CHAPTER 2.4.7.

INFECTION WITH *XENOHALIOTIS CALIFORNIENSIS*

1. Scope

Intracytoplasmic infections with *Xenohaliotis californiensis*, a rickettsial bacterium, in gastrointestinal epithelia causes disease (termed withering syndrome [13]) in wild and farmed abalones, *Haliotis* spp. (Vetigastropoda: Mollusca [6]). Gross signs of the disease include pedal atrophy, mottled digestive gland, anorexia, weakness, and lethargy (3, 6, 12).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

Xenohaliotis californiensis is an intracellular bacterium in the family Anaplasmataceae (5) and is closely related to members of the genera *Ehrlichia, Anaplasma* and *Cowdria* (6). The disease caused by this bacterium is known as withering syndrome (7, 13) and may be more appropriately termed abalone rickettsiosis. No information exists on the presence of strain variations of this bacterium. The dimorphic rod-to-spherical-shaped bacterium measures an average of 332 × 1550 nm in the bacillus form and an average of 1405 nm in the spherical morphotype. The bacterium reproduces within intracytoplasmic vacuoles 14–56 µm in diameter within gastrointestinal epithelia (6).

2.1.2. Survival outside the host

Although *X. californiensis* is thought to be an obligate intracellular organism, the bacterium may survive outside the host for an undetermined period of time as evidenced by water-borne transmission studies (3, 4, 7, 11, 19).

2.1.3. Stability of the agent (effective inactivation methods)

Based on successful decontamination in the laboratory, this bacterium is readily inactivated by immersion in <10% bleach. In addition, exposure of seawater containing the bacterium to >10 mg litre^{-1} [ppm] calcium hypochlorite and disinfection of equipment in a bath of 1% tamed iodine in freshwater for 1 hour are effective disinfectants based on the use of these disinfection methods at a marine laboratory with flow-through seawater and a lack of detection of this pathogen in adjacent abalone populations (8, Friedman, pers. obs.).

2.1.4. Life cycle

The bacterium divides by binary fission (6) and has direct, horizontal transmission (4, 7, 17). Although not typically observed in farmed abalones until they are in grow-out conditions (>2.5 cm maximum size), polymerase chain reaction (PCR) examination of exposed 6-week-old abalones suggested that 1–2 mm abalones may become infected (Moore *et al.,* unpublished observations).

2.2. Host factors

2.2.1. Susceptible host species

Xenohaliotis californiensis infects members of the genus *Haliotis* and natural infections have been observed in black abalones (*H. cracherodii*), white abalones (*H. sorenseni*), red abalones (*H. rufescens*), pink abalones (*H. corrugata*), green abalones (*H. fulgens*), the small abalone (*H. diversicolor supertexta;* 23), the European abalone (3) in the wild or culture facilities, as well as flat (*H. wallalensis*) and Japanese abalones (*H. discus-hannai*) in laboratory challenges (Friedman, unpublished observations). Other abalone species have not been tested.

2.2.2. Susceptible stages of the host

While all post-larval life stages have been demonstrated susceptible to infection with *X. californiensis*, clinical disease is typically observed in animals >1 years of age in farmed abalones (Friedman, unpublished observations) and all abalone size classes observed in wild populations surveyed to date (e.g. 3, 4, 9, 13, 22, 23).

2.2.3. Species or subpopulation predilection (probability of detection)

Probability of detection increases with increasing abalone size. Animals less than 10 mm in size have a reduced probability of detection using histology but equal probability of detection using PCR (11, 19).

2.2.4. Target organs and infected tissue

Xenohaliotis californiensis infects the gastrointestinal epithelial cells of the posterior oesophagus, digestive gland and, to a lesser extent, intestine (6).

2.2.5. Persistent infection with lifelong carriers

Infections may persist for long periods without the development of clinical disease when the host is maintained at cool water temperatures (e.g. 15°C for red abalones), and exposure to elevated seawater temperatures (e.g. >17°C for red, black and white abalones) typically results in clinical disease (8, 16, 20). Recent data suggest that some species, especially those inhabiting warmer waters may harbour the bacterium without the development of clinical disease (23).

