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First report of *Streptococcus parauberis* in a cultured freshwater ornamental fish, the ram cichlid *Mikrogeophagus ramirezi* (Myers & Harry, 1948)

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Running title: *Streptococcus parauberis* infection in the ram cichlid

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Since the first report of an outbreak of a streptococcal infection in rainbow trout (*Oncorhynchus mykiss*) in Japan in 1958 (Hoshina *et al.* 1958), streptococcosis has been responsible for significant mortalities resulting in considerable losses to the aquaculture industry (Salati 2006; Noga 2010). Numerous species from the family Streptococcaceae have been identified as etiological agents of streptococcosis in fish (Toranzo *et al.* 2005; Salati 2006; Noga 2010), susceptibility to which was documented in both food (Inglis *et al.* 1993) and ornamental fish species (Russo *et al.* 2006). *Streptococcus parauberis* is a coccoid, non-motile, alpha-hemolytic Gram-positive bacterium belonging to the *Streptococcacea* family (Nho *et al.* 2011) and has been reported as the etiological agent of streptococcosis in a few fish species, including turbot (*Scophthalmus maximus*), olive flounder (*Paralichthys olivaceus*), sea bass (*Sebastes ventricosus*) and striped bass (*Morone saxatilis*) (Domeénech *et al.* 1996; Mata *et al.* 2004; Baeck *et al.* 2006; Park *et al.* 2009; Haines *et al.* 2013; Oguro *et al.* 2014). *S. parauberis* has been previously identified as the etiologic agent of bovine mastitis (Bradley 2002). It was formerly known as *Streptococcus uberis* Type II until comparative analysis of the sequence data of *Streptococcus uberis* Types I and II showed that both were phylogenetically distinct, and the new species *Streptococcus parauberis* was proposed (Williams and Collins 1990).

This report describes the first occurrence of septicemic disease associated with *S. parauberis* in a cultured freshwater ornamental fish, the ram cichlid (*Mikrogeophagus ramirezi*). This small, colorful omnivorous fish is popular among aquarists. The histopathological changes associated with the infection are presented, as well as the

46 preliminary bacteriological characteristics of this first isolate of *S. parauberis* from a
47 freshwater ornamental fish.

48 Mortalities had been reported following a routine sorting procedure at a commercial
49 fish farm culturing the ram cichlid in Southern Israel in January 2014. Fish were seen to be
50 exhibiting apparent signs of sickness that included weakness, loss of equilibrium, skin redness
51 and ecchymotic hemorrhaging as well as lepidorthosis and exophthalmia (Supplementary
52 material 1). Fish were brought for examination to the Fish Health Laboratory at The Jacob
53 Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev (Midreshet Ben-
54 Gurion, Israel) monthly between January and June of 2014 and a total of around 30 fish were
55 examined. From amongst these, around 20 fish underwent a direct microscopic examination
56 of wet mounts and aseptic bacterial isolation and around 10 fish were processed for
57 histopathological analysis. For bacteriological examination, sterile swabs from the liver and
58 kidney were streaked onto tryptone soy agar (Oxoid, Hampshire, UK) and the plates were
59 incubated at 25˚C for 24 h. Biochemical analyses were performed with API 20 STREP and API
60 50 CH test (API system, La Balme les Grottes, France). The isolate was sent to Hy Laboratories
61 Ltd. (Rehovot, Israel) for 16s rRNA gene sequencing and the resulting sequence was
62 subjected to comparative phylogenetic analysis. Whole fish were fixed in formalin for 48 h
63 and stored in 70% ethanol until processing by routine histological techniques.

64 Histopathological analysis revealed infiltration of macrophages, which was mostly
65 evident in liver, kidney, and muscle (Fig. 1a-e). Gram staining demonstrated the presence of
66 densely packed, Gram-positive bacteria in the infiltrating macrophages (Fig 1b, d). Focal

necrosis occurred in muscle fibers (Fig. 1f) and vacuolization was seen in the liver (Fig. 1c). There was no evident damage to kidney tubules or stroma (Fig. 1a).

