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Evidence of fish and human pathogens associated with doctor fish (*Garra rufa*, Heckel, 1843) used for cosmetic treatment

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1 **Evidence of fish and human pathogens associated with doctor fish (*Garra rufa*, Heckel,**  
2 **1843) used for cosmetic treatment**

3 Running title: Zoonotic pathogen detection in fish spa

4  
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25

26 **Abstract**

27 Doctor fish (*Garra rufa*, Heckel, 1843) are increasingly used for cosmetic treatment raising  
28 particular concerns regarding the potential transmission of infections to clients.

29 Investigations of microbial causes undertaken in two outbreaks of mortality among *G. rufa*  
30 used for cosmetic treatment revealed the presence of multiple bacteria, including both fish and  
31 human pathogens such as *Aeromonas veronii*, *A. hydrophila*, *Vibrio cholerae*, *Shewanella*  
32 *putrefaciens*, *Mycobacterium marinum* and *M. goodii*. This range of bacteria indicates an  
33 intense microbial proliferation involving multiple pathogens, most likely induced by the poor  
34 health condition of the fish.

35 Most of the detected pathogens are well-known agents of zoonosis. Indeed, *M. goodii* is an  
36 emerging nosocomial human pathogen that has never been detected in fish to date, nor in  
37 other animals. This first detection of *M. goodii* associated with fish infection points out a new  
38 zoonotic potential for this pathogen.

39 These findings point out that handling, poor environmental conditions and the presence of fish  
40 pathogens, that can compromise the immune system of fish, can result in a mixed microbial  
41 proliferation and increase the spread of water-borne bacteria, including zoonosis agents.

42 Accordingly, the microbiological surveillance of fish used for cosmetic treatment is extremely  
43 important, particularly in association with mortality outbreaks.

44

45 **Keywords:** *Garra rufa*; Fish spa; *Mycobacterium* spp.; Public health; *V. cholerae*; Zoonotic  
46 bacteria

## 47 **1. Introduction**

48 The use of doctor fish (*Garra rufa*, Heckel, 1843) for cosmetic treatment is increasingly  
49 common in European countries. *G. rufa* is a non-migratory, freshwater species belonging to  
50 the family Cyprinidae (Ruane et al., 2013). In particular, this fish species is widely used in  
51 pedicures, nibbling off dead skin and offering both aesthetic and psychological benefits to  
52 clients. Nevertheless, certain concerns have arisen given that the same water and fish may be  
53 used for subsequent customers. Zoonotic agents may be transmitted either directly from the  
54 affected fish, or indirectly through the water. Moreover, human pathogens can be transmitted  
55 from one person to another through either the water or the fish hosting the pathogen (HPA,  
56 2011; Gauthier, 2015). The welfare of fish should also be considered, both for ethical and  
57 sanitary reasons. In fact, the maintenance of stress conditions for fish, such as poor water  
58 quality, overcrowding and rough handling, can promote the microbial proliferation of both  
59 fish and human pathogens (HPA, 2011).

60 Only scattered citations of episodes of mortality affecting this species are available (Baeck et  
61 al., 2009; Majtan et al., 2012; Ruane et al., 2013; Lee et al., 2016), and episodes of *G. rufa*  
62 mortality indicate bacteria such as *Citrobacter freundii*, *Aeromonas sobria*, *Shewanella*  
63 *putrefaciens* and *Streptococcus agalactiae* as potential causative agents (Baeck et al., 2009;  
64 Majtan et al., 2012; Verner-Jeffreys et al., 2012; Lee et al., 2016). Furthermore, pathogenic  
65 viral agents of the genus *Aquabirnavirus* have been identified in *G. rufa* collected during a  
66 mortality outbreak, although no clinical signs of disease attributable to this pathogen were  
67 found, with the authors positing that the mortalities were most likely due to transport-related  
68 stress, exacerbated by the presence of the pathogens (Ruane et al., 2013).

69 Investigations of the presence of human pathogens in *G. rufa* and/or the water of fish spas are  
70 even less common, and those results that do exist contrast significantly (Verner-Jeffreys et al.,  
71 2012; Schets et al., 2015; Pennazio et al., 2016). A survey conducted in *G. rufa* batches

72 imported into the United Kingdom showed the presence of several pathogens, including *A.*  
73 *veronii*, *Shewanella* spp., *Vibrio vulnificus*, *V. cholerae*, mycobacteria and *S. agalactiae*  
74 (Verner-Jeffreys et al., 2012). The authors did not report whether the fish showed any clinical  
75 signs, but due to the nature of surveillance on moving batches, the fish were assumed healthy.  
76 On the other hand, an Italian survey of fish collected from beauty centres indicated the  
77 negativity of all microbiological, parasitological and mycobacterial analyses conducted on  
78 each sample (Pennazio et al., 2016). Furthermore, microbial investigations have been  
79 conducted on the water in fish spas with *G. rufa*, such as in the Netherlands with the presence  
80 of *Aeromonas* spp., *Vibrio* spp., including *V. cholerae*, *Pseudomonas aeruginosa*,  
81 *Mycobacterium* spp. including *M. fortuitum*, *M. conceptionense*, *M. abscessus* and *M.*  
82 *chelonae* (Schets et al., 2015).