2.2.6. Vectors

Although no alternate, non-haliotid hosts have been identified, it has been suggested that some colonial ascidians may concentrate the bacterium (based on PCR evidence). Thus, the possibility of such species acting as a vector for the bacterium exists, but further investigation of possible vectors are warranted (J.D. Moore, unpublished observation).

2.2.7. Known or suspected wild aquatic animal carriers

None.

2.3. Disease pattern

Disease (withering syndrome) occurs at elevated water temperatures (~18°C and above) in abalones with moderate to severe infections (6, 7, 16). The incubation period of withering syndrome is prolonged and typically ranges between 3 and 7 months (3, 4, 7, 9, 16, 17). Clinical disease is characterised by morphological changes in the digestive gland, which vary between species and may include degeneration (atrophy of tubules, increase in connective tissues and inflammation) and/or metaplasia of the digestive tubules. Metaplasia involves the replacement of terminal secretory/absorptive acini with absorptive/transport ducts similar in appearance to the post-oesophagus. These morphological changes are accompanied by anorexia, depletion of glycogen reserves, followed by use of the foot muscle as an energy source and subsequent death (3, 4, 9, 11, 14, 16, 17). The foot of affected abalones contain fewer and less organised muscle bundles, abundant connective tissue and may contain more cerous cells than unaffected individuals (11, 16, 22). Surviving abalones appear to remain infected, even in low water temperature environments, such as in northern California (8).

2.3.1. Transmission mechanisms

Transmission of *X. californiensis* is horizontal and is postulated to be via a faecal–oral route. Exposure of abalones to seawater containing infectious material is sufficient for transmission of the bacterium, and no direct animal contact is required (3, 4, 7, 16, 17). Temperatures below 13°C have been demonstrated to limit transmission of the bacterium (i.e. less than 1% transmission) relative to those held at ~18°C (72–94% transmission) (4).

2.3.2. Prevalence

Table 2.1. *Variation in the prevalence of Xenohaliotis californiensis among species and location*

Species	PCR prevalence		Histology prevalence		References
	Wild	Farmed	Wild	Farmed	
Haliotis rufescens	ND	0–100%	1–75%[1]	0–100%[2]	8, 16, Friedman, unpublished obs.
Haliotis cracherodii	ND	NA	74–98%	NA	8
Haliotis sorenseni	0	0–100%	0	0–100%	11, Moore *et al.,* unpublished obs.
Haliotis fulgens	ND	ND	44–100%	ND	21
Haliotis corrugata	ND	ND	62–63%	ND	21
Haliotis walallensis	NA	NA	0	NA	J.D. Moore, unpublished obs.
Haliotis discus-hannai	ND	0	0	0	C.S. Friedman, unpublished obs.
Haliotis diversicolor supertexta	ND	61%	ND	53%	23[3]
Haliotis tuberculata					3

[1]Prevalences of 1–17% have been observed in northern California and up to 75% in central and southern California. [2]Larger abalones typically have a higher prevalence of infection.
[3]Only 36 animals were sampled from one farm in Thailand. ND = no data: NA = not applicable.

2.3.3. Geographical distribution

Xenohaliotis californiensis occurs along the south-west coast of North America in California, USA and Baja California, Mexico. However, as infected abalones have been transported to Chile, China (People's Rep. of), Chinese Taipei, Iceland, Ireland, Israel, Japan, Spain, and Thailand and possibly other countries, the geographical range of the aetiological agent is suspected to be broad where California red abalones, *Haliotis rufescens*, are cultured or areas where native species have been exposed to red abalones (e.g. see ref. 23).

