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9. *Vibrio harveyi* group

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9.1. Introduction

Vibrio harveyi and related species are referred to as the *Harveyi* clade (Sawabe *et al*., 2007; Sawabe *et al*., 2013). There is no definitive consensus on which species comprise the *Harveyi* clade, and up to 11 species have been included in this clade following MLSA analysis: *V. harveyi, V. alginolyticus, V. campbellii, V. mytili, V. natriegens, V. parahaemolyticus, V. rotiferianus, V. azureus* (Yoshizawa *et al*., 2009), *V. sagamiensis* (Yoshizawa *et al*., 2010), *V. owensii* (Cano-Gómez *et al*., 2010), *V. jasicida* (Yoshizawa *et al*., 2012). Members of this clade share a high degree of genetic and phenotypic similarity and are commonly found in marine and estuarine water and sediments or as commensal, opportunistic or primary pathogens of marine species. Among vibrios of the *Harveyi* clade, four species (*V. harveyi, V. campbellii, V. rotiferianus* and *V. owensii)* known as the *V. harveyi* group, are well-known pathogenic agents in marine reared fish, crustaceans and shellfish, being responsible for high mortality rates in commercial farms worldwide (Cano-Gómez *et al*., 2011).

9.2. Aetiology of *V. harveyi*

*Vibrio harvey*i (Johnson and Shunk, 1936) is a Gram-negative bacterium of the family *Vibrionaceae* (Gammaproteobacteria) that has been recognized as an emerging pathogen for global marine aquaculture (Austin and Zhang, 2006). It has been reported with increasing frequency in finfish species reared in subtropical regions (groupers, barramundi, flatfish, pompano), in sharks and in gastropods (abalone), and it is a well-known problem in the husbandry of post-larvae stages of penaeid shrimps (luminous vibriosis). In Europe and particularly in the Mediterranean basin this microorganism is gaining importance as a primary pathogen of European seabass (*Dicentrarchus labrax*) and flatfish (*Solea* spp.).

Type strains of the previously recognized species *Vibrio carchariae* (Grimes *et al.,* 1985) and *Vibrio trachuri* (Iwamoto *et al*., 1995) were determined to be synonyms of *V. harveyi* based on molecular studies (Pedersen *et al*., 1998; Thompson *et al*., 2002). *V. harveyi* is found in a free-living state in aquatic environments and as a part of the normal flora of marine animals (Makemson and Hermosa, 1999). *V. harveyi* isolates from the environment or invertebrate hosts (crustacean) are frequently characterized by luminescence.

V. harveyi causes, in the majority of teleost fish, cutaneous, ophthalmic or gastroenteric infections that frequently generalize in septicaemia (Austin and Zhang, 2006). Cutaneous lesions appear as erosions, haemorrhaging at the base of the fins, ulcerations or necrotic vesicles of the dermis. Ocular lesions encompass keratitis, corneal opacities or panophthalmitis frequently related to secondary infection after traumatic or parasitic lesions (Pakingking *et al*., 2018; Minami *et al*., 2016). Gastrointestinal infections show serous or serous-catarrhal enteritis with marked dilatation of the intestinal lumen (proximal tract) and accumulation of yellowish exudate (Lee *et al*., 2002; Yii *et al*., 1997). *V. harveyi* has been isolated in sharks from cutaneous ulcers and in septicemic forms characterized by vasculitis (Grimes *et al*., 1985).

In *D. labrax*, *V. harveyi* has been isolated during mortality outbreaks characterized by lethargy, anorexia and ataxia, occurring mainly during the grow-out phase (40-160 g) and in juveniles housed in hatcheries. Affected specimens showed cutaneous or ophthalmic lesions, enteric inflammation and encephalic congestion.

9.3. Sampling

9.3.1. Preparation and shipment of samples from fish

Symptomatic or moribund fish or recently dead specimens (less than 2-3 h) should be preferred and sent refrigerated to the laboratory as soon as possible (no later than 24-36 h). If possible, for example when fingerlings are affected, live fish should be sent inside a double plastic bag (one part water and two parts air/oxygen). When feasible, collect the animals before the administration of any antimicrobial treatment in order to avoid false-negative results.