83 For these reasons, disease and mortality outbreaks in fish spas must be monitored and  
84 controlled in order to improve fish health, prevent microbial dissemination and pave the way  
85 to establishing proper legislative rules.

86 In this study, the microbial causes of two mortality outbreaks involving *G. rufa* used for  
87 cosmetic treatments are investigated. The study shows that handling and poor water quality  
88 can lead not only to outbreaks of disease and mortality among fish, but also to the increase of  
89 zoonotic bacteria that are potentially risky to public health.

90

## 91 **2. Materials and methods**

### 92 2.1 Clinical history and sampling

93 In the summer of 2016, a disease outbreak occurred in a fish spa located in Italy. *G. rufa* fish  
94 were held in 100 L glass tanks with a stocking density of 1.2 fish/L and thermostated at 29°C.  
95 The outbreak resulted in a chronic mortality (daily mortality 3-4%) of fish a few days after  
96 their arrival, and lasted for several weeks. Cumulative mortality reached 60% in four weeks.

97 Fish, 6 cm in length, were collected from the tank where mortality was observed (batch  
98 AUG2016) and were carried alive to the laboratories for diagnostic investigations.  
99 After the tanks were emptied, disinfected with hypochlorite and subjected to fallowing, a new  
100 stock of fish was placed in the fish spa in April 2017, and the sampling of asymptomatic fish  
101 was conducted at the time of placement (batch APR2017). At the same time, a dead fish was  
102 collected from a tank (same batch APR2017). Several days following placement, the fish  
103 started to die in three of the five tanks, with a daily mortality of 3-7% and a cumulative  
104 mortality of 58, 75 and 83% in six weeks in the three tanks involved in the outbreak,  
105 respectively. Samples was conducted from the two tanks that showed the highest mortality  
106 (batch MAY2017).

107 The fish collected for sampling were euthanised with an overdose of tricaine  
108 methanesulfonate (200 ppm) and immediately sampled for diagnostic investigations using  
109 clean, sterile dissecting instruments to prevent contamination between the outside and inside  
110 of the fish and between the tissues.

111

## 112 2.2 Gross examination and histopathology

113 Five fish collected during the first sampling (AUG2016), three asymptomatic fish and one  
114 dead fish collected in the second sampling (APR2017), and ten fish collected during the third  
115 sampling (MAY2017) were fixed *in toto* in 10% buffered formalin and processed for routine  
116 histology. Histological sections of 3 µm were cut and stained with hematoxylin and eosin  
117 (H&E).

118 When granulomas were evidenced, they were classified on the basis of their histological  
119 features and timing of lesions (Antuofermo et al., 2017) as “early stage” granulomas  
120 (presence of groups of macrophages with a granular eosinophilic cytoplasm, round to ovoid  
121 pale nuclei arranged in a spherical pattern), “intermediate” granulomas (central core of

122 coagulative necrosis surrounded by a thin inner layer of flat cells and externally  
123 macrophages), or “late stage” granulomas (several layers of intensely eosinophilic necrotic  
124 material surrounded by a layer of fibroblasts).

125 Furthermore, when granulomatous lesions were detected, histochemical staining for the  
126 detection of acid-fast bacteria was performed (Zeehl-Neelsen Kit, Histoline Laboratories,  
127 Milan, Italy).

128

### 129 2.3 Immunohistochemistry

130 Immunohistochemistry was carried out on all formalin-fixed paraffin-embedded (FFPE)  
131 samples with a polyclonal antibody against *Mycobacterium bovis* (Bacillus Calmette-Guerin,  
132 BCG, code no. B 0124 Dako, Denmark). This polyclonal antibody was previously applied  
133 successfully to immunolabel *M. marinum* in fish (López et al., 2018). Endogenous peroxidase  
134 inhibition was made with 3% H<sub>2</sub>O<sub>2</sub> methylene solution. The antigen retrieval was made using  
135 a microwave oven at 750W, 2 cycles x5 min. Pre-incubation with a blocking solution (10%  
136 Normal Goat Serum and PBS) was performed for 30 min, and then incubated overnight at 4°C  
137 with a primary antibody at 1:3000 dilution in a blocking solution. The secondary anti-rabbit  
138 antibody was incubated at 1:200 dilution for 30 min, followed by a revelation system with an  
139 ABC kit developed with diaminobenzidine (DAB) chromogen for 90 sec and countercoloured  
140 with Papanicolau hematoxylin.