2.3.4. Mortality and morbidity

Susceptibility varies with species, since the bacterium is known to cause disease in black (up to 99% mortality; 18), white (up to 100% mortality; Friedman & McCormick, unpublished observations), red (up to 35% mortality; 16, 17), pink (also called yellow) and green (also called blue) abalones (21). Unlike the other abalone species studied to date, the magnitude of abalone mortality is not well documented in pink and green abalones. However, in Baja California, Mexico, up to 100% of green (blue) and 63% of pink (yellow) abalones may be infected, with up to 43% of the green and 71% of the pink abalones having microscopic signs of disease (degenerated or metaplastic digestive gland; 21). The incubation period varies with temperature but typically involves a prolonged 3–7 month prepatent period. Mortality typically occurs 1–2.5 months after the onset of visible clinical signs (9). *Xenohaliotis californiensis* was recently observed, based on histological and molecular data, in several Asian countries including China (People's Rep. of), Chinese Taipei and Thailand (23). Prevalence has not been well documented but up to 61% of *H. diversicolor supertexta* were infected at a farm in Thailand, however, like the European abalone, *H. tuberculata*, no abalones exhibited clinical signs of withering syndrome (3, 23).

2.3.5. Environmental factors

Disease (withering syndrome) occurs at elevated water temperatures (~18–25°C in abalones with moderate to severe infections (4, 6, 7, 16, 19). Parasite transmission is enhanced in fed (94%) as opposed to starved (72%) abalones (3, 4). Subclinical infections have been observed in *H. diversicolor supertexta* raised at 27–29°C (23). As abalones are obligate marine species, salinity tolerances of the *Rickettsia*-like organism (RLO) have not been investigated.

2.4. Control and prevention

The most effective prevention is avoidance of the pathogen. Should infection occur, holding abalones at ≤15°C may reduce RLO transmission and subsequent disease transmission (4). Application of oxytetracycline (see Section 2.4.2 below) reduces losses.

2.4.1. Vaccination

Vaccination is not a viable option in controlling infection with *X. californiensis*.

2.4.2. Chemotherapy

Reducing densities and application of an oxytetracycline-medicated diet may reduce losses (10, 11, 19). Oral administration of 12–19% TM-100 (90–100 mg kg^{-1}) in a medicated diet for 10 or 20 days provides protection against bacterial re-infection for several months. Recent data suggest that a single day oral administration of 12% TM-100 can reduce bacterial infections from 80% to 10% prevalence and mean infection intensity from 1.4 to 0.1 on a scale of 0–3 (6, 11). Based on the observation of oxytetracycline in unmedicated abalones that received seawater from treated animals, it is thought that either intake of seawater containing the drug or absorption may be the key route of uptake for this therapeutant (Friedman *et al.,* unpublished data)

2.4.3. Immunostimulation

No data exists on immunostimulation as a control measure for this disease.

2.4.4. Resistance breeding

Interest in selecting for resistant abalones, particularly for restoration purposes, is increasing. Wild black abalones are recruiting along the California Channel Islands and some recruits survive, suggesting that these individuals may be more resistant to this rickettsial disease (VanBlaricom, pers. obs.). Recent laboratory trials have demonstrated enhanced disease resistance in progeny of black abalones that survived *X. californiensis* relative to those from non-disease selected populations (Friedman *et al.* unpublished data).

2.4.5. Restocking with resistant species

No data currently exists and one must consider the relative merits of culturing alternate species.

2.4.6. Blocking agents

No data.

2.4.7. Disinfection of eggs and larvae

No attempts to disinfect eggs and larvae have been undertaken. Abalone larvae are non-feeding and it is unlikely that transmission occurs prior to settlement and metamorphosis after which feeding begins (Moore & Friedman, unpublished data).

2.4.8. General husbandry practices

Husbandry practices to reduce problems caused by *X. californiensis* are typical of those for any bacterial disease and include the purchase of inspected seed (devoid of evidence of infection), maintaining separate families or groups (i.e. avoid high grading and mixing of disparate groups), rinsing hands and equipment in freshwater or iodinated water and drying them in between uses. Isolation of infected groups is recommended if possible. If oxytetracycline treatment is employed, therapeutic application under federal guidelines (e.g. FDA-CVM[1] in the USA or EMEA[2] in Europe) prior to the warm water season may reduce losses and infection. Typically, only a single application during the second or third year of growth is required during a typical 3–4 year culture cycle.

1 United States Food and Drug Administration – Center for Veterinary Medicine.
2 European Medicines Agency.

3. Sampling

3.1. Selection of individual specimens

For routine sampling, random or haphazard selection of individuals is recommended, and the optimal number of samples collected will vary with the population size and level of detection desired (e.g. *n*=60 for a population >2000 individuals). To optimise detection (targeted sampling), selection of abalones exhibiting the clinical sign of reduced weight (atrophied pedal muscle) is recommended. If possible, animals should be sampled after exposure to a period (e.g. 30 days) of warm water (e.g. >18°C).

