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

Running title: Zoonotic pathogen detection in fish spa

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Abstract

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

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

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

These findings point out that handling, poor environmental conditions and the presence of fish pathogens, that can compromise the immune system of fish, can result in a mixed microbial proliferation and increase the spread of water-borne bacteria, including zoonosis agents. Accordingly, the microbiological surveillance of fish used for cosmetic treatment is extremely important, particularly in association with mortality outbreaks.

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1 Clinical history and sampling

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

Fish, 6 cm in length, were collected from the tank where mortality was observed (batch AUG2016) and were carried alive to the laboratories for diagnostic investigations.

After the tanks were emptied, disinfected with hypochlorite and subjected to fallowing, a new stock of fish was placed in the fish spa in April 2017, and the sampling of asymptomatic fish was conducted at the time of placement (batch APR2017). At the same time, a dead fish was collected from a tank (same batch APR2017). Several days following placement, the fish started to die in three of the five tanks, with a daily mortality of 3-7% and a cumulative mortality of 58, 75 and 83% in six weeks in the three tanks involved in the outbreak, respectively. Samples was conducted from the two tanks that showed the highest mortality (batch MAY2017).

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

2.2 Gross examination and histopathology

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

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

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

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

2.3 Immunohistochemistry

Immunohistochemistry was carried out on all formalin-fixed paraffin-embedded (FFPE) samples with a polyclonal antibody against *Mycobacterium bovis* (Bacillus Calmette-Guerìn, BCG, code no. B 0124 Dako, Denmark). This polyclonal antibody was previously applied successfully to immunolabel *M. marinum* in fish (López et al., 2018). Endogenous peroxidase inhibition was made with 3% H_2O_2 methylene solution. The antigen retrieval was made using a microwave oven at 750W, 2 cycles x5 min. Pre-incubation with a blocking solution (10% Normal Goat Serum and PBS) was performed for 30 min, and then incubated overnight at 4°C with a primary antibody at 1:3000 dilution in a blocking solution. The secondary anti-rabbit antibody was incubated at 1:200 dilution for 30 min, followed by a revelation system with an ABC kit developed with diaminobenzidine (DAB) chromogen for 90 sec and countercoloured with Papanicolau hematoxylin.

2.4 Bacteriological examination

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

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

2.4.1 Phenotypic characterisations

The following were utilised as screening tests: Gram-stain and cell morphology determination; oxidase production (OXIBIOSWAB, Biolife); glucose fermentation, utilisation of lactose and H_2S (hydrogen sulfide) production on Kligler Iron Agar (Oxoid); motility and indole production on SIM (sulphide–indole-motility) medium (Oxoid); reduction of nitrate (Zen-Yoji et al., 1973). When non-halophilic *Vibrio* sp. was suspected, growth on TCBS (Thiosulfate-Citrate-Bile salts-Sucrose) Agar was also tested.

2.4.2 Genotyping

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

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

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

2.6 Molecular investigation for mycobacteria

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

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

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

for 10 min, and DNA extraction was subsequently undertaken using the aforementioned kit. The DNA was used soon after extraction, or stored at −20°C until use.

Mycobacteria presence was investigated through two PCR methods targeting *Mycobacterium* sp. 16S rDNA and HSP65 genes, respectively (Kirschner, et al., 1993; Telenti et al., 1993). The PCR products of at least one sample per batch was sequenced as previously described (see Bacteriological examination paragraph) and subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for species identification.

3. Results

3.1. Gross findings

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

3.2. Histopathology and immunohistochemistry

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

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

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

3.3. Bacteriological findings

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

A. veronii and *A. hydrophila* colonies appeared white, convex and circular on the TSA medium and were composed of Gram-negative, oxidase positive, glucose fermenting, nitrate positive, motile, indole positive and negative to H2S production bacteria. Sequencing of the gyrase subunit beta (*gyr*B) gene fragment confirmed the affiliation of the putative *A. veronii* isolates with reference strain *A. veronii* strain ATCC 9071 (GenBank accession number AY101775) on the similarity of > 97.4%, whereas the fragment obtained from the putative *A. hydrophila* colony showed 99.3% nucleotide identity with *A. hydrophila* reference strain ATCC 7966 (GenBank accession number AF417622).