Gram positive cocci were isolated from symptomatic fish. On TSA plates, morphological characteristics of the colony of around 1 mm in diameter included whitish-to-yellowish coloration, a circular shape with a raised cross sectional elevation and a smooth surface. The isolate was molecularly identified as *S. parauberis* and, from here onwards will be referred to as *S. parauberis* RC. The partial 16s rRNA sequence was deposited in GenBank under accession no. MF102143. Partial sequences of several *S. parauberis* isolates from aquatic and terrestrial environments were retrieved from the National Center for Biotechnology Information (NCBI) database to perform phylogenetic and molecular evolutionary analyses in Phylogeny.fr (Dereeper *et al.* 2008). The *S. parauberis* RC was closely related to other *S. parauberis* strains of aquatic origin (Fig. 2a) although it formed a separate clade from the rest of the group. In the API 20 STREP test, *S. parauberis* RC was Voges-Proskauer, hippuric acid and esculin positive. All other tests were negative. Furthermore, the isolate was able to metabolize a number of carbohydrates including galactose, glucose, fructose, mannosen-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, cellobiose, maltose, lactose, saccharose, trehalose, amidon, glycogen, and gentiobiose. The isolate was able to grow in a wide range of temperatures (17-33˚C), though growth (OD_{620}) was affected in temperatures lower than 21˚C (Fig. 2b). It was found to thrive at various NaCl concentrations (0-40 ppt) in the culture media, however, growth was negatively affected at the highest salinity tested (*i.e.* 40 ppt) (Fig. 2c).

We evaluated the susceptibility of *S. parauberis* RC to several antibiotics including *SXT*: trimethoprim/sulphamethoxazole; *T30*: oxytetracycline; *N30*: neomycin; *NOR1*: norfloxacin; *FFC30*: florfenicol by the disc diffusion method. An overnight bacterial inoculum (approx. 10^8 CFU ml^{-1}) was applied onto the surface of Mueller-Hinton agar plate before placement of the antibiotic discs (BBL™ Sensi-Disc™, BD, NJ). *Streptococcus parauberis* RC was resistant to T30 but susceptible to SXT, N30, NOR1 and FFC30. A strain of *S. parauberis* from olive flounder (*Paralichthys olivaceus*) had similarly been previously identified to be resistant to tetracycline (Park *et al.* 2009). Based on the results of the biogram, on-farm treatment with florfenicol was applied through medicated feed. The treatment reduced the mortalities, but the infection reoccurred when treatment was withdrawn. After four cycles of repeated antibiotic treatments and reoccurrence of the disease, the farm started feeding the fish with a diet supplemented with rosemary (*Rosmarinus officinalis*). Rosemary has been previously reported to be effective against *Streptococcus iniae* and *Streptococcus agalactiae* (Abutbul *et al.* 2004; Zilberg *et al.* 2010). Bacteria could not be isolated from fish during and soon after the application of rosemary, but infection reoccurred once rosemary supplementation was withdrawn.

Basic factors contributing to bacterial virulence were comparatively analyzed in our *S. parauberis* RC isolate and the most common causative agents of streptococcosis in fish, including *S. iniae* and *S. agalactiae*. Intra-community (*i.e.* biofilm, autoaggregation) and inter-community interactions (*i.e.* co-aggregation) are common mechanisms of bacterial survival in nature and have been identified to play a part in the virulence of pathogens, including in the streptococci (Cvitkovitch *et al.* 2003; Khemaleelakul *et al.* 2006). Many aquatic bacteria are

capable of forming a biofilm, a dense aggregate of surface-adherent microorganisms embedded in an exopolysaccharide matrix (Cvitkovitch *et al.* 2003; Branda *et al.* 2005). Biofilm-forming ability was determined by a modified crystal violet assay protocol (Lazado *et al.* 2010). *S. parauberis* RC was shown to be capable of forming biofilms under static (Fig. 2d) or mobile (Fig. 2e) conditions. The biofilm forming potential of *S. parauberis* RC was similar to that of *S. iniae* at both static and mobile conditions, and to *S. agalactiae* under mobile conditions (Fig. 2d). Auto-aggregation allows cell-cell interactions to occur and has properties similar to those of biofilms, providing protection from the host defense factors and from external treatments, such as antibiotics (Aparna and Yadav 2008; Lazado *et al.* 2010). A spectrophotometric-based assay was adopted to evaluate this feature (Lazado *et al.* 2011). *Streptococcus parauberis* RC auto-aggregating index was calculated to be 23.4±5.68% (Lazado *et al.* 2011), indicating that around 23% of the individual bacteria clumped together. Comparing to the other pathogenic streptococci, the capability is 19% higher than *S. iniae* but 43% lower than *S. agalactiae*. The ability of *S. parauberis* RC to aggregate provides insight to the documented re-occurrence of infection following treatment withdrawal, *i.e.* this ability may have provided protection and allowed the bacteria to survive the treatment. Interestingly, *S. parauberis* RC was also capable to co-aggregating with the 2 pathogenic streptococci (Rickard *et al.* 2003), with *S. iniae* 41.8±13.8% and with *S. agalactiae* 41.7±5.68%. This interaction suggests the potential for co-infection to occur.

