the Division chairs: Dr Tim Inglis, Dr Christopher Peacock, Dr Allison Imrie and Dr Harry Sakellaris in addition to the Scientific Program chair, Dr Megan Lloyd, for all the long hours and hard work putting this high quality program together.

We are also indebted to the Rubbo Trust for supporting the Rubbo oration which was awarded to Dr Anne Kelso, the CEO of the NHMRC, who presented her current analysis of the challenges facing research funding in Australia. We also thank the Nancy Millis bequest which supported a range of outstanding social activities for students and early career researchers including a round table on career advice which was exceptionally well received by all who attended. We also thank Dr Barry Marshall, the director of the Marshall Center for Infectious Disease Research and Training, who donated prize money for the three 3 minute thesis and four ASM

poster awards. Thanks also to all those who participated in the judging of these awards.

We also presented nine major ASM awards to 18 participants! Each awardee is listed below with their details. We believe these awards reveal the depth and breadth of the talent within the Society and look forward to watching the careers of the student awardees over the coming years! We thank our industry supporters, Becton Dickenson and bioMérieux for their continued contributions to these activities.

Lastly I would like to thank the remaining local organising committee members, Dan Knight (secretary and advertising), Shakeel Mowlaboccus (student social activities), Chris Mullally (social media) and Rod Bowman (trade rep) in addition to ASN events (Kara Taglieri, Phil McShane and Jarrad Thessman) for their excellent support over the past two years in organising this conference. I was very honoured to chair this event!

At our closing event we heard a presentation from our colleagues in Tasmania who now carry the banner into the next year! They have already organised a stellar cast of plenary speakers and I urge everyone to have a look at the ASM website and save the date!

Honorary Life Membership of the Australian Society for Microbiology

Professor Lyn Gilbert was awarded Honorary Life Membership of ASM. Her certificate was presented by Julian Rood and Tania Sorrell.

New Fellows of the Australian Society for Microbiology

David Whiley, Pathology Queensland & University of Queensland

Mark Turner, University of Queensland

Peter Speck, Flinders University

Matt Payne, University of Western Australia

ASM Distinguished Service Awards

Fran Morey, Danilla Grando, Sue Coloe and Helen Cain.

Chris Ossowicz, SA/NT, Danilla Grando, VIC, Fran Morey, SA/NT and Sue Coloe, VIC were acknowledged with ASM Distinguished Service Awards.

Clinical Travel Award: Pei Vern Fong

Pei is a junior scientist with just over 5 years of working experience in both private and public pathology. Her interests lies in organisms that are weird, wonderful and stinky. She is currently finishing her final Masters project in Actinomyces spp.

ASM Jim Pittard Award: Jaclyn Pearson

Jaclyn Pearson joined the laboratory of Professor Elizabeth Hartland at Melbourne University in 2009 to undertake a PhD on virulence mechanisms of pathogenic *E. coli*. Since the start of her research career in 2009, she has a total of 16 publications, as well as another four in review/preparation including a first author paper in review at

Nature, and a corresponding author paper in preparation. She has made a number of discoveries that have advanced knowledge and practices within the immediate and broader field of her research. Her most recent research project describes a novel family of bacterial cysteine proteases that cleave all Rip homotypic interaction motif (RHIM)-domain proteins, both mammalian and viral.

Millis Colwell Postgraduate Award: Deanna Deveson

Deanna completed her BSc (Hons) in the Department of Microbiology at Monash University in 2004. Her honours project involved investigating regulation of virulence factors in *Clostridium perfringens* and was based in the Rood Laboratory. Following honours, Deanna worked at CSL Animal Health and Pfizer Veterinary Medicine Research and Development (VMRD) in the Bacteriology and Bioprocess Improvement Groups, developing, and trouble-shooting vaccines for domestic and livestock animals. Deanna undertook her PhD, 'The bovine immune response to *Leptospira borgpetersenii* serovar Hardjo', in collaboration with Pfizer VMRD and the ARC Centre of Excellence in Structural and Functional Microbial Genomics, Adler Laboratory, Department of Microbiology, Monash University, which she completed in 2014.