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

246 fermenting, nitrate positive, motile, H2S negative and indole positive bacteria. *V. cholerae*
247 colonies were positive in the species-specific PCR assay targeting *tox*R 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 H2S 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

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

4. Discussion

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

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

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

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

S. putrefaciens was isolated on media without NaCl, confirming this halophilic marine species' adaptability to a large variety of environments. Indeed, it plays an important role in the spoilage process of food products, and is also considered an opportunistic pathogen for 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-

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

Conflict of interest statement

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

References

Antuofermo, E., Pais, A., Polinas, M., Cubeddu, T., Righetti, M., Sanna, M.A., Prearo, M., 2017. Mycobacteriosis caused by Mycobacterium marinum in reared mullets: first evidence from Sardinia (Italy). Journal of Fish Diseases 40, 327-37. doi: 10.1111/jfd.12515.

Baeck, G.W., Kim, J.H., Choresca, C.J., Gomez, D.K., Shin, S.P., Han, J.E., P ark, S.C., 2009. Mass mortality of doctor fish (*Garra rufa obtusa*) caused by Citrobacter freundii infection. Journal of Veterinary Clinics 26, 150-154.

Bhattacharya, S., 2016. Doctor fish *Garra rufa*: health and risk. Journal of FisheriesSciences.com 10, 1-3.

Caccamo, D., Di Cello, F.P., Fani, R., Gugliandolo, C., Maugeri, T.L., 1999. Polyphasic approach to the characterisation of marine luminous bacteria. Research in Microbiology 150, 221-230. doi: 10.1016/S0923-2508(99)80039-4

Dutta, D., Chowdhury, G., Pazhani, G.P., Guin, S., Dutta, S., Ghosh, S., Rajendran, K., Nandy, R. K., Mukhopadhyay, A. K., Bhattacharya, M. K., Mitra, U., Takeda, Y., Nair, G. B., Ramamurthy, T., 2013. Vibrio cholerae non-O1, non-O139 serogroups and cholera-like diarrhea, Kolkata, India. Emerging Infectious Diseases 19, 464–467. doi: 10.3201/eid1903.121156

Gauthier, D.T., 2015. Bacterial zoonoses of fishes: A review and appraisal of evidence for linkages between fish and human infections. The Veterinary Journal 203, 27-35.

Gauthier, D.T., Rhodes, M.W., 2009. Mycobacteriosis in fishes: A review. The Veterinary Journal 180, 33-47.

Gutiérrez Cancela, M.M., García Marín, J.F., 1993. Comparison of Ziehl-Neelsen staining and immunohistochemistry for the detection of Mycobacterium bovis in bovine and caprine tuberculous lesions. Journal of Comparative Pathology 109, 361-370.

Health Protection Agency (HPA), 2011. Guidance on the management of the public health risks from fish pedicures. London: HPA. 31 Aug 2011. Available from: https://www.gov.uk/government/ uploads/system/uploads/attachment_data/file/322420/Fish_ Spa_guidance.pdf