V. harveyi can be isolated from swabbing from skin ulcers or internal organs (head kidney), swabs embedded in Amies transport medium and maintained at a temperature lower than 10°C (not frozen).

All materials should be placed in leak-proof containers and precisely labelled. Please refer to Chapter 2.2. for specific instructions on shipping biological substances.

9.4. Diagnostic procedures for *V. harveyi*

9.4.1. Primary cultivation of bacteria

V. harveyi can be retrieved by standard bacteriological sampling from head kidney, spleen, brain, cutaneous lesions or ocular lesions.

If submitted specimens are smaller than 3-4 cm (juveniles or larvae), rinse the animals with three or four washes of sterile saline solution, mince the fish with sterilized blades and with a sterile loop inoculate the appropriate media.

V. harveyi can be easily isolated in blood agar (BA), tryptone soya agar (TSA) supplemented with 2% NaCl, marine agar or in liquid mediums (TSB 2% NaCl or Marine broth) maintained at 22-25°C. Colonies can be observed after 24-48 hours. Direct isolation in a selective and differential medium like TCBS is a viable option.

9.4.2. Screening of pure cultures

Macroscopically *V. harveyi* colonies do not show any discriminant features on BA, except luminescence (for some isolates) when observed in the dark; they generally appear as greyish-white, slightly translucent, non swarming colonies. Haemolytic activity (α or β-haemolysis) can be observed on BA (≈50% of isolates) after 24-48 h. Luminescence is relatively infrequent in isolates from fish (10% of isolates) but, if present, is easier to observe in colonies cultivated on BA than TSA 2%NaCl.

V. harveyi isolates from finfish typically metabolize sucrose and appear yellow on TCBS agar, while their coloration may vary on CHROMagar Vibrio™. Most frequently isolates appear bicolour (lilac and white): colonies at high densities appear pale lilac or rose, while isolated colonies appear white. Other colorations are uniform pale rose or pale lilac or milky white (Pretto, 2018).

V. harveyi is a Gram-negative short rod, slightly curved, pleomorphic, facultative anaerobic, motile by single polar flagella with dimensions that vary according to author: 1.0-1.6 × 0.5-0.7 µm (Buller, 2014); 1.9-3.0 × 1.2-1.4 µm (Shen *et al.,* 2017), 1.6-2.2 × 1.0-1.1 µm (Tu *et al.*, 2017). It is capable of oxidative and fermentative metabolism, oxidase and catalase positive. *V. harveyi* is halophilic and grows at NaCl concentration between 1-7%, temperature between 10-40°C; no growth is observed at 42°C. It can swarm on TSA 2% NaCl (≈50% of isolates) or marine salt agar but not on BA, TCBS, MacConkey agar (Buller, 2014) or CHROMagar Vibrio™. *Vibrio harveyi* is sensitive to vibriostat agent O/129 (2, 4- diamino-6, 7-di-iso-propylpteridine phosphate) at high concentrations (150 µg), whereas results may vary at low concentrations (10 µg) (Buller, 2014).

9.4.3. Identification of the strain

9.4.3.1. API

API®20E™ (bioMérieux) test gives an effective identification for *V. harveyi*; it should be performed with inoculum at 0.5 McFarland (bacteria suspended in a solution with 2% NaCl) maintained at 25°C and evaluated after 24 and 48 hours. The most discriminating tests for *V. harveyi* isolated from teleost fish are related to aminoacid metabolism (ADH -; LDC +; ODC +), citrate (CIT+), hydrogen sulphide (H2S -), β-galactosidase (ONPG -), tryptophan deaminase (TDA -), indole (IND +), Voges-Proskauer test (VP -) and gelatinase (GEL +, 80%). Citrate metabolism and gelatinase may appear later (after 48-72 h). Sugar metabolism may vary for sorbitol (SOR +; 60%) and sucrose (SAC +; 95%) and appears positive for glucose, mannose and amygdalin and negative for inositol, rhamnose, melibiose and arabinose (Pretto, 2018).