141

### 142 2.4 Bacteriological examination

143 Three to five fish for each sampling were examined for the presence of bacteria. The kidney  
144 was sampled by loop and immediately streaked onto TSA (Tryptone Soy Agar, Oxoid, UK).  
145 In case of skin lesions in batch MAY2017, bacteriological investigation was also conducted in  
146 the affected area. Plates were incubated at 28°C for three days. Randomly chosen colonies

147 were purified onto TSA and TSA NaCl 3% respectively, and incubated at 28°C for 24 h to  
148 obtain pure cultures for further phenotypic and genotypic characterisations.

149

#### 150 2.4.1 Phenotypic characterisations

151 The following were utilised as screening tests: Gram-stain and cell morphology  
152 determination; oxidase production (OXIBIOSWAB, Biolife); glucose fermentation, utilisation  
153 of lactose and H<sub>2</sub>S (hydrogen sulfide) production on Kligler Iron Agar (Oxoid); motility and  
154 indole production on SIM (sulphide–indole-motility) medium (Oxoid); reduction of nitrate  
155 (Zen-Yoji et al., 1973). When non-halophilic *Vibrio* sp. was suspected, growth on TCBS  
156 (Thiosulfate-Citrate-Bile salts-Sucrose) Agar was also tested.

157

#### 158 2.4.2 Genotyping

159 Molecular identification through 16S rDNA amplification and sequencing was performed. In  
160 brief, DNA was extracted from the colonies through the boiling method, and amplification of  
161 16S rDNA was performed with primers P0F and P6R using 1 µl of each lysed cell suspension  
162 according to the procedure described previously (Caccamo et al., 1999). Polymerase chain  
163 reaction products were purified and sequenced to confirm the bacterial identity. When  
164 *Aeromonas* sp. was suspected, species identification was conducted by *gyrB* gene  
165 amplification and sequencing (Bio-Fab Sequencing Service, Rome, Italy). Sequences were  
166 aligned and compared with reference strain sequences using Clustal W in BioEdit software  
167 (bioedit.software.informer.com). The percentage of similarity of pairwise distances was  
168 calculated with BioEdit software. When *Vibrio* sp. was suspected, species identification was  
169 conducted by a multiplex PCR assay targeting species-specific genes for *V. cholerae*, *V.*  
170 *parahaemolyticus* and *V. vulnificus* (Passalacqua et al., 2016).

171

172 2.5 Molecular investigation for Infectious pancreatic necrosis virus, Aquabirnavirus,  
173 Birnaviridae

174 The presence of *Infectious pancreatic necrosis virus* (IPNV) RNA was investigated in two  
175 fish for each sampling. In order to investigate the presence of IPNV, RNA was extracted from  
176 approximately 25 mg of tissue (visceral specimens) using NucleoSpin RNA II (Macherey-  
177 Nagel, Duren, Germany) according to the manufacturer's instructions. The RNA samples  
178 were subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) analysis to  
179 detect IPNV with a protocol previously described (Pinheiro et al., 2016).

180

181 2.6 Molecular investigation for mycobacteria

182 Due to the presence of granulomas indicated by histopathology investigation, a retrospective  
183 survey was conducted on samples stored frozen, if available, or on those formaldehyde-fixed  
184 paraffin embedded (FFPE).

185 Visceral specimens stored frozen (-20°C) of batch AUG2016 (n=4) and MAY2017 (n=2)  
186 were processed for DNA extraction using the Purelink Genomic DNA kit (Invitrogen,  
187 Carlsbad, USA) following the manufacturer's instructions.