421 Höller, C., Hörmansdorfer, S., Schramek, N., Moritz, J., 2013. [Sanitary, veterinary and legal
422 aspects on the use of *Garra rufa* for humans]. Hygiene & Medizin 38, 306-311. [German].
423
424 Igbinosa, I.H., Igumbor, E.U., Aghdasi, F., Tom, M., Okoh, A.I., 2012. Emerging Aeromonas
425 species infections and their significance in public health. Scientific World Journal 625023, 1-
426 13. doi: 10.1100/2012/625023.
427
428 Jagoda, S.S., Wijewardana, T.G., Arulkanthan, A., Igarashi, Y., Tan, E., Kinoshita, S.,
429 Watabe, S., Asakawa, S., 2014. Characterization and antimicrobial susceptibility of motile
430 aeromonads isolated from freshwater ornamental fish showing signs of septicaemia. Diseases
431 of Aquatic Organisms 109, 127-37. doi: 10.3354/dao02733.
432
433 Khajanchi, B.K., Fadl, A.A., Borchardt, M.A., Berg, R.L., Horneman, A.J., Stemper, M.E.,
434 Joseph, S.W., Moyer, N.P., Sha, J., Chopra, A.K., 2010. Distribution of virulence factors and
435 molecular fingerprinting of Aeromonas species isolates from water and clinical samples:
436 suggestive evidence of water-to-human transmission. Applied and Environmental
437 Microbiology 76, 2313-25. doi: 10.1128/AEM.02535-09.
438
439 Kirschner, P., Springer, B., Vogel, U., Meier, A., Wrede, A., Kiekenbeck, M., Bange, F.C.,
440 Böttger, E.C., 1993. Genotypic identification of mycobacteria by nucleic acid sequence
441 determination: report of a 2-year experience in a clinical laboratory. Journal of Clinical
442 Microbiology 31, 2882-2889.
443

Lee, J.Y., Gang, N.I., You, J.S., Ko, C.Y., Lee, K.W., Han, W.M., Kim, E., 2016. Bacteriological study about the death of cultured doctor fish, *Garra rufa* in the aquarium. Journal of Marine Life Science 1, 18-24.

López, V., Risalde, M.A., Contreras, M., Mateos-Hernández, L., Vicente, J., Gortázar, C., de la Fuente, J., 2018. Heat-inactivated Mycobacterium bovis protects zebrafish against mycobacteriosis. Journal of Fish Diseseas 41, 1515-1528.

Majtàn, J., Cerny, J., Ofùkana, A., Takàc, P., Kozànek, M., 2012. Mortality of therapeutic fish *Garra rufa* caused by Aeromonas sobria. Asian Pacific Journal of Tropical Biomedicine 2, 85-87. doi: 10.1016/S2221-1691(11)60197-4

Passalacqua, P.L., Zavatta, E., Bignami, G., Serraino, A., Serratore, P., 2016. Occurrence of Vibrio parahaemolyticus, Vibrio cholerae and Vibrio vulnificus in the clam Ruditapes philippinarum (Adams & Reeve, 1850) from Emilia Romagna and Sardinia, Italy. Italian Journal of Food Safety 5, 5709. doi: 10.4081/ijfs.2016.5709

Paździor, E., 2016. Shewanella putrefaciens – a new opportunistic pathogen of freshwater fish. Journal of Veterinary Research 60, 429-434. doi: 10.1515/jvetres-2016-0064

Pennazio, R., Prearo, M., Righetti, M., Serracca, L., Verzuri, A., Nante, N., Pastorino, P., Briata, M.P., 2016. Fish doctor and beauty center: a possible marriage in respect of public health. European Journal of Public Health 26, 77-8. doi.org/10.1093/eurpub/ckw166.012