Urease test appears frequently negative (URE -; 95%), whereas positive results are obtained with Christensen agar slant.

The most frequent API®20E™ code for *V. harveyi* isolates in *D. labrax* are 4346525, 4346125, 4344125; less frequently: 4356525, 4354525, 4346105, 4344525, 4246525, 4344125 (Pretto 2018).

The interpretation of the API®20E™ (bioMérieux) results via Apiweb™ does not effectively identify *V. harveyi*, because the bioMérieux database does not contain a profile for this species in the API 20E system. Consequently, the identification of codes obtained from *V. harveyi* strains is assigned to *Vibrio alginolyticus*, which is the most phenotypically similar species between the Vibrionaceae of the database.

9.4.3.2. Mass spectrometry

The identification of isolates belonging to the species *V. harveyi* can be effectively obtained through MALDI-TOF analyses, generally with good identification scores. It is important to evaluate the presence of all the species considered belonging to the *Harveyi* clade in the database of the instrument and if necessary integrate the database with reference strains of the species missing or improve the database with more than one isolate for each species.

9.4.3.3. PCR

Molecular analysis can be performed on isolated bacterial colonies. DNA extraction is performed on a single isolate (clonal growth after 24 h in BA or TSA 2%NaCl) collected with a loop and mixed in a suitable amount with the lysis buffer of the DNA extraction kit selected, following the manufacturer's instructions. Extracted DNA should be standardized at 100 ng/µl.

Identification of *V. harveyi* can be performed by diagnostic laboratories with two different approaches:

(i) Amplification of the housekeeping gene uridine monophosphate kinase (*pyrH*) followed by sequencing and phylogenetic analysis in order to confirm the identification of the isolate at the species level through the comparison with the sequences available in the literature.

(ii) Amplification of the *toxR* gene in its hypervariable region by *V. harveyi* species-specific primers (Pang *et al.*, 2006).

(i): end-point PCR for the *pyrH* gene, following (Sawabe *et al.,* 2007; Pascual *et al.,* 2010).

Forward primer	**pyrH80F**	5'- GATCGTATGGCTCAAGAAC-3'
Reverse primer	**pyrH530R**	5'-TAGGCATTTTGTGGTCACG-3'

PCR mix contains the following reagents:

Reagent	Quantity
Water (molecular biology grade)	34.20µl
10X Buffer (-MgCl2)	5 µl
50mM MgCl2	1.5 µl
10 mM dNTPs	4 µl
10 µM primer pyrH80F	2 µl
10 µM primer pyrH530	2 µl
Platinum Taq polymerase 5 U/µl	0.3 µl
DNA samples	1 µl
Total volume	50 µl

Thermal profile:

Initial polymerase activation	Denaturation	Annealing	Extension	Final extension
95°C 2 min	94°C 1 min	53°C 2 min 15 sec	72°C 75 sec	72°C 7 min
	40 cycles			

Expected amplified product is 449 bp long.

(ii): end-point PCR *V. harveyi*, species-specific (*toxR* gene) following (Pang *et al.*, 2006).

Forward primer	**toxRF1**	5' - GAAGCAGCACTCACCGAT-3'
Reverse primer	**toxRR1**	5'- GGTGAAGACTCATCAGCA-3'

PCR mix contains the following reagents:

Reagent	Quantity
Water (molecular biology grade)	34.20 µl
10X Buffer (-MgCl2)	5 µl
50mM MgCl2	1.5 µl
10 mM dNTPs	4 µl
10 µM primer toxRF1	2 µl
10 µM primer toxRR1	2 µl
Platinum Taq polymerase 5 U/µl	0.3 µl
DNA samples	1 µl
Total volume	50 µl

Thermal profile (Pretto, 2018), modified from (Pang *et al.*, 2006):

Initial polymerase activation	Denaturation	Annealing	Extension	Final extension
94°C 1 min	94°C 1 min	61°C 1 min 15	72°C 1 min	72°C 10 min
	30 cycles			

Expected amplified product is 382 bp long.