We are speculating two probable causes of the presence of *S. parauberis* on the farm where the bacterium was isolated. One likely scenario was that the bacteria originated from incoming fish. Phylogenetic relationship of *S. parauberis* RC with other aquatic-derived

strains lends support to such a speculation. The farm personnel reported that there was no delivery of fish to the farm for a long time period prior the outbreak, but its correlation with fish handling could suggest that the bacterial infection was latent, and the resulting stress may have caused an outbreak. Another likely scenario is the possibility of transmission of the infection from an adjacent dairy facility.

The only reported fish-derived *S. parauberis* isolate (GenBank accession no. JQ780604) in Israel was from a diseased broomtail wrasse (*Cheilinus lunulatus*). However, this is the first report to discuss the histopathological changes associated with the infection caused by an *S. parauberis* isolate from Israel in a freshwater ornamental fish. In addition, some of the fundamental microbiological features characterized in *S. parauberis* RC may offer insights in the subsequent study of the virulence and pathogenesis associated with this pathogen.

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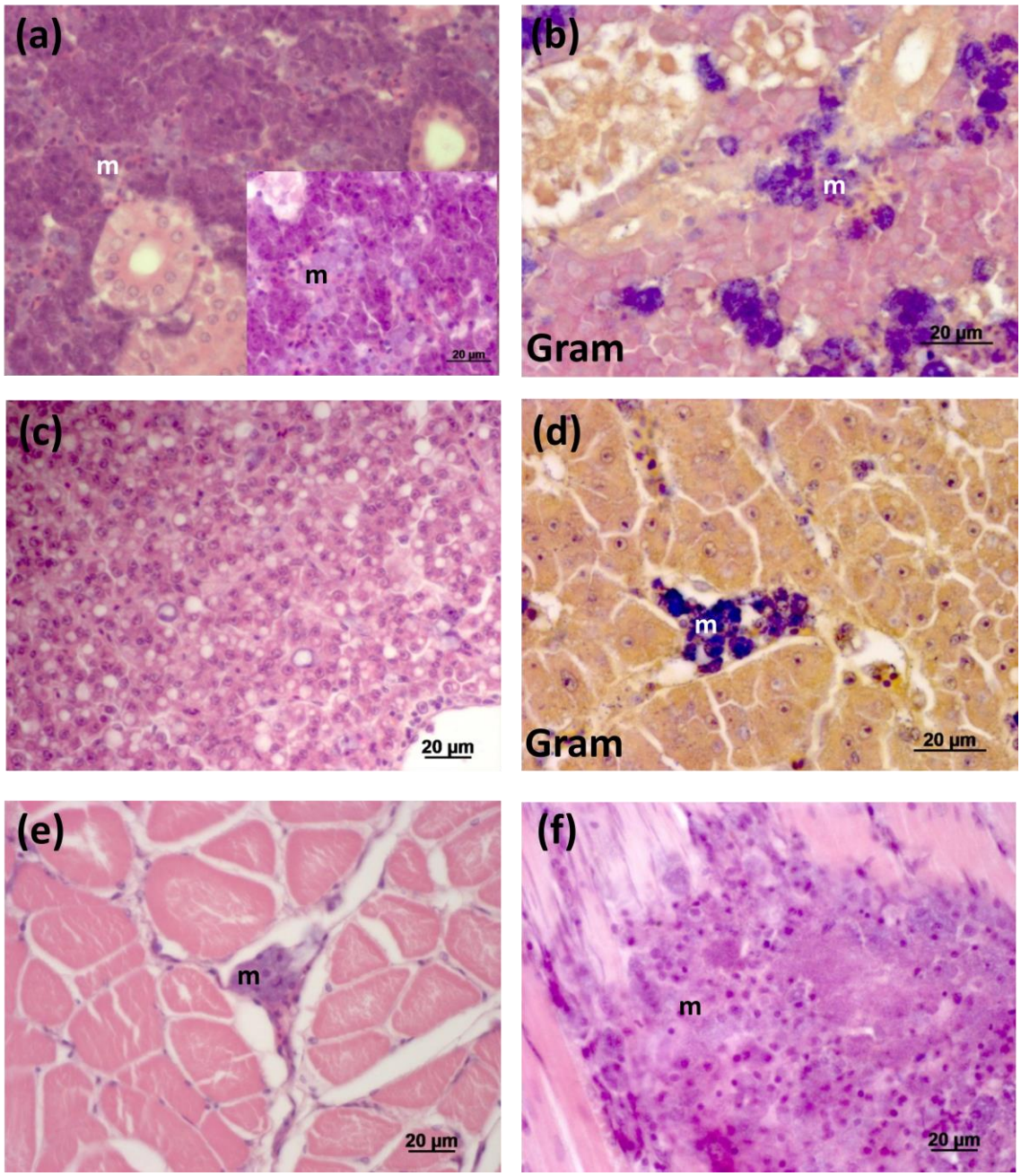
List of figure legends

