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Biofilm is associated with chronic streptococcal meningoencephalitis in fish

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ABSTRACT

Biofilms are aggregates of attached microbial organisms whose existence on tissues is often recognised as a mechanism for the establishment of most chronic diseases. Herein we investigated the ability of piscine *Streptococcus agalactiae*, an important aquatic pathogen, for adaptation to this sessile lifestyle *in vitro* and in the brain of a tilapia fish model. Piscine *S. agalactiae* exhibited a weak attachment to polystyrene plates and expressed a low biofilm phenotype under the study conditions. Furthermore, fluorescent *in situ* hybridization and confocal laser scanning microscopy revealed discrete aggregates of attached *S. agalactiae* within brain tissues and around meningeal surfaces. They were embedded in an exopolysaccharide containing matrix, intractable to inflammatory response and showed some level of resistance to penicillin despite proven susceptibility on sensitivity test. Intracellular bacterial aggregates were also observed, moreover, antibody mediated response was not demonstrated during infection. Nucleated erythrocytes appear to facilitate brain invasion possibly via the Trojan horse mechanism leading to a granulomatous inflammation. We have demonstrated that biofilm is associated with persistence of *S. agalactiae* and the development of chronic meningoencephalitis in fish.

1. Introduction

Streptococcus agalactiae (group b streptococcus, GBS) is an important pathogen of a broad range of hosts, including domestic and wild, terrestrial and aquatic animal species, as well as humans [1–3]. The disease caused by GBS is of common interest to both human and animal health. In humans, it particularly leads to septicaemia, a problem in neonates that acquire an infection at birth from the maternal GBS colonisation of genital mucosa [4–6]. Biofilm formation was demonstrated as a putative mechanism of GBS persistence in these adult females [4,7]. Streptococcal septicaemia is associated with poor water quality, extreme temperatures and high mortality in teleost fish [8–10]. Moreover, piscine GBS (PiGBS) also circumvents the immune system of teleost and is often isolated in apparently healthy fish [11,12]. In the biofilm mechanism of persistence, bacteria grow on tissue surfaces as large microcolonies juxtapose within an exopolymeric matrix of polysaccharides, proteins, nucleic acids and lipids [13,14]. They are then able to defy host defence, resist antimicrobial therapies and establish chronic infection [15,16].

PiGBS attaches to and penetrates intestinal mucous membrane from where it hematogenously advances to establish an infection in the brain of fish as chronic meningoencephalitis [17,18]. Although, its ability to cross the blood-brain barrier (BBB) is suggested to be through a Trojan horse or a modified Trojan horse approach [11], the mechanism of persistence within brain tissue has been in obscurity. Indeed after tissue localisation, the closely related *Streptococcus pyogenes* and *Streptococcus suis* induce biofilm-associated tissue persistence and diseases [19,20]. Precisely, the expression of biofilm phenotype by *S. suis* was identified as a virulence factor that may be linked to meningitis and endocarditis in pigs [20]. On the other hand, *S. pyogenes* biofilm is associated with asymptomatic infection, pyoderma and necrotising fasciitis in humans [19,21,22]. We therefore, hypothesise that biofilm may be the leading attribute of PiGBS persistence in the brain tissues of apparently healthy fish.

So far, it has been observed that expression of pili type 2a, presence of glucose and environmental pH influences GBS biofilm formation [4,7,23]. While PiGBS have the ability to develop fluppy

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pellets suggestive of an extracellular polysaccharides (EPS) production [24]. In this study, we adopted previously proposed diagnostic criteria for discerning the role of biofilms in diseases to investigate its involvement in the pathogenesis of chronic streptococcal meningoencephalitis in fish [14,25]. The criteria include attachment to tissue surfaces, organised bacterial aggregation, confinement within an area in tissues, tolerance to antimicrobial drug and evasion of inflammatory responses. Our findings highlight the potential role of biofilm in the pathogenesis of PiGBS in fish.

2. Materials and methods

2.1. Microtiter plate biofilm formation assay

An *in vitro* quantification of biofilm was done in 96 well flat bottom polystyrene plates as described previously with some modifications [7]. The piscine strain of GBS (SA2K, biotype 1) originally isolated from an outbreak in a cage cultured tilapia [26] and a *Staphylococcus aureus* ATCC 12600 were grown to mid-exponential phase in brain heart infusion broth (BHIB) supplemented with 1% glucose (Sigma Ultra, USA). The broth cultures were diluted to 1:20 with fresh media and 100 μL of each suspension was put into wells of the microtiter plates. The plates were then incubated for 24 h at 37 °C. Media containing planktonic cells were discarded and plates rinsed three times with distilled water to remove loosely attached cells. Each well was stained with 0.5% crystal violet (BDH Chemical Ltd., UK) for 10–15min. A 30% glacial acetic acid was used to solubilize the dye from adherent cells. Solubilized dye was transferred into new plates and the absorbance measured at a wavelength of 490 nm using an ELISA reader.