188 FFPE samples of the APR2017 (n=4) were processed for DNA extraction using the Purelink  
189 Genomic DNA kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions  
190 with minor modifications. Particularly, unstained sections, serial to sections showing  
191 granulomas were used to identify the Mycobacteria species associated to tissue showing  
192 granulomas. Excess paraffin was removed from samples using sterile scalpels, and 5-10 mg of  
193 sliced FFPE tissue was placed in 1 ml of xylene (J.T. Baker, the Netherlands). As a pre-  
194 extraction step to remove paraffin from the sample, a described method was applied (Sirri et  
195 al., 2018). In brief, the sample was deparaffinised in xylene for 5 min. Following  
196 centrifugation, the sample was twice washed with ethanol 100%. The pellet was dried at 37°C

197 for 10 min, and DNA extraction was subsequently undertaken using the aforementioned kit.  
198 The DNA was used soon after extraction, or stored at  $-20^{\circ}\text{C}$  until use.  
199 Mycobacteria presence was investigated through two PCR methods targeting *Mycobacterium*  
200 sp. 16S rDNA and HSP65 genes, respectively (Kirschner, et al., 1993; Telenti et al., 1993).  
201 The PCR products of at least one sample per batch was sequenced as previously described  
202 (see Bacteriological examination paragraph) and subjected to BLAST analysis  
203 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for species identification.

204

### 205 **3. Results**

#### 206 3.1. Gross findings

207 At gross examination, the main macroscopic findings in batch AUG2016 were multifocal  
208 petechial cutaneous haemorrhages. Although no significant mortality was reported in batch  
209 APR2017, some fish showed the same haemorrhagic cutaneous findings. In the MAY2017  
210 batch, more severe gross cutaneous lesions were present, characterised by large erosive-  
211 ulcerative lesions in the oral part of the head and in the lateral-caudal part of the body (Figure  
212 1a).

213

#### 214 3.2. Histopathology and immunohistochemistry

215 Nineteen animals were available for histopathology evaluation. Granulomatous lesions were  
216 observed in 10 fish collected in all three batches (n=4 AUG2016; n=2 APR2017; n=4  
217 MAY2017). Granulomas were multifocal expansive lesions that primarily affected the kidney  
218 (Figure 1b) and almost totally replaced the perivisceral adipose tissue. All animals in batches  
219 AUG2016 and MAY2017 had “late stage” granulomas, whereas fish of batch APR2017  
220 revealed “intermediate” granulomas. In the majority of fish (n=2 AUG2016; n=2 APR2017;  
221 n=2 MAY2017), the kidney was primarily involved, followed by the perivisceral adipose

222 tissue (n=2 AUG2016; n=1 APR2017; n=3 MAY2017). More rarely, the spleen (n=1  
223 AUG2016), liver (n=1 MAY2017) and adipose and subcutaneous tissue infiltrating muscle  
224 (n=1 APR2017) were also affected (Figure 1c). A final diagnosis of visceral granulomatosis  
225 was formulated.

226 Unexpectedly, Ziehl-Neelsen staining did not reveal the presence of acid-fast bacteria in the  
227 sections examined. However, at immunohistochemistry, all of the investigated cases (n=19)  
228 showed a granular, intracytoplasmic immunoreactivity to anti-Mykobacterium antibody,  
229 particularly centred on the areas of the granulomas (Figure 1d).

230

### 231 3.3. Bacteriological findings

232 Phenotypic and genetic characterisations (Table 1) of the isolated bacteria showed the  
233 presence of *Aeromonas veronii* and *Vibrio cholerae* in batch AUG2016, and *A. veronii* and  
234 *Shewanella putrefaciens* in batches APR2017 and MAY2017. From the skin lesion of one fish  
235 of batch, MAY2017, *Aeromonas hydrophila* was also isolated.

236 *A. veronii* and *A. hydrophila* colonies appeared white, convex and circular on the TSA  
237 medium and were composed of Gram-negative, oxidase positive, glucose fermenting, nitrate  
238 positive, motile, indole positive and negative to H<sub>2</sub>S production bacteria. Sequencing of the  
239 gyrase subunit beta (*gyrB*) gene fragment confirmed the affiliation of the putative *A. veronii*  
240 isolates with reference strain *A. veronii* strain ATCC 9071 (GenBank accession number  
241 AY101775) on the similarity of > 97.4%, whereas the fragment obtained from the putative *A.*  
242 *hydrophila* colony showed 99.3% nucleotide identity with *A. hydrophila* reference strain  
243 ATCC 7966 (GenBank accession number AF417622).

244 *V. cholerae* colonies appeared white, convex and circular on the TSA medium and yellow on  
245 TCBS (sucrose utilisation). They were composed of Gram-negative, oxidase positive, glucose

246 fermenting, nitrate positive, motile, H<sub>2</sub>S negative and indole positive bacteria. *V. cholerae*  
247 colonies were positive in the species-specific PCR assay targeting *toxR* gene (Figure 2).  
248 *S. putrefaciens* colonies appeared white, convex and circular on the TSA medium and were  
249 composed of Gram-negative, oxidase positive, glucose non-fermenting (oxidising), motile and  
250 H<sub>2</sub>S producing bacteria. Sequencing of the 16S rDNA gene fragment confirmed the affiliation  
251 of these isolates with reference strain *S. putrefaciens* ATCC 8071TM (Genbank accession  
252 number X82133) on the similarity of >98%.  
253 Table 1 reports the results of phenotypic and genetic characterisations of isolated bacteria.  
254 The sequence data obtained in this study have been submitted to the GenBank database under  
255 accession numbers (NCBI; GenBank accession numbers: MN450803–MN450808).