Figure 1. Histopathology from *S. parauberis*-infected ram cichlid. Infiltrating macrophages in kidney tissue **(a)** containing Gram positive bacteria **(b)**. Liver appears vacuolated **(c)** with focally occurring infiltrating macrophages, containing Gram positive bacteria **(d)**. Infiltrating macrophages in the muscle **(e)** and focally occurring necrosis in muscle fibers **(f)**. Sections are stained with H&E **(a, c, e, f)** and Gram stain **(b, c)**; m, macrophages.

Figure 2. *S. parauberis* RC: Phylogeny, growth characteristics and biofilm formation. **(a)** Phylogram *of S. parauberis* from ram cichlid and other isolates of terrestrial and aquatic (*with red arrowhead*) origins. The isolate with an arrowhead shaded in red and outlined in black was previously isolated in Israel. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. The alignment in MUSCLE was curated in Glocks 0.91b to include a total of 635 positions, representing 40% of the alignment. The curated alignment was used for phylogenetic anylysis in PhyML and the tree was rendered by TreeDyn. Culture conditions, including **(b)** temperature and **(c)** NaCl concentration**,** affecting the growth of *S. parauberis* RC. Biofilm formation at 25˚C either in **(d)** static or **(e)** mobile conditions were analyzed in a microplate. For mobile conditions, the plate was incubated with shaking (80 rpm). Values presented in **b**, **c**, **d** and **e** are mean ± SE of observations from three independent experiments each with three replicate set-ups. Column bars with different letters indicate significant difference ($P<0.05$) as tested by one-way ANOVA followed by Tukey's multiple comparison tests.



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241 Figure 1.