David White Excellence in Teaching Award: Helen Cain

Helen Cain has been a contracted (and later continuing) member of the staff of the Department of Microbiology & Immunology since March 2003. From 1992–2003 she was employed on a casual basis as a practical class demonstrator for microbiology practical classes and as a Problem Based Learning tutor for the undergraduate medical course. She also taught a Medical Microbiology unit in the Diploma of Animal and Biological Sciences course at Box Hill TAFE in 2001, and taught a unit on Introduction to Microbiology in the Certificate of Sterilisation and Disinfection at the Mayfield Education Centre, Hawthorn between 2000 and 2008.

bioMérieux ASM Identifying Resistance Award: Anton Peleg

Despite having a consistent clinical load, Professor Peleg has established himself as an international expert in the field of antibiotic resistance and hospital-acquired infections. The majority of these publications are on the study of bacterial resistance to antimicrobials in a clinical setting. He has published in the highest quality general medical or scientific journals. He has also written book chapters on Gram-negative resistance for leading reference texts in Medicine: Harrisons Textbook of Medicine and Kucer's, The Use of Antibiotics. He has made several pioneering studies in the area of antibiotic resistance. He has been at the forefront of developing molecular methods for direct detection of antimicrobial resistance for *Neisseria gonorrhoeae*, an organism now ranked as an 'urgent' antimicrobial resistance threat, the highest level designated by the CDC and shared with only two other organisms, *Clostridium difficile* and carbapenem-resistant Enterobacteriaceae. He has 17 years' experience in clinical microbiology research and is a leading authority in molecular detection and characterisation of infectious diseases, particularly sexually transmitted infections and novel or emerging agents.

ASM Frank Fenner Award: Scott Beatson and Kate Seib

Scott Beatson is an Associate Professor at The University of Queensland and is a member of the Australian Infectious Diseases Research Centre and the Australian Centre for Ecogenomics. He leads a research group in bacterial pathogenomics at the School of Chemistry and Molecular Biosciences. He was awarded a PhD in Genetics from the University of Queensland for his work on *Pseudomonas aeruginosa* pathogenesis in 2002 and holds Masters degrees in

Microbiology and Bioinformatics from the Universities of Otago and Manchester, respectively. Competitive fellowships from the Royal Commission for the Exhibition of 1851 and MRC (UK) supported his postdoctoral study of bacterial genomics at the Universities of Oxford and Birmingham, UK, before he returned to Australia in 2006 as a NHMRC Howard Florey Fellow.

Dr Kate Seib's research is focused on understanding the processes involved in host colonisation and disease, with the aim to identify vaccine targets for mucosal pathogens including *Neisseria meningitidis, Neisseria gonorrhoeae* and *Moraxella catarrhalis*. Dr Seib completed a PhD in microbiology in 2004 from the University of Queensland (Brisbane, Australia) and worked briefly as a Postdoctoral Researcher in Australia. She then spent 6.5 years at Novartis Vaccines (Siena, Italy) as a Postdoctoral Researcher and Project Leader. Dr Seib returned to Australia in 2012 and is a Group Leader and NHMRC Career Development Fellow at the Institute for Glycomics, Griffith University (Gold Coast). She is the current Chair of the Queensland Branch of the ASM.

ASM Lyn Gilbert Award: David Smith

BD ASM Student Travel Awardees

Victoria

Joshua Newsome, University of Melbourne

South Australia

Felise Adams, Flinders University

New South Wales

Caitlin Abbott, University of Sydney

Tasmania

Neeraj Singh, University of Tasmania

Queensland

Leah Roberts, University of Queensland

Western Australia

Daniel Knight, University of Western Australia

Chronic rhinosinusitis: a microbiome in dysbiosis and the search for alternative treatment options




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Chronic rhinosinusitis (CRS) is a common chronic disease. While CRS is a multifactorial disease, many cases involve an imbalance in the sinus bacterial microbiome. This article reviews the composition of the healthy human sinus microbiome compared to the microbiome of CRS patients. Issues with current treatment options, particularly antibiotics, are discussed. Insights into the future of CRS treatment are also explored, principally with regards to probiotics.