3.2. Preservation of samples for submission

Samples should be placed in 80–95% non-denatured ethanol (1:9 [v/v] tissue:ethanol) and stored between 4–20°C. Samples may also be flash frozen in liquid nitrogen and stored at –80°C until analysed. Samples in ethanol should be sent to the laboratory on ice according to national or international shipping standards for flammable materials, as applicable. Frozen samples should be sent on dry ice according to national or international shipping standards, as applicable.

3.3. Pooling of samples

Ideally, samples should be excised and stored individually. Should pooling be desired as a cost-saving measure, it is recommended that samples are pooled (*n*=5/pool) for DNA extraction and subsequent PCR analyses. To account for possible pooling-related dilution of target DNA, PCRs should be run in triplicate reactions.

3.4. Best organs or tissues

The best target tissue is the posterior oesophagus and the second best tissue is the digestive gland/intestine complex.

3.5. Samples/tissues that are not suitable

Non-digestive tissues do not contain rickettsial DNA and should be avoided.

4. Diagnostic methods

Gross signs of the disease include pedal atrophy, mottled digestive gland, anorexia, weakness, and lethargy. The disease is characterised by intracytoplasmic bacterial inclusions within the posterior oesophagus, intestine and absorptive/transport epithelia of the digestive gland, whereas moderate to advanced infections are typically associated with degenerative or metaplastic changes within the digestive gland, followed by pedal muscle atrophy in susceptible species.

4.1. Field diagnostic methods

4.1.1. Clinical signs

Abalones with *X. californiensis* infections may be subclinically infected during the prepatent period or at water temperatures ≤15°C. Infected individuals may be slightly to severely emaciated (atrophied) under permissive water temperatures.

4.1.2. Behavioural changes

During an epidemic, affected abalones will often cling to horizontal (as opposed to vertical or inverted) substrates and appear weak (easily removed from the substrate by hand) and emaciated (withered) (13). Farmed abalones will also be anorexic. In addition, the presence of an abnormally high number of fresh shells may also indicate disease.

4.2. Clinical methods

4.2.1. Gross pathology

Clinical characterisation of *X. californiensis* disease relies on a combination of tissue morphological changes in conjunction with the presence of the agent. Morphological changes include an atrophied foot

muscle that is visible at the gross and microscopic level (histology). As a direct result of pedal catabolism, infected abalones excrete substantially higher levels of ammonia than do unaffected individuals (14). If moribund abalones are found, the observation of a mottled digestive gland (dark brown with small foci of tan coloured tissue) indicative of metaplastic changes provides further presumptive evidence of this disease (6).

4.2.2. Clinical chemistry

A standard glycogen assay can be used (3) to test for loss of glycogen in the digestive gland and pedal muscle. However, a decrease in glycogen is related to the anorexia and inability to digest food and, thus, is not specific to this disease (i.e. it is similar to effects of starvation [3]).

4.2.3. Microscopic pathology

The presence of basophilic, oval intracytoplasimic bacterial inclusions in digestive epithelia (posterior oesophagus, transport ducts and metaplastic epithelia of the digestive gland, and or intestine. Metaplasic changes in the digestive gland that include the transformation of the terminal secretory acini into absorptive/transport epithelia is amplified in abalones infected with *X. californiensis* (3, 4, 7, 16, 17). Although metaplasia has been observed in all affected species examined to date, the response to infection may vary between hosts. Red abalones and white abalones, for example, typically respond with a metaplastic change (3, 4, 16), while black abalones generally respond with a combination of metaplasia, digestive tubule degeneration and inflammation (7, 9). Affected individuals contain less pedal glycogen and fewer muscle bundles than do unaffected individuals (3, 4, 12). In some abalones, an increase in cerous cells may be observed in the foot muscle (22), but these signs are not pathognomonic for this disease.