468 Pinheiro, A.C.A.S., Volpe, E., Principi, D., Prosperi, S., Ciulli, S., 2016. Development of a
469 multiplex RT-PCR assay for simultaneous detection of the major viruses that affect rainbow
470 trout (Oncorhynchus mykiss). Aquaculture International 24, 115-125. doi: 10.1007/s10499-
471 015-9912-9
472
473 Puk, K., Banach, T., Wawrzyniak, A., Adaszek, Ł., Zieztek, J., Winiarczyk, S., Guz, L., 2018.
474 Detection of Mycobacterium marinum, M. peregrinum, M. fortuitum and M. abscessus in
475 aquarium fish. Journal of Fish Diseases 41, 153-156. doi.org/10.1111/jfd.12666
476
477 Ruane, N.M., Collins, E.M., Geary, M., Swords, D., Hickey, C., Geoghegan, F., 2013.
478 Isolation of Streptococcus agalactiae and an aquatic birnavirus from doctor fish *Garra rufa* L.
479 Irish Veterinary Journal 66:16. doi: 10.1186/2046-0481-66-16
480
481 Salas, N.M., Klein, N., 2017. Mycobacterium goodii: an emerging nosocomial pathogen a
482 case report and review of the literature. Infectious Diseases in Clinical Practice 25, 62-65. doi:
483 10.1097/IPC.0000000000000428
484
485 Sarli, G., Morandi, F., Zanoni, R.G., Brunetti, B., Mandrioli, L., Florio, D., Prearo M., 2005.
486 Comparison of immunohistochemistry and Ziehl-Neelsen for the detection of Mycobacterium
487 infection in sea bass (Dicentrarchus labrax). Bulletin of European Association of Fish
488 Pathologists 25, 182-185. doi: 10.1016/S0021-9975(08)80299-X
489
490 Schets, F.M., van den Berg, H.H., de Zwaan, R., van Soolingen, D., de Roda Husman, A.M.,
491 2012. The microbiological quality of water in fish spas with *Garra rufa* fish, the Netherlands,
492 October to November 2012. Euro surveillance 20, (19):pii=21124.

Sirri, R., Ciulli, S., Barbé, T., Volpe, E., Lazzari, M., Franceschini, V., Errani, F., Sarli, G., Mandrioli, L., 2018. Detection of Cyprinid Herpesvirus-1 DNA in cutaneous squamous cell carcinoma of koi carp (Cyprinus carpio). Veterinary Dermatology 29, 60-e24. doi: 10.1111/vde.12482.

Sirri, R., Zaccaroni, A., Di Biase, A., Mordenti, O., Stancampiano, L., Sarli, G., Mandrioli, L., 2013. Effects of two water disinfectants (chloramine T and peracetic acid) on the epidermis and gills of *Garra rufa* used in human ichthyotherapy. Polish Journal of Veterinary Sciences 16, 453-461.

Sreedharan, K., Philip, R., Singh, I.S., 2013. Characterization and virulence potential of phenotypically diverse Aeromonas veronii isolates recovered from moribund freshwater ornamental fishes of Kerala, India. Nederlandse Vereniging voor Microbiologie Stichting Antonie van Leeuwenhoek 103, 53-67.

Sugimoto, K., Frei, R., Graber, P., 2013. Methicillin-resistant Staphylococcus aureus foot infection after fish pedicure. Infection 41, 1013-1015. doi: 10.1007/s15010-013-0437-8.

Sunil, V., Harris, A. W., Sinem B., Holtm A. M., Noseworthy, A. L., Sider, D., Jamieson, F. B., White, S., Johnston, C., & Spohn, O. (2018). Investigation of a community cluster of cutaneous Mycobacterium marinum infection, an emerging zoonotic pathogen in aquaculture industry, Haliburton, Kawartha, PineRidge District Health Unit, Ontario, Canada, July–August 2015. Zoonoses Public Health, [Epub ahead of print] doi: 10.1111/zph.12521