Rhinosinusitis is a condition characterised by paranasal sinus and nasal inflammation. Symptoms include nasal blockage/obstruction/congestion, facial pain/pressure, reduction or loss of smell, and rhinorrhoea; when these continue for over 12 weeks, the condition is classified as chronic rhinosinusitis (CRS)[1]. CRS is one of the most prevalent chronic diseases worldwide, conservatively affecting 5–6% of US adults[2] and 8.5% of Australian adults[3]. The economic burden of CRS in the US alone is approximately US$22 billion annually[4].

Like many chronic diseases, CRS has a complex etiology, with interplay between microorganisms (bacteria, fungi and viruses), environmental disturbances (e.g. pollutants or smoking) and host factors (e.g. the immune system and underlying diseases)[5]. This article explores the role that bacteria play in CRS by examining recent research suggesting that disturbances to the sinus microbiome are involved in CRS pathophysiology.

CRS and the sinus microbiome

A microbiome is a collective term for all of the microorganisms present in an environment. Various groups of microorganisms can cause disturbances to the sinus microbiome, which can sometimes contribute to the development of CRS. For instance, an acute viral infection is the cause for many cases of sinus microbiome disturbance, but this is a short-term disturbance[1,6]. Also, while fungi are not considered primary etiologic agents of CRS, allergies to some fungi can result in a distinct condition with similar symptoms as CRS called Allergic Fungal Rhinosinusitis[5]. However, the group of microorganisms that are the biggest players in CRS are bacteria[1,5].

In the past, healthy sinuses were considered sterile environments, and CRS developed when bacteria colonised these sinuses[7]. Nowadays, it is recognised that healthy sinuses house diverse microbiomes with both commensal bacteria and potentially pathogenic bacteria; the pathogens present in these healthy sinuses are present at levels too low to cause disease[6,7]. The commensal microbes form a symbiotic relationship with the host, such as by forming a barrier against incoming external pathogens[8]. An imbalance of the sinus microbiome – termed microbiome dysbiosis – is seen in many cases of CRS, as seen by an overabundance of opportunistic pathogens and loss of key commensals[5,6,8,9]. The immune system is then activated due to pathogen invasion through epithelial tight

junctions and release of various immunostimulatory molecules, thus resulting in inflammation[1,5] (Figure 1).

Determining differences in the bacterial makeup of CRS or non-CRS sinuses is a relatively novel area of research. This is due to both the recent change in our understanding of CRS pathophysiology and recent advances in microbiome characterisation methods. Traditional culture-dependent methods fail to truly represent the sinus microbiome[9,15], so truly accurate insights into CRS versus healthy microbiomes are from a currently limited number of molecular-based studies[9–12,15–18].

Further adding to the complexity of CRS is that there does not seem to be a 'model' CRS microbiome; that is, each CRS patient has a unique sinus microbiome composition[15,16]. Also, even within a single CRS patient, the microbiome of each sinus is different[19]. However, after taking these sources of variation into consideration, there are still certain features that can distinguish between CRS and non-CRS microbiomes, as described in the next two sections.

Balance of bacterial taxa

Compared to healthy sinuses, CRS sinuses have decreased bacterial diversity (the total number of bacterial taxa) and evenness (the relative proportion of each taxon)[8,10–12]; in other words, healthy sinuses have many different types of bacteria present in similar numbers, while CRS sinuses have few types of bacteria present, and of those some are overabundant while others are barely present (Figure 1). In ecology terms, these decreases are mainly due to selective enrichment of certain 'disease-producing' species and depletion of other 'protective' species.

Mostly commensal taxa are depleted in CRS patients; notably, decreases in *Bacteroidetes* spp., *Prevotella* spp.[11], *Lactobacillus* spp.[10], *Peptoniphilus* spp., *Propionibacterium acnes*[9], *Acinetobacter johnsonii* and *Corynebacterium confusum*[18] have been observed.