4.2.4. Wet mounts

Although not recommended, cytoplasmic inclusions can be viewed via phase contract or DIC illumination of posterior oesophagus tissues; morphology of the digestive gland makes wet mounts difficult to interpret.

4.2.5. Smears

Stained smears of digestive epithelia may be used to observe bacterial inclusions. However, examination of small pieces of posterior oesophagus that have been dried onto a slide and stained with a DNA fluorochrome may be easier to interpret than stained smears.

4.2.6. Electron microscopy/cytopathology

Transmission electron microscopy (TEM) can be used to confirm the presence of RLO. However this is not confirmatory for this agent because of the lack of unique morphological characteristics. If used, TEM will reveal intracellular colonies of rod-shaped, ribosome-rich prokaryotes with trilaminar cell walls within membrane-bound vacuoles in the cytoplasm of gastrointestinal epithelial cells. The dimorphic rod-to-spherical-shaped bacterium measures an average of 332 × 1550 nm in the bacillus form and an average of 1405 nm in the spherical morphotype. The bacterium reproduces within intracytoplasmic vacuoles 14–56 µm in diameter (6).

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

Several methods to identify and observe *X. californiensis* in tissue samples or extracted DNA are outlined in the sections below.

4.3.1.1. Microscopic methods

4.3.1.1.1. Cytological examination: tissue imprints

Tissue imprints may be used to detect moderate to high intensities of infection of *X. californiensis*. However, histology is more sensitive than tissue imprints.

a) *Stain method:* Excise a section of posterior oesophagus and blot on to slide. Fix the smear in methanol and stain with a modified Giemsa. Dry and observe under oil immersion for rickettsial inclusions or coverslip and examine at ×200–400 magnification.

b) *Fluorescent method:* Excise a section of the post-oesophagus, mince and lay on a slide, dry with a hair dryer for ~20 minutes. Stain the slides using a fluorescent stain for nucleic acid such as

propidium iodide or Hoechst 33258 (13). Incubate in the dark for 3 minutes and view by epifluorescence at ×200 magnification. Bacterial inclusions are differentiated from host nuclei by size and frequency. However, if the sample slides are to be retained for future examination, they should be thoroughly dried and stored desiccated until staining.

Inclusions of the parasite, 14–56 µm in diameter, appear interspersed with the smaller host nuclei. An observation time of 5 minutes per slide is sufficient at ×200 magnification (15). This method is less sensitive than histology and bacterial morphology cannot be differentiated. It is best employed as a rapid examination method within the known range of this disease. The test is not commercially available.

4.3.1.1.2. Histology

The histological procedure is detailed in Chapter 2.4.0 of this *Aquatic Manual*. Remove the shell and cut several 3–5 mm cross sections that contain posterior oesophagus (post-oesophagus), digestive gland, and foot muscle. Place excised tissue into Davidson's or Carson's solutions (see Chapter 2.4.0 of this *Aquatic Manual*) for 24 hours and process for routine paraffin histology. Cross sections are most easily handled when placed in cassettes prior to fixation. The ratio must be no more than one volume of tissue to ten volumes of fixative.

Deparaffinised 3–5 µm sections should be stained with haematoxylin and eosin and viewed by light microscopy for bacterial inclusions (oblong, basophilic intracytoplasmic vacuoles 14–56 µm in diameter [6]) in the post-oesophagus and digestive gland, and morphological changes in the digestive gland and foot. It is recommended that sections should be examined at ×200 or ×400 magnification.

Xenohaliotis californiensis may be morphologically similar to other marine rickettsial bacteria. In Californian abalones, up to three morphologically distinct intracytoplasmic bacteria have been observed (Friedman *et al.,* unpublished data). Definitive diagnosis of the bacterium may include molecular tools (e.g. *in-situ* hybridisation [ISH]). Definitive diagnosis of withering syndrome by histology must include the presence of the bacterium and morphological changes to the digestive gland, metaplasia and/or degeneration, and may include those of the foot muscle.

Where losses have been observed within the known geographical range of withering syndrome, visualisation of intracellular bacterial foci within digestive epithelia, by histological examination, may be considered to be a confirmatory method and is considered the gold standard for this disease. However, confirmation by using histology in conjunction with PCR and sequence analysis or ISH is recommended to verify the identity of the rickettsial bacteria in abalone species previously not known to be susceptible to the bacterium or infection in a new geographical location.