9.4.4. *In vitro* susceptibility testing

The Kirby Bauer Disk diffusion test can be effectively performed on Mueller Hinton agar supplemented with 2% NaCl evaluated at 25°C after 24 h. Minimum Inhibitory Concentration can be performed following the CLSI protocol (CLSI, 2014) applying a suspension of 18-24 h young culture in cation-adjusted Mueller Hinton broth (CAMHB) with a final concentration of 5×10^5 ufc/ml. Incubation is best performed at 22°C for 24 h.

V. harveyi isolated from farmed *D. labrax,* collected in the Mediterranean basin, evidenced a limited number of antibiotic resistance. Susceptibility to florfenicol, tetracycline, flumequine and sulfamethoxazole+trimethoprim has been observed in the majority of isolates (Pretto, 2018). Resistance is observed to ampicillin and colistin.

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Photos

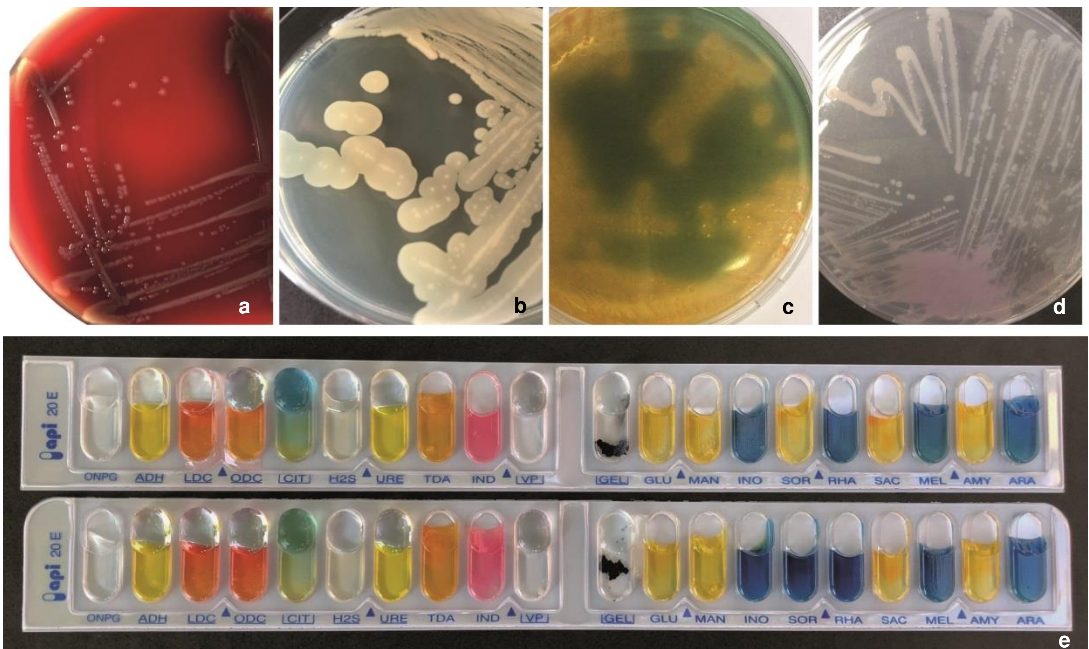

Fig. 9.1. a) *V. harveyi* growth on blood agar with greyish slightly translucent colonies; b) colonies of *V. harveyi* on TSA 2%NaCl may show some swarming; c) yellow colonies of *V. harveyi* on TCBS; d) bicolour growth on CHROMagar Vibrio™, with pink and white colonies; e) most frequent *V. harveyi* results of API®20E™ after 24 h; gelatinase and citrate should be evaluated after 48 h.

Fig. 9.2. a) Cutaneous erosions and ulcers in *D. labrax* juveniles (arrows); b) meningeal and encephalic congestion of blood vessels in *D. labrax* juvenile; c) serous-catarrhal enteritis with marked dilatation of the intestinal lumen (arrows).