Other bacterial taxa are found to be enriched in CRS microbiomes. Increases in *Pseudomonas* spp.[16], *Corynebacterium* spp.[10,16], certain *Streptococcus* spp., *Staphylococcus aureus*, *Propionibacterium acnes* and *Haemophilus influenzae*[15,16] have been reported in CRS. Abreu *et al.*[10] notably found enrichment of a novel sinopathogen *Corynebacterium tuberculostearicum*, typically a commensal when present on skin. Of particular importance to CRS is *S. aureus*. Most microbiome studies found that *S. aureus* was enriched in CRS patients; some even found it to be the most abundant organism in CRS sinuses[9,11,12,16,18]. Here it is important

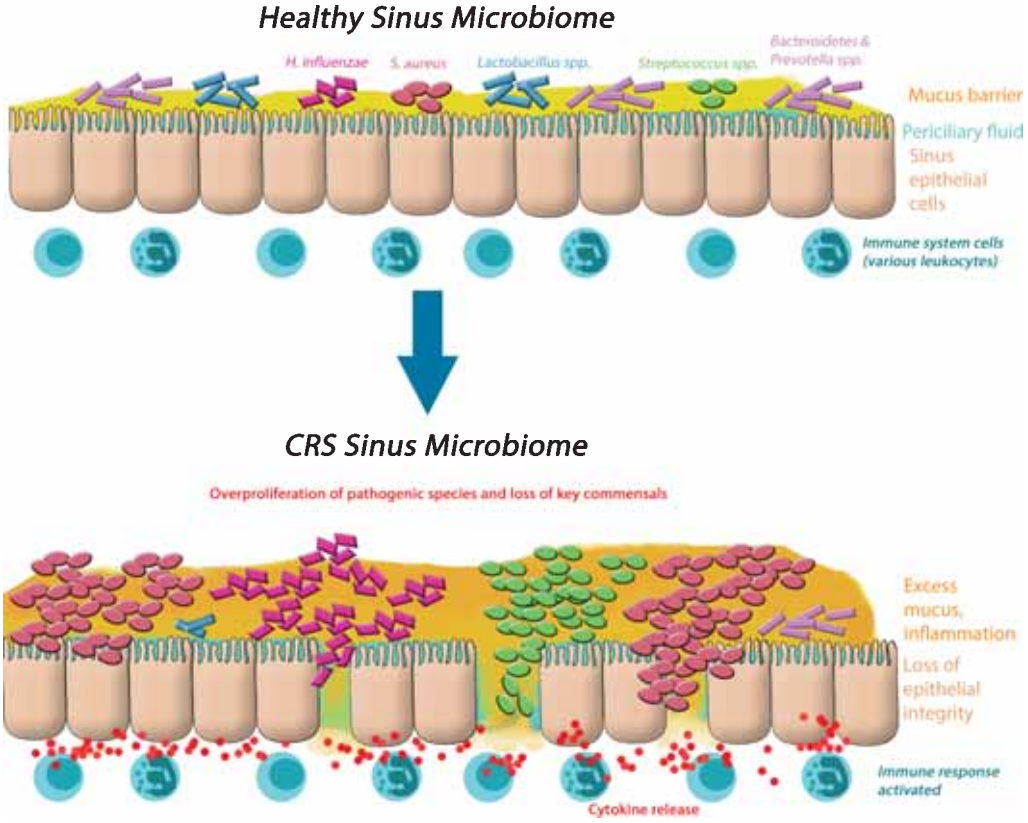

Figure 1. Nasal mucosa microbiomes of healthy versus CRS patients. The normal nasal mucosa is colonised by a highly diverse dynamic mix of commensal microbes, and some pathogenic microbes at low abundances. Perturbations to the microbiome can lead to microbiome dysbiosis; now the sinuses have low species diversity and evenness, with loss of critical commensal species and selected enrichment of pathogens. This then leads to a loss of epithelial integrity, immune activation and sinus inflammation. The bacterial taxa presented here are a few of the commensal and pathogenic species that have been implicated in CRS disease progression. This figure is adapted from the information in the following references[5,6,8–16].

to emphasise the problem of 'correlation vs causation', as seeing increases of certain taxa in a disease state is insufficient evidence to conclude that those taxa are causing the disease. With particular regard to *S. aureus*, no study has been carried out to explicitly determine whether or not an increased sinus level of *S. aureus* will cause CRS. However, current research on *S. aureus* in CRS[12,18,20,21] indicates that *S. aureus* increases the severity of CRS, and is at the very least involved in CRS development.