518 Telenti, A., Marchesi, F., Balz, M., Bally, F., Böttger, E.C., Bodmer, T., 1993. Rapid
519 identification of mycobacteria to the species level by polymerase chain reaction and
520 restriction enzyme analysis. Journal of Clinical Microbiology, 31, 175-178.
521
522 Thirunavukkarasu, S., Plain, K, M., de Silva, K., Marais, B. J., & Whittington R. J. (2017).
523 Applying the One Health Concept to Mycobacterial Research – Overcoming Parochialism.
524 Zoonoses and Public Health, 64, 401-422. doi: 10.1111/zph.12334
525
526 Veraldi, S., Nazzaro, G., Cuka, E., 2014. Staphylococcus aureus infection of the feet
527 following fish pedicure. Infection 42, 925-926. doi: 10.1007/s15010-014-0622-4.
528
529 Verner-Jeffreys, D.W., Baker-Austin, C., Pond, M.J., Rimmer, G. S. E., Kerr, R., Stone, D.,
530 Griffin, R., White, P., Stinton, N., Denham, K., Leigh, J., Jones, N., Longshaw, M., Feist,
531 S.W., 2012. Zoonotic disease pathogens in fish used for pedicure. Emerging Infectious
532 Diseases 18, 1006-1008. doi: 10.3201/eid1806.111782.
533
534 Vignier, N., Barreau, M., Olive, C., Baubion, E., Theodose, R., Hochedez, P., Cabie, A.,
535 2013. Human infection with Shewanella putrefaciens and S. algae: report of 16 cases in
536 Martinique and review of the literature. The American Journal of Tropical Medicine and
537 Hygiene 89, 151-156. doi: 10.4269/ajtmh.13-0055.
538
539 Wildgoose, W.H., 2012. A review of fish welfare and public health concerns about the use of
540 *Garra rufa* in foot spas. Fish Veterinary Journal 13, 3-16.
541

542 Zen-Yoji, H., Le Clair, R. E., Ohta, K., Montagle, T.S., 1973. Comparison of Vibrio
543 parahaemolyticus cultures isolated in the United States with those isolated in Japan. The
544 Journal of Infectious Diseases 127, 237-241. https://www.jstor.org/stable/30108816

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_2S † production	
Vibrio cholerae	AUG2016	negative	positive	fermenting	positive	motile	positive	negative	Positive to the species-specific PCR assay targeting *tox*R gene.
Aeromonas veronii	AUG2016 APR2017 MAY2017	negative	positive	fermenting	positive	motile	positive	negative	*gyrB* gene > 97.4% with ATCC 9071 (GenBank accession number AY101775)
Shewanella putrefaciens	APR2017 MAY2017	negative	positive	oxidizing	positive	motile	negative	H_2S † production	16S rRNA gene >98% with ATCC 8071TM (Genbank accession number X82133).
Aeromonas hydrophila	MAY2017 (skin lesion)	negative	positive	fermenting	positive	motile	positive	negative	*gyrB* gene 99.3% with ATCC 7966 (GenBank accession number AF417622)