256

#### 257 3.4. Molecular investigation for IPNV, Aquabirnavirus, Birnaviridae

258 No PCR products were obtained for *Infectious pancreatic necrosis virus* from batches  
259 AUG2016, APR2017 and MAY2017.

260

#### 261 3.5. Molecular investigation for mycobacteria

262 All tested samples of the three batches were positive to the PCR for *Mycobacterium* spp.  
263 demonstrating the presence of these pathogens in the frozen visceral specimens and also in the  
264 FFPE unstained sections, serial to sections showing granulomas.

265 BLAST analysis of sequences of PCR products obtained from the three batches for 16S rDNA  
266 and HSP65 gene revealed the presence of *M. marinum* in all three batches and *M. goodii* in  
267 one sample of batch APR2017. In particular, the *M. marinum* strains showed a nucleotide  
268 identity of 99.4% and 99.2% for 16S rDNA and HSP65 genes, respectively, when compared  
269 with reference strain *M. marinum* ATCC 927 (Genbank accession number AY299134). The  
270 *M. goodii* strain detected in batch APR2017 showed a nucleotide identity of 99.1% for 16S

271 rDNA with reference strain *M. goodii* ATCC 700504 (Genbank accession number  
272 AY458071). The sequence data have been submitted to the GenBank databases under  
273 accession numbers [We are waiting for Genbank numbers].

274

#### 275 **4. Discussion**

276 The use of *G. rufa* for cosmetic treatments in fish spas is becoming increasingly  
277 commonplace. Fish must be kept in captivity and managed using appropriate procedures for  
278 their health and welfare. Unfortunately, such conditions are often not met, raising issues of  
279 fish health. Furthermore, relatively few reports investigating the causes of mortality in this  
280 species are present in the literature (Baeck et al., 2009; Majtan et al., 2012; Ruane et al., 2013;  
281 Lee et al., 2016), rendering it difficult to prevent and manage health problems that should rely  
282 first and foremost on health screening so that fish can be healthy from their arrival at the spas.  
283 This study documents the presence of pathogenic bacteria, including zoonotic agents, not only  
284 in sick fish during outbreaks of mortality, but also in seemingly asymptomatic carriers that  
285 had recently arrived at the spa.

286 In the episodes described, the animals primarily showed multifocal petechial cutaneous  
287 haemorrhages, which in some fish evolved into erosive and ulcerative lesions of the skin,  
288 followed by sudden death. At histopathology, the main finding was instead represented by a  
289 visceral granulomatosis. This is a well-known condition in fish pathology as an aspecific  
290 tissue response to numerous etiologic agents, of which *M. marinum*, *M. chelonae* and *M.*  
291 *fortuitum* are most recognised and described in the literature (Gauthier and Rhodes, 2009; Puk  
292 et al, 2018). In this study, granulomas were detected in all of the batches analysed, including  
293 in asymptomatic fish collected immediately following their arrival. Despite no acid-fast  
294 bacteria have been detected, *Mycobacterium* antigens have been immunohistochemically  
295 labeled in all batches, confirming the presence of Mycobacteria within granulomas. Molecular

296 investigation permitted not only to identify the mycobacterial species, but also to detect the  
297 mycobacterial DNA in tissues with granulomas, when conducted on unstained sections, serial  
298 to sections showing granulomas. In particular, *M. marinum* was detected in all three tested  
299 batches and *M. goodii* in one batch. Mycobacteria and especially *M. marinum* are well known  
300 causes of disease in humans through contact with fish and the aquatic environment (Verner-  
301 Jeffreys et al., 2012; Thirunavukkarasu, 2017; Sunil et al., 2018). To the best of our  
302 knowledge, *M. goodii* has not been detected in fish to date; on the contrary, it is an emerging  
303 nosocomial human pathogen (Salas and Klein, 2017).