Bacterial load

Bacterial load is the total number of bacteria in a microbiome[8]. There is currently disagreement in the literature on the correlation between CRS and bacterial load. Boase *et al.*[9] and Choi *et al.*[11] found an increased bacterial load in CRS patients and suggested that a rise in bacteria, possibly from external sources, causes CRS. However, Abreu *et al.*,[10], Feazel *et al.*[12] and Ramakrishnan *et al.*[13] found no difference in burden between the two groups. Feazel *et al.*[12] pointed out that pathogens are present in low abundances in healthy patients, and are selectively enriched in CRS patients, and suggested that shifts in the existing bacterial community, rather than influxes of external pathogenic bacteria, cause CRS[12]. While this hypothesis is currently favoured[5,8], more studies are required to establish a causal link.

Current CRS treatment

If a patient presents to a clinic with over twelve weeks of the rhinosinusitis symptoms as mentioned previously, a preliminary test is to check for any allergy. If positive for allergy, the condition is termed allergic rhinosinusitis[1], which is out of the scope of this article. On the other hand, if the rhinosinusitis is not caused by allergy, it is diagnosed as CRS and treated accordingly. Treatment options are initially saline nasal irrigation, antibiotics, followed by topical or oral corticosteroids[1]; if these treatments fail to improve symptoms, sinus surgery may be necessary[1]. Despite their only partial success rate, antibiotics are the current most widely used treatment option for CRS (50–70% of CRS diagnoses result in the prescription of an antibiotic) due to their ease of access, low costs and low complexity of intervention[22,23]. However, using antibiotics to treat CRS seems to be a short-term solution with long-term problems. Managing a chronic condition with ongoing antibiotic administration creates conditions particularly favourable for the rise of antibiotic resistant bacteria[23,24]. Penicillin-resistant pathogens have been found in extensively antibiotic-treated CRS patients since the 1980s[25]. Furthermore, Bhattacharyya and Kepnes[24] found that extensively treated sinus microbiomes have increased abundances of methicillin-resistant *S. aureus* (MRSA). They found that 3.6% of all bacterial isolates from CRS sinuses were MRSA, which is much higher than the general population[24]. Bhattacharyya and Kepnes[24] also found that resistance rates against erythromycin, another key antibiotic, increased in CRS microbiota from 30% to 69% over five years.

This issue of emerging resistance against the most common treatment for CRS has encouraged several researchers to investigate alternative treatment options. One approach that has been recently garnering interest is the use of probiotics to restore a healthy microbiome in CRS patients.

Probiotics in CRS

A probiotic is defined as '...a live microorganism that, when administered in adequate amounts, confers a health benefit on the host'[26]. This benefit often involves restoring a healthy commensal microbiome by competing with pathogenic taxa, either by direct attack or by better-filling a niche[6,27]. Probiotics have been used against several diseases, such as traveller's diarrhoea, otitis media, and irritable bowel syndrome, as reviewed by Goldin and Gorbach[28]. Using probiotics to treat CRS is a very novel research area. While research is limited, the studies currently available show promise for various probiotic species that can reduce colonisation of different sinus pathogens.

For instance, Cleland *et al.*[29] co-inoculated mice with *Staphylococcus epidermidis* (potential probiotic) and *S. aureus* (CRS pathogen). They found that these mice had lower goblet cell counts (a marker for airway inflammation) compared to *S. aureus* only inoculated mice, suggesting that the *S. epidermidis* interfered with the pathogenicity of *S. aureus*. Further, Abreu *et al.*[10] found that *Lactobacillus sakei* (potential probiotic) reduced the colonisation levels of *C. tuberculostearicum* (CRS pathogen) in microbiome-depleted mice sinuses, again suggesting that the probiotic had an inhibitory effect on the pathogen. Finally, Uehara *et al.*[30] repeatedly administered a commensal *Corynebacterium* species into the nares of healthy human participants who were natural nasal carriers of *S. aureus*. This treatment eradicated *S. aureus* in the nares of 13 out of 17 participants.