This test is not commercially available.

4.3.1.1.3. Transmission electron microscopy examination

Transmission electron microscopy procedures are described in Chapter 2.4.0 of this *Aquatic Manual*. Rod-shaped, ribosome-rich prokaryotes with trilaminar cell walls accumulated into intracellular colonies within membrane-bound vacuoles in the cytoplasm of gastrointestinal epithelial cells are observed. The dimorphic rod-to-spherical-shaped bacterium measures an average of 332 × 1550 nm in the bacillus form and an average of 1405 nm in the spherical morphotype. The bacterium reproduces within intracytoplasmic vacuoles 14–56 µm in diameter (6). This test is not commercially available.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

None developed.

4.3.1.2.2. Antibody-based antigen detection methods

None developed.

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. Polymerase chain reaction

A positive PCR amplification is only a presumptive diagnosis because it detects DNA and not necessarily a viable pathogen. Other techniques, preferably histology and ISH, must be used to visualise the pathogen. When used in conjunction with histology, PCR may be used for confirmation. Examination of the amplified sequence is recommended when examining a new host species or a new

geographical area. Sequences must be consistent with the known 16S rDNA sequence of this bacterium (GenBank Accession AF133090; [1]).

Samples for PCR should be excised from the post-oesophagus or digestive gland and processed using the classical phenol-chloroform extraction method or the commercial DNA extraction kits designed to remove inhibitors (e.g. stool or soil DNA extraction kits), which are present in abalone digestive gland. Post-oesophagus tissue is recommended because infections are consistently more intense than in digestive gland tissue. A positive control reaction should always be included in the PCR and should consist of genomic DNA extracted from a known infected individual or the use of a plasmid containing an insert of the amplified product. If a plasmid positive control is to be used, it is recommended that an insert of ~100 bp should be added to the cloned fragment to alleviate concerns over cross contamination of aerosolised plasmid DNA. In addition, in situations where low copy numbers of the target DNA are present, this plasmid may out compete the target. A negative control consisting of master mix without the addition of template should also be included in each PCR. All reactions should be run in duplicate. Observation of a 160 bp band in tissue samples and positive control reactions, as well as no bands in the negative control reactions, characterise a successful test. The sensitivity and specificity of this test are in the process of being formally assessed by the OIE Reference Laboratory (Friedman *et al.,* unpublished data.). No commercial tests are currently available.

The PCR primers developed for *X. californiensis* detection specifically amplify a 160 bp segment of the *Rickettsia*-like pathogen. Primers are currently designated as: RA 5-1 (5'-GTT-GAA-CGT-GCC-TTC-AGT-TTA-C-3') and RA 3-6 (5'-ACT-TGG-ACT-CAT-TCA-AAA-GCG-GA-3'). They target small subunit ribosomal DNA and have been shown to be sensitive and specific for this pathogen (1). PCR amplification is performed in a standard 20 µl reaction volume containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 400 ng ml^{-1} BSA, 200 µM of dNTPs, 0.5 µM of each primer, 1.6 units of Taq polymerase, and 100 ng template DNA. The reaction mixtures are cycled in a thermal cycler. The programme for the amplification reaction is: Initial denaturation at 95°C for 5 minutes, 40 cycles at 95°C for 1 minute, 62°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. An aliquot of each PCR reaction is checked for the 160 base pair amplification product by agarose gel electrophoresis and ethidium bromide staining.

This test is not commercially available

4.3.1.2.3.2 Sequence analysis

Analysis of amplified DNA is needed to confirm sequence identity with the target bacterium. This may be accomplished via standard cloning and sequencing of multiple clones after PCR amplification of sample DNA. (Note: both forward and reverse sequencing is recommended.) Direct sequencing of PCR products may also be employed.

4.3.1.2.3.3 In-situ *hybridisation*

ISH is the method of choice for confirming identification because it allows visualisation of a specific probe hybridised to the target organism. However, DNA probes must be thoroughly tested for specificity and validated in comparative studies before they can be used for confirmatory identification.