304 The development of mycobacterial granulomatosis is typically a chronic process. The  
305 classification of granulomas using the method described by Antuofermo et al., (2017)  
306 permitted the lesions to be timed and indicated that the granulomas were initially staged as  
307 “intermediate”, before rapidly progressing to the “late” stage. The presence of “late” stage  
308 lesions could explain the absence of acid-fastness at Ziehl-Neelsen staining and a weak  
309 immunohistochemical staining. Previous studies have indicated that Ziehl-Neelsen stains only  
310 “perfectly preserved” organisms, whereas IHC detects mycobacterial antigens, fragments and  
311 living or dead organisms, even with “defective” cell walls (Gutiérrez Cancela & García  
312 Marín, 1993; Sarli et al., 2005).

313 Furthermore, other bacteria such as *A. veronii*, *V. cholerae* and *S. putrefaciens* have been  
314 isolated from the same fish in which granulomas were detected.

315 *Aeromonas* spp. are common pathogens in fish that can induce ulcerative and hemorrhagic  
316 cutaneous lesions (Sreedharan et al., 2013; Jagoda 2014). The role of *Aeromonas* sp. has been  
317 highlighted as a cause of mass mortality in *G. rufa* (Majtan et al, 2012). Indeed, *Aeromonas*  
318 spp. are responsible for human gastroenteritis, soft-tissue and muscle infections, septicaemia  
319 and skin diseases (Igbinosa et al., 2012). Furthermore, particular strains of *A. veronii*-*A.*  
320 *sobria* have been related to water-to-human transmission (Khajanchi et al, 2010).

321 In this study, the presence of multiple bacteria co-infecting the fish was demonstrated. In  
322 particular, *A. veronii* was found in all batches, alongside the mycobacterial granulomas. In the  
323 batch of 2016, *V. cholerae* was isolated. In both batches of 2017, *S. putrefaciens* was detected  
324 in addition to *A. veronii* and *M. marinum*. Moreover, *A. hydrophila* was isolated from a skin  
325 lesion of a fish in the MAY2017 batch. This variety of bacteria is indicative of an intense  
326 mixed microbial proliferation, most likely induced by the poor health condition of the fish.  
327 This scenario suggests that the most probable cause of mortality outbreaks comprised the  
328 association between stress conditions (movement, captivity) and bacterial co-infections. In  
329 particular, the poor management of fish debilitated by mycobacterial infection may have  
330 triggered a bacterial septicaemia due to opportunistic bacteria such as *A. veronii* and *S.*  
331 *putrefaciens*. These agents have in fact been associated with fish haemorrhagic septicaemia  
332 outbreaks (Sreedharan et al., 2013; Jagoda 2014; Lee et al., 2016). *A. veronii* especially  
333 resulted in the most frequent *Aeromonas* spp. associated with freshwater ornamental fish  
334 showing signs of septicaemia (Jagoda 2014).

335 Moreover, the isolation of *V. cholerae* and *S. putrefaciens* denotes the poor hygienic  
336 conditions of the fish tanks used for cosmetic treatment. Both these bacteria raise a concern  
337 for human health. *V. cholerae* isolates were not characterised to the toxigenic level, and even  
338 if this aspect requires further investigation, it should be noted that *V. cholerae* O1 and O139,  
339 the agents of epidemic cholera, are not alone in being harmful to humans. Indeed, serogroups  
340 other than O1 and O139 have been identified as responsible for human infection (Dutta et al.,  
341 2013).

342 *S. putrefaciens* was isolated on media without NaCl, confirming this halophilic marine  
343 species' adaptability to a large variety of environments. Indeed, it plays an important role in  
344 the spoilage process of food products, and is also considered an opportunistic pathogen for  
345 freshwater fish and humans (Paździor, 2016). *S. putrefaciens* human infections are

346 additionally reported worldwide, affecting the soft tissue, ear, abdominal and biliary tract, and  
347 are frequently associated with underlying conditions and complications (Vignier et al., 2013).  
348 Although our investigation aimed to clarify the causes of fish mortality, most of the bacteria  
349 detected in the fish are also zoonotic pathogens and present serious issues for public health.  
350 Accordingly, our findings indicate the extent of the risk posed to public health related to  
351 cosmetic treatments that use fish, at least in the case of people with underlying medical  
352 conditions or who are immunocompromised. In fact, the use of fish for pedicures has already  
353 been associated with zoonotic infection (Sugimoto et al., 2013; Veraldi et al., 2014).  
354 However, in spite of the widespread use of fish for cosmetic treatment, few surveys have been  
355 conducted to investigate the presence of human pathogens in fish, and these have produced  
356 widely contrasting results. Accordingly, to our results Verner-Jeffreys et al. (2012) and Schets  
357 et al. (2012) also showed the presence of several zoonotic pathogens in *G. rufa* and in fish spa  
358 water respectively.