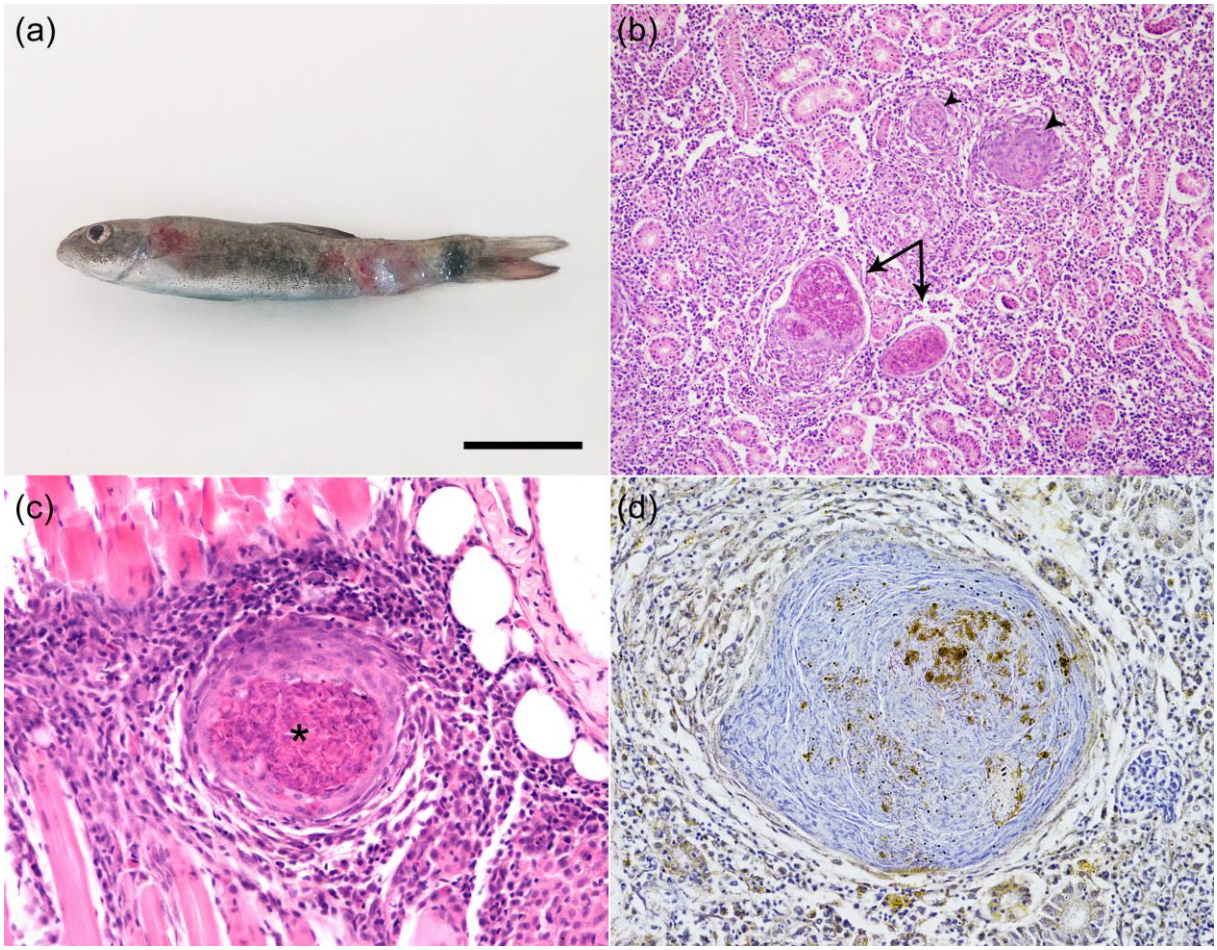
548 † Hydrogen sulfide production

Figure legends

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

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

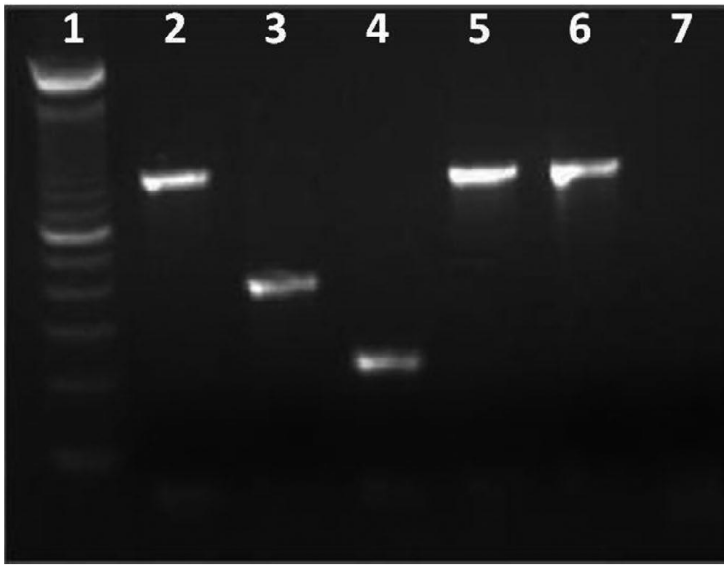
574 Figure 1



575

576

577 Figure 2



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