ISH has been developed to detect Rickettsiales-like prokaryotes in tissue sections (2). Specific labelled oligonucleotide probes hybridise with the small subunit ribosomal RNA of the bacterium. This hybridisation is detected by an antibody conjugate that recognises the labelled probes. Substrate for the antibody conjugate is added, causing a colorimetric reaction that enables visualisation of probe–parasite DNA hybridisations. Although this method has not been formally validated, tests for specificity using several bivalve and fish rickettsial organisms suggested that the test was specific for *X. californiensis* (2).

The procedure of ISH is conducted as follows. Positive (known infected tissues) and negative (uninfected or those infected with a different bacterium) controls must be included in the procedure. The probe is made by PCR using a PCR DIG Probe Synthesis Kit. Before using the DIG labelled probe, denature the probe at 95°C for 3 minutes and immediately place on ice for ~30 minutes to separate the double stranded DNA. Store at –20°C or –80°C until use. The sequences of the probes designated as RA 5-1, RA 3-6, RA 3-8 and RA 5-6 (2) are, respectively: 5'-GTT-GAA-CGT-GCC-TTC-AGT-TTA-C-3', 5'-ACT-TGG-ACT-CAT-TCA-AAA-GCG-GA-3', 5'-CCA-CTG-TGA-GTG-GTT-ATC-TCC-TG-3', and 5'-GAA-GCA-ATA-TTG-TGA-GAT-AAA-GCA-3'.

i) After removing the shell, a transverse section (3–5 mm) is cut so that it contains posterior oesophagus (post-oesophagus), digestive gland, and foot muscle and placed in Davidson's AFA fixative (glycerin [10%], formalin [20%], 95% ethanol [30%], dH_2O [30%], glacial acetic acid [10%]) for 24–48 hours, then transferred to 70% ethanol until processed by histological procedures (step ii). The ratio must be no more than 1 volume of tissue to 10 volumes of fixative.

ii) The samples are subsequently embedded in paraffin by conventional histological procedures. Sections are cut at 5–6 µm and placed on positively charged slides or 3-aminopropyl-triethoxylane-coated slides. Histological sections are then dried overnight in an oven at 40°C. Note: the drying step may be omitted if using positively charged slides.

iii) The sections are deparaffinised by immersion in xylene or other less toxic clearing agent for 10 minutes. The solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each and rehydrated by immersion in an ethanol series. The sections are then washed twice for 5 minutes in Tris buffer (pH 7.2; 0.2 M Tris/HCl, 2.0 mM CaCl).

iv) The sections are treated with proteinase K, 50 µg ml^{-1} in Tris buffer, at 37°C for 45 minutes. The reaction is then stopped by washing the sections in PBS three times for 10 minutes each.

v) The sections are prehybridised for 10 minutes to 1 hour at 37°C in prehybridisation buffer (4 × SSC, 50% formamide).

vi) The prehybridisation solution is then rinsed off in 2× SSC and briefly dried prior to being replaced with prehybridisation buffer containing the digoxigenin-labelled probes (1:373, probe:buffer [v:v]). The sections may be denatured* by being placed on a heating block at 100°C for 10 minutes and then covered with ISH plastic cover-slips. The slides are then hybridised overnight at 53°C in a humid chamber. *This step may be omitted if desired.

vii) Carefully remove cover slips from the section by immersing slides for 5-10 minutes in 2× SSC at room temperature. The sections are washed twice for 15 minutes in 2 × SSC at 40°C, three times for 15 minutes in 1 × SSC at 40°C, and once for 15 minutes in 0.5 × SSC at 40°C. The sections are then placed in Buffer 1 (100 mM Tris/HCl, 10 mM NaCl, pH 7.5) for 10 minutes.

The sections are placed in Buffer 1 (see step vii) supplemented with 0.3% Triton X-100 and 2% sheep serum for 1 hour at room temperature (do not let slides dry out).