359 In order to meet the welfare needs of fish and to limit the risks to public health, several  
360 procedures are suggested (Wildgoose et al., 2012; Höller et al., 2013; Sirri et al., 2013;  
361 Bhattacharya, 2016). Some countries have defined standards for the management of public  
362 health risks from the cosmetic use of fish pedicures (HPA, 2011), whilst other countries have  
363 opted to ban this practice (Sugimoto et al., 2013). Nevertheless, in most countries this practice  
364 remains unregulated.

365

## 366 **5. Conclusions**

367 In conclusion, this study has highlighted the presence of several bacterial pathogens,  
368 including zoonotic agents, associated with *G. rufa* used for cosmetic treatments. Pathogens  
369 were detected in both asymptomatic and sick animals during two mortality outbreaks. The  
370 handling of the animals, which was probably inadequate, led to the progression of already-

371 extant pathological processes and to intense mixed microbial proliferation. Most of these  
372 pathogens have a zoonotic potential and presented a severe concern for public health. The  
373 presence of these pathogens associated with the water and fish used for cosmetic treatment  
374 could represent a serious risk to clients, especially those with underlying health conditions or  
375 those who are immunocompromised. In order to protect human health and fish welfare, the  
376 use of fish for cosmetic treatment should be regulated by rules that must include at the very  
377 least: 1) the use of disease-free fish; 2) good husbandry practices; and 3) periodic analysis of  
378 the water and fish kept in the spa.

379

#### 380 **Conflict of interest statement**

381 The authors declare that the research was conducted in the absence of any commercial or  
382 financial relationships that could be construed as a potential conflict of interest.

383

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545 **Table 1.** Results of phenotypic and genetic characterisations of isolated bacteria.

546

547

Isolates	Batch	Phenotypic characterizations							Genetic identification
		GRAM	oxidase	glucose utilization	nitrate reduction	motility	indole	H <sub>2</sub> S <sup>†</sup> production	
<i>Vibrio cholerae</i>	AUG2016	negative	positive	fermenting	positive	motile	positive	negative	Positive to the species-specific PCR assay targeting <i>toxR</i> gene.
<i>Aeromonas veronii</i>	AUG2016 APR2017 MAY2017	negative	positive	fermenting	positive	motile	positive	negative	<i>gyrB</i> gene > 97.4% with ATCC 9071 (GenBank accession number AY101775)
<i>Shewanella putrefaciens</i>	APR2017 MAY2017	negative	positive	oxidizing	positive	motile	negative	H <sub>2</sub> S <sup>†</sup> production	16S rRNA gene >98% with ATCC 8071TM (Genbank accession number X82133).
<i>Aeromonas hydrophila</i>	MAY2017 (skin lesion)	negative	positive	fermenting	positive	motile	positive	negative	<i>gyrB</i> gene 99.3% with ATCC 7966 (GenBank accession number AF417622)

548 <sup>†</sup> Hydrogen sulfide production

549 **Figure legends**

550

551 Figure 1. (a) Large ulcerative lesions in the lateral-caudal part of the body (bar = 1cm). (b)  
552 Multifocal expansive granulomas that partially replaced the kidney tissue. Some initial  
553 granulomas (arrow heads) and intermediate stage granulomas (arrows), 20x magn. (c)  
554 Granuloma (necrotic core, asterisk) infiltrating the skeletal tissue, 40x magn. (d) Granular,  
555 intracytoplasmic immunoreactivity to anti-Mycobacterium antibody centred on the areas of  
556 the granulomas, 40x magn.

557

558 Figure 2. Results of the multiplex PCR assay targeting species-specific genes for *V. cholerae*,  
559 *V. parahaemolyticus* and *V. vulnificus*. Lane 1: 100 bp molecular marker (Invitrogen). Lane 2  
560 *V. cholerae* positive control. Lane 3 *V. parahaemolyticus* positive control. Lane 4 *V.*  
561 *vulnificus* positive control. Lane 5-6 PCR products obtained from isolated colonies of batch  
562 AUG2016. Lane 7 negative control.