It is important to acknowledge that this research field is still in its infancy. Each study reviewed here only explored one probiotic and its effect on one pathogen; as CRS is a complex disease involving whole microbiome dysbiosis, more comprehensive studies looking at multiple probiotic/commensal species interactions are certainly called for. However, the results of these studies at least show enough promise to warrant future research in this area.

Conclusion

Overall, it is clear that the etiology of CRS is not as simple as infection by pathogenic bacteria. Rather, bacteria play a role in the

development of CRS through the dysbiosis of the sinus microbiome. Compared to a healthy sinus microbiome, a CRS microbiome has a decrease in bacterial diversity and evenness, with a loss of some commensal species and overabundance of some pathogenic species. With the emergence of antibiotic resistant bacteria, researchers are starting to explore other treatment options, such as probiotics. These treatments aim to restore the sinus microbiome to normal, which may contribute to improving the symptoms of CRS.

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Biographies

Professor Anders Cervin is a Professor in Otolaryngology (Rhinology) and a Senior Staff Specialist at the Department of ENT, Head and Neck Surgery, Royal Brisbane and Women's Hospital. Professor Anders Cervin's expertise includes endoscopic sinus surgery as well as endoscopic anterior skull base surgery. Research focus of our group is on the role of the microbiota in health and disease in chronic airway disease and the possibility to modify disease expression by modifying the microbiome of the nasal cavity and sinuses.

Dr Hanna Sidjabat is a Research Officer of Infection and Immunity Theme at the University of Queensland, UQ Centre for Clinical Research. Dr Sidjabat is working within Professor Anders Cervin's group, leading the microbiology work of microbiome of upper respiratory tract of chronic rhinosinusitis (CRS) study and healthy participants as well as otitis media in the Australian Indigenous population. Dr Sidjabat has been leading the *in vitro* development of probiotic strains that can be used to treat airway diseases.

Miss Amanda Bordin is an Honours student of Dr Sidjabat and Professor Cervin. Her honours project is on genomic and proteomic features of *Lactobacillus* spp. and *Staphylococcus aureus* with applications to chronic rhinosinusitis.

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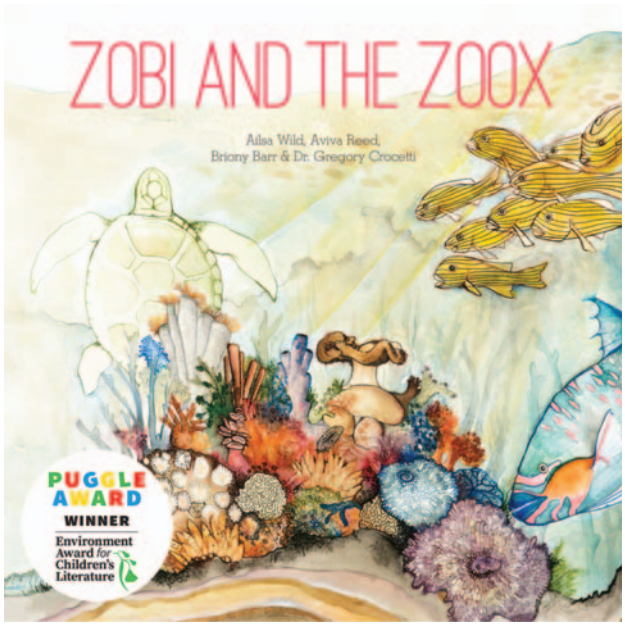

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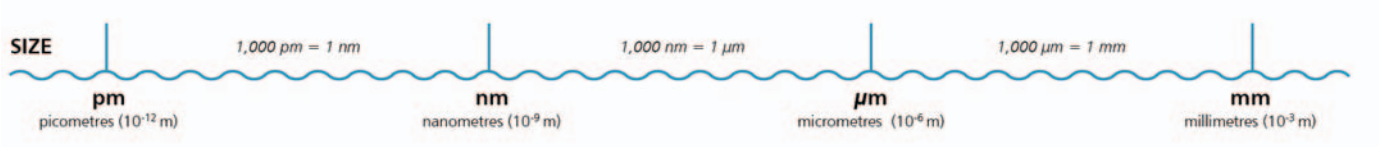

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