Anti-digoxigenin alkaline phosphatase antibody conjugate is diluted 1:1000 (or according to the manufacturer's recommendations) in Buffer 1 supplemented with 0.3% Triton X-100 and 1% sheep serum and applied to the tissue sections. The sections are covered with ISH cover-slips and incubated for 2 hours at room temperature in the humid chamber.

x) The slides are washed twice in Buffer 1 for 10 minutes each (see step vii) and twice in Buffer 2 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 10 minutes. Colour development solution (add 45 µl nitroblue tetrazolium, 35 µl 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt [BCIP]) to 10 ml Buffer 2 for 30 minutes to 1 hour in a humid chamber in the dark.

xi) The slides are then rinsed 3× in sterile distilled water (dH_2O). The sections are counterstained with Bismarck Brown Y for 3 minutes, rinsed in dH_2O, then 70% ethanol, followed by a brief rinse in 100% ethanol prior to being air dried and cover-slips applied using a mounting medium. The presence of the pathogen is demonstrated by the purple-black labelling of the parasitic cells.

This test is not commercially available.

4.3.2. Serological methods

Not developed.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of *X. californiensis* are listed in Table 5.1 below. The designations used in the table are as follows: a = this method is the recommended method due to availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

***Table 5.1**. Methods for targeted surveillance, detection and diagnosis*

Method	Targeted surveillance			Presumptive diagnosis	Confirmatory diagnosis
	Larvae	Juveniles	Adults		
Gross signs	d	c	c	c	d
Bioassay	d	d	c	c	c(a)[1]
Tissue imprint – Giemsa stain	d	c	c	b	b(c)[2]
Histopathology	d	b	b	a	a[3]
Transmission EM	d	d	d	b	c
DNA probes – *in situ*	d	c	c	a	a
PCR	d	a	a	a	c(a)[4]
SSU rDNA sequence	d	d	d	a	a

[1]For valuable broodstock, it is possible to use polymerase chain reaction (PCR) of faeces as a first screen and, if negative, subsequently to use the bioassay method in combination with histology (See Section 6). [2]Tissue imprints should be used in combination with PCR and possibly sequencing to confirm the agent. [3]In new cases, such as a new geographical location, PCR and sequencing are recommended to confirm identity of the bacterium. [4]PCR alone is not confirmatory but when used in combination with histology, it may be considered confirmatory. EM = electron microscopy; SSU rDNA = small subunit ribosomal DNA.

6. Test(s) recommended for targeted surveillance to declare freedom from infection with *Xenohaliotis californiensis*

The method for targeted surveillance to declare freedom from *X. californiensis* is histology in combination with PCR and sequence analysis, or via ISH. Given the chronic nature of the disease and influence of temperature it is recommended that animals in the nursery and grow out are examined during the warm water season for the site. Abalones held in cooler waters may be chronically infected without showing any signs of disease. It is also recommended that PCR examination of faeces or bioassay of smaller abalones (e.g. 1–4 cm) commingled with broodstock for at least 6 weeks at >17°C is also used.

7. Corroborative diagnostic criteria

In accordance with the *Aquatic Code*, all cases in other species should be referred immediately to the appropriate OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.

7.1. Definition of suspect case

A suspect case of *X. californiensis* infection and associated clinical disease (withering syndrome) may include the observation of gross clinical signs (weakness, lethargy, anorexia, pedal atrophy, and mottled digestive gland) and mortality in association with warm water conditions, particularly within the known geographical range of this disease. In farmed abalones, anorexia may be a first sign of disease. These clinical signs in combination with either microscopic observation of an atrophied foot muscle, inclusion bodies in gastrointestinal epithelia, or PCR evidence (without sequence confirmation) also represent a suspect case.

7.2. Definition of confirmed case

Confirmation of *X. californiensis* infection relies on observation of the agent using histology and PCR with sequence analysis, or ISH. Gross signs and tissue imprints alone cannot be used for confirmatory diagnosis and must be supported by histology, ISH or PCR with sequence analyses.

Confirmation of withering syndrome relies on both the presence of the agent and the presence of microscopic signs of the disease. As a minimum, digestive gland metaplasia or degeneration, as evidenced on histological examination, must accompany *X. californiensis* infection to diagnose clinical withering syndrome.

8. References

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NB: There is an OIE Reference Laboratory for infection with *Xenohaliotis californiensis* (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).