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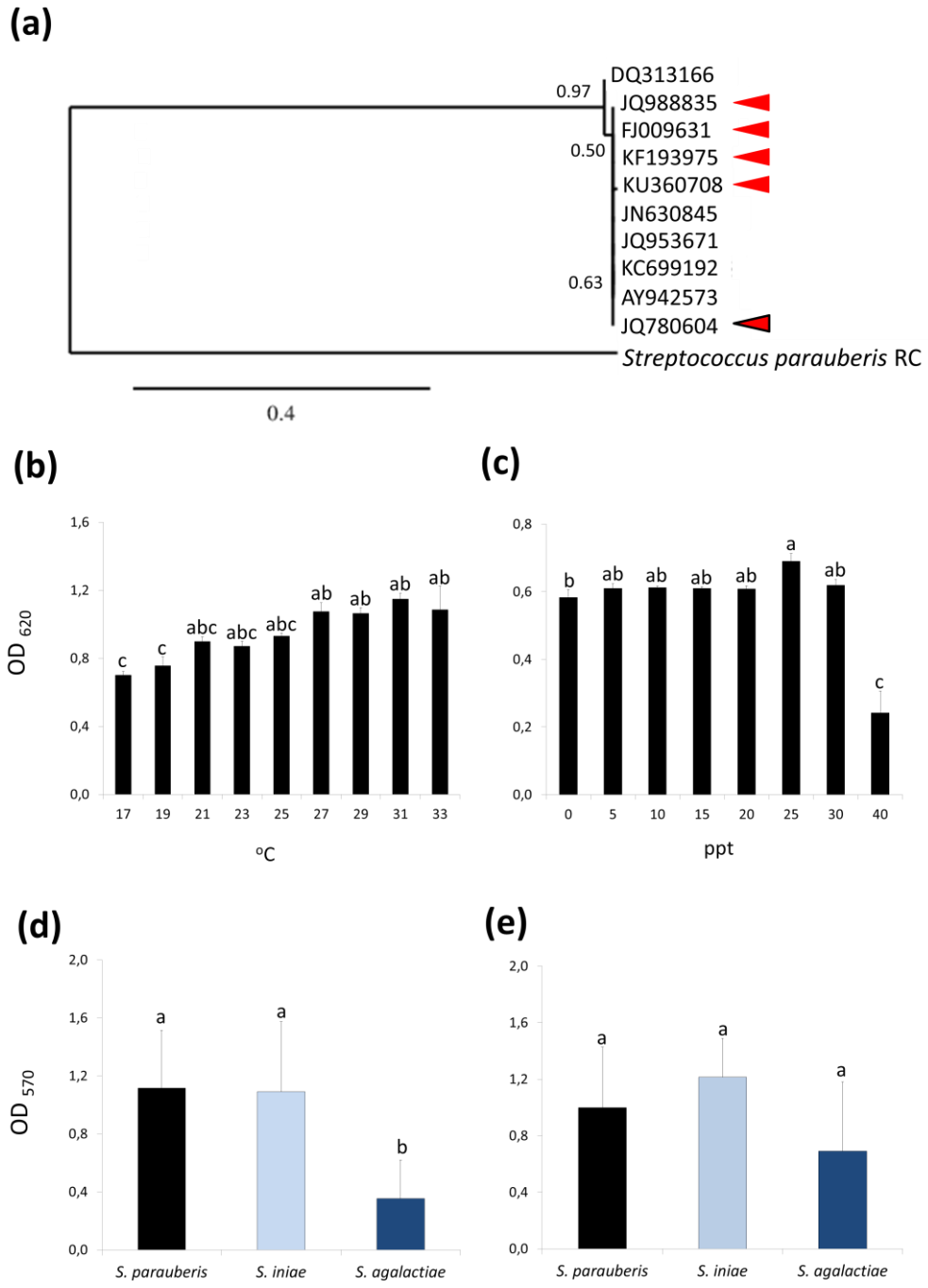

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244 Figure 2.

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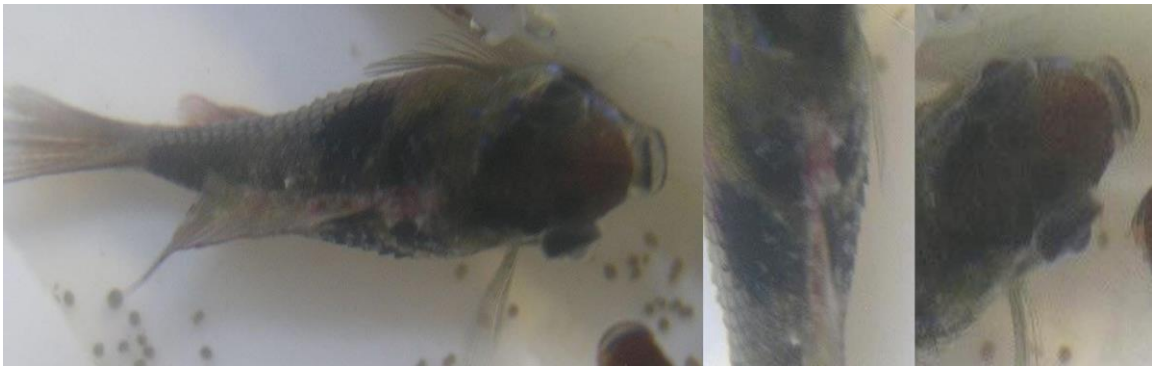
Supplementary information



Supplementary material 1. Gross pathology of ram cichlid (*Mikrogeophagus ramirezi)* infected with *Streptococcus parauberis.*