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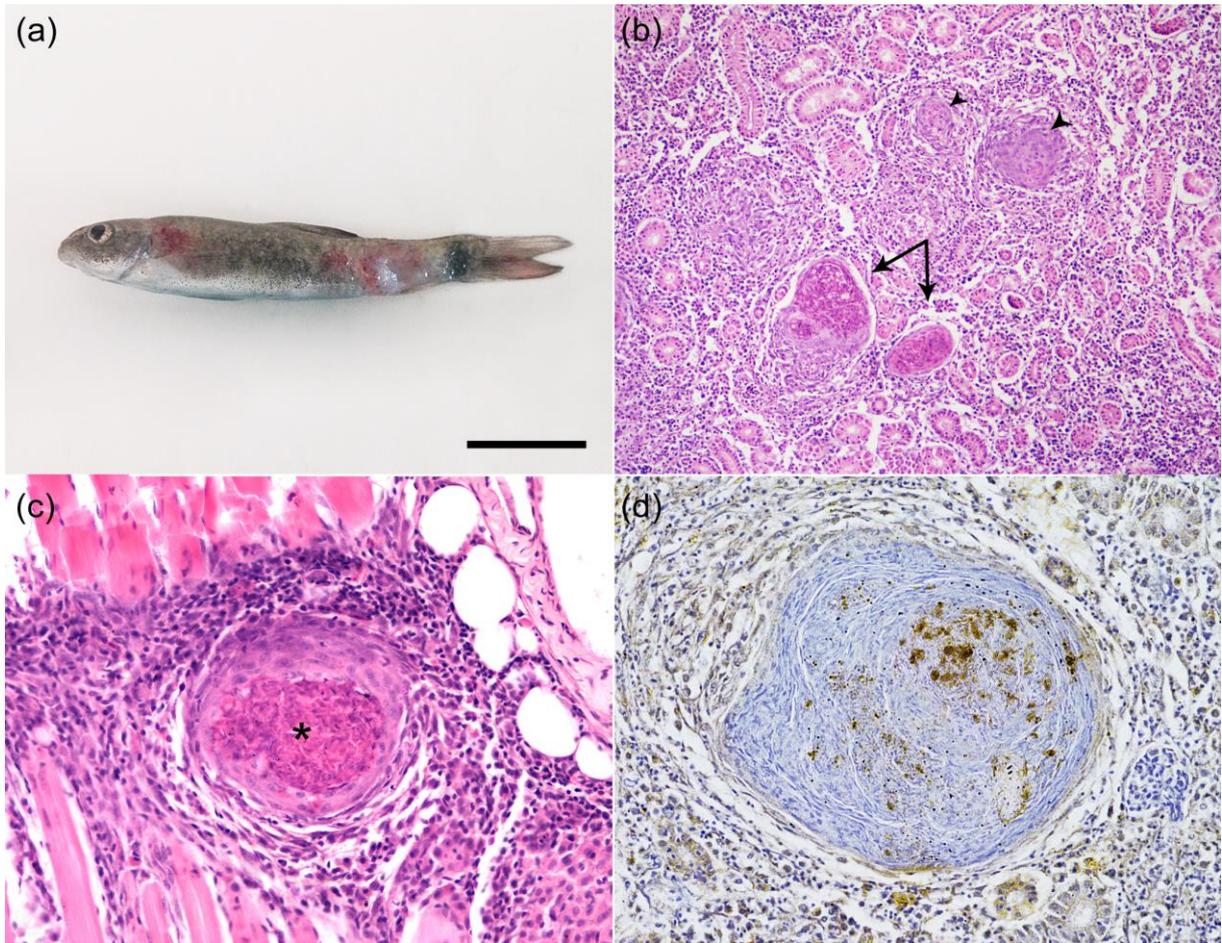
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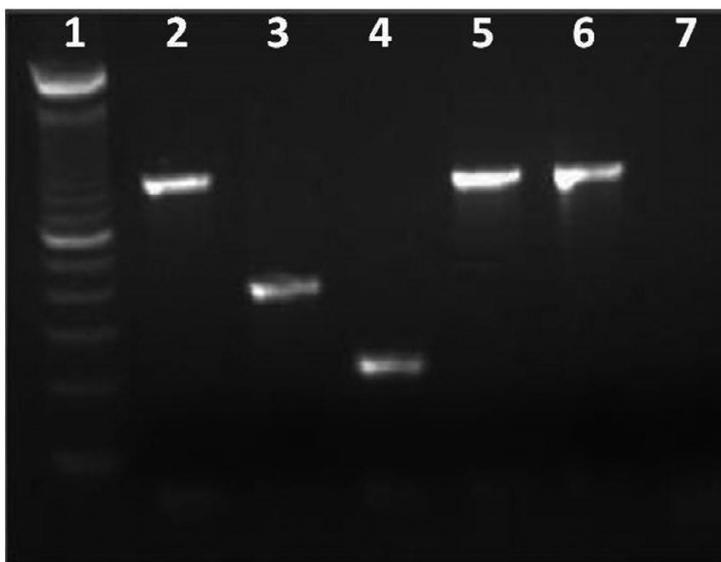
574 Figure 1



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576

577 Figure 2



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