2.2. Experimental design

Forty red hybrid tilapia fish with an average weight of 38.4 g were divided into infected (n = 30) of two subgroups, and the control non-infected group (n = 10). They were kept in 60 L glass aquaria with constant aeration. Dissolved oxygen (*M*: 4.20, *SD*: 1.46 ppm) and temperature (*M*: 28.02, *SD*: 0.54 °C) were frequently monitored. Fish were fed with commercial feed pellet to satiety and about 60% of water was changed daily for 3 weeks duration of the study. PiGBS on blood agar plate was submitted for antibiotic sensitivity test (Ref. No. M2015/625) to the Bacteriology Laboratory of the Faculty of Veterinary Medicine, Universiti Putra Malaysia. Fish were orally exposed to PiGBS and the control group was given phosphate buffered saline (PBS). Samples of blood and brain were taken after every four days from day one. Both groups were treated with an appropriate antimicrobial on the 14th day of the experiment (See details below).

2.3. Inoculum preparation

PiGBS SA2K from glycerol stock stored at −20 °C was streaked on tryptic soy agar, readapted into the tilapia host by intra-abdominal inoculation for about 72 h and isolated from the eye onto a blood agar. Few colonies from the blood agar were then subcultured in BHIB and incubated at 37 °C for 12 h, i.e., early logarithmic phase [27]. One milliliter (1 mL) of broth culture was then centrifuged at 10,000 g for 15 min and the resulting pellets washed twice with PBS. The cell pellet was 10 fold serially diluted in PBS to an absorbance of OD_{600} = 0.02. Colonies were counted and expressed as colony forming units per milliliter (CFU/mL).

2.4. Piscine GBS chronic infection model

Red tilapia (*Oreochromis* sp.) was selected as a model species for GBS infection in fish, because it is currently one of the most susceptible to this pathogen. The fish were sedated using tricaine methanesulfonate (MS 222) in water at 150 mg/L. A low exposure dose (1.9×10^4 CFU/0.1 mL) of inoculum was then introduced gently into the gastrointestinal tract using 20G IV indwelling cannula (Vasofix® Braunüle®) as described in previous studies of experimental streptococcosis [28,29]. Tilapia were observed for clinical signs of streptococcosis throughout the experimental period. Benacillin injection at the dose rate of 100 mg/kg was administered with an insulin syringe through the pectoral fin muscle. Benacillin (Ilium) contains 150 mg/mL procaine penicillin, 150 mg/mL benzathine penicillin and 20 mg/mL procaine hydrochloride. Penicillin was selected based on the result of antibiotic sensitivity test (Table 1).

2.5. Post-mortem procedure and tissue processing

Apparently healthy fish were sedated as above and blood was drawn from the caudal vein using a 3 mL syringe, 25G 1″ needle. Serum was harvested and kept at −20 °C until used. Cervical pitting using a fine scalpel blade was employed for euthanasia. A dorsal rectangular incision was made on the skull and the brain exposed using the blade. A scissors was used to separate the metencephalon from the myelencephalon and to severe attachment of the brain from the base of the skull. The brain was then removed using a thumb forceps and submerged into a 10% buffered formalin for fixation or into a clean 1.5 mL Eppendorf tube kept at −80 °C for bacterial cell viability. Internal organs were exposed by a ventral midline incision using clean scissors. The organs were then examined *in situ* and individually for gross pathological changes.

Tissues of the brain were processed through dehydration with an increasing concentration of alcohol (80%, 95%, and 100%), cleared in chloroform and impregnated with paraffin wax in a semi-enclosed bench top tissue processor (Leica TP 1020). The processed tissues were then sectioned at 4–5 μm using a microtome (Leica 2045 Multicut) and the resulting ribbons put into a pre-warmed (42 °C) floatation bath (TBS®, USA). The sections were picked up on plain slides for haematoxylin and eosin (H&E) staining and silane coated slides for *in situ* hybridization, immunoperoxidase and histofluorescence.

2.6. Tissue staining

Acridine orange and propidium iodide (AOPI) stains were used for determination of bacterial cell viability as previously performed [30]. Propidium iodide was the exclusion dye, which is only able to cross damaged bacterial cell membranes. A piece of frozen brain tissue was embedded in a freezing medium for cryosectioning. Sections (10 μm) were obtained at −15 °C or above using a cryostat (Leica). A 5 μg/mL AOPI solution (Sigma-Aldrich) was prepared from stock solution, overlaid on tissues and incubated at room temperature for 10 min. Tissues were then washed with water and allowed to dry. Slides were covered with a glass coverslip before

Table 1
Antibacterial sensitivity test[a] result for piscine *Streptococcus agalactiae* (PiGBS).

Antibacterial drug	Resistance	Intermediate susceptibility	Sensitive
Amoxycilin	−	+	−
Ampicilin	+	−	−
Chloramphenicol	−	+	−
Gentamycin	+	−	−
Tetracyclin	+	−	−
Penicillin G	−	−	+

[a] Disc diffusion method. + = yes, − = nil.