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New Approaches for Controlling *Saprolegnia parasitica*, the Causal Agent of a Devastating Fish Disease

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Abstrak: Oomycetes patogenik mempunyai keupayaan untuk menjangkiti banyak tumbuhan dan haiwan perumah. Saprolegniasis merupakan penyakit yang memberi kesan terhadap telur ikan dan ikan juvenil di pusat penetasan seluruh dunia, dan berpunca daripada oomycete patogenik *Saprolegnia parasitica*. Penyakit ini menunjukkan miselium berfilamen bertompok kelabu-putih di atas badan atau sirip ikan dan berkaitan dengan kerosakan tisu yang membawa kepada kematian haiwan tersebut. Dahulunya, saprolegniasis dikawal menggunakan Malachite green; namun demikian bahan kimia ini diharamkan pada tahun 2002 kerana kesan-kesan karsinogenik dan toksikologi. Akibat daripada ini, kebelakangan ini terdapat peningkatan saprolegniasis di industri akuakultur, yang membawa kepada kerugian ekonomik seluruh dunia. Maka, ada keperluan segera untuk mencari kaedah alternatif bagi mengawal patogen ini. Kami akan membincangkan penggunaan pendekatan molekular dalam kajian saprolegniasis, yang dijangka membolehkan perkembangan vaksin ikan yang berkesan dan potensi perkembangan kaedah baru untuk mengawal penyakit ini.

Kata kunci: Saprolegniasis, *Saprolegnia parasitica*, Oomycete, Patologi

Abstract: Pathogenic oomycetes have the ability to infect a wide range of plant and animal hosts and are responsible for a number of economically important diseases. Saprolegniasis, a disease affecting fish eggs and juvenile fish in hatcheries worldwide, is caused by the pathogenic oomycete *Saprolegnia parasitica*. This disease presents as greyish-white patches of filamentous mycelium on the body or fins of fish and is associated with tissue damage leading to death of the animal. Traditionally, saprolegniasis was controlled using Malachite green; however, this chemical was banned in 2002 due to its carcinogenic and toxicological effects. As a direct result of this ban, there has been a recent resurgence of saprolegniasis in the aquaculture industry, leading to economic losses world-wide. Hence, there is an urgent need to find alternative methods to control this pathogen. We discuss the use of molecular approaches for the study of saprolegniasis, which are anticipated to enable the development of effective fish vaccines and the potential for the development of new methods to control this devastating disease.

Keywords: Saprolegniasis, *Saprolegnia parasitica*, Oomycete, Pathology

INTRODUCTION

Traditionally, oomycetes have been classified in the kingdom Fungi due to their filamentous growth and other fungal-like characteristics; however, recent molecular and biochemical analysis have classified oomycetes within the group

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Stramenopiles, which includes kelp and diatoms (Kamou, 2003; Phillips *et al.* 2008). Oomycetes are divided into three subclasses, Saprolegniomycetidae, Rhipidiomycetidae and Peronosporomycetidae, all of which are able to infect a wide range of hosts, including economically important plants and vertebrate animals (van West 2006; Phillips *et al.* 2008). Fish and animal pathogenic oomycetes belonging to the order Saprolegniales of the subclass Saprolegniomycetidae contain three main genera, *Saprolegnia*, *Achlya* and *Aphanomyces* (van West 2006). Species within the genus *Saprolegnia* have been classified according to sexual and morphological characteristics; however, recent molecular characterisation of the ribosomal DNA (rDNA) repeat has demonstrated that *Saprolegnia* is a phylogenetically diverse genus (Molina *et al.* 1995; Ke *et al.* 2009). Recognisable *Saprolegnia* species include *S. diclina*, *S. ferax*, *S. australis* and *S. parasitica* (Molina *et al.* 1995; Hussein *et al.* 2001; Stueland *et al.* 2005; Dieguez-Uribeondo *et al.* 2007; Fernandez-Beneitez *et al.* 2008; Petrisko *et al.* 2008; Ke *et al.* 2009; Ghiasi *et al.* 2010).

S. parasitica represents a serious problem in the aquaculture growth industry (Molina *et al.* 1995; van West 2006; Phillips *et al.* 2008). Saprolegniasis caused by *S. parasitica* affects aquaculture broodfish and incubating eggs. It is estimated that 10% of all hatched salmon succumb to saprolegniasis, causing major financial loss in an industry accounting for approximately 30% of the global fish production for consumption (Molina *et al.* 1995; Murray & Peeler 2005; van West 2006; Fregeneda-Grandes *et al.* 2007; Phillips *et al.* 2008). The incidence of saprolegniasis extends to Asian tropical aquaculture systems where over 80% of fish produced by aquaculture comes from the area (Karunasagar *et al.* 2003). Malaysia is one of the largest producers of cultured fish, notably Seabass, through its immense expansion in cage aquaculture (Alongi *et al.* 2002). Though responsible for the decline in aquaculture fish populations, *S. parasitica* has also been found in natural populations of salmonids and other fresh water fish species, and it is endemic to all fresh water habitats across the globe (van West 2006). Until 2002, *S. parasitica* was kept under control through the use of Malachite green; however, due to its carcinogenic and toxicological effects, treatment with this chemical has been banned internationally (Torto-Alalibo *et al.* 2005; van West 2006; Fugelstad *et al.* 2009; Robertson *et al.* 2009). To develop effective controls, it is necessary to better understand the molecular and physiological pathways underlying the development, pathogenicity and host specificity of saprolegniasis.

The asexual life stages of *S. parasitica* are responsible for saprolegniasis (Andersson & Cerenius 2002; Robertson *et al.* 2009). Sporulation is induced when there is a local decrease in nutrients, and asexual sporangia are induced to form on the hyphal tips apically releasing biflagellate, motile, and primary zoospores that disperse and in some cases may cause primary infection of host fish (Torto-Alalibo *et al.* 2005; van West 2006; Robertson *et al.* 2009). Primary zoospores may also encyst on a host, forming primary cysts and subsequently laterally releasing biflagellate, highly motile, secondary zoospores (Torto-Alalibo *et al.* 2005; van West 2006; Robertson *et al.* 2009). Secondary zoospores are considered the infective stage of *S. parasitica* and will encyst on host fish and form secondary cysts that will release the next generation of laterally biflagellate

zoospores (Torto-Alalibo *et al.* 2005; van West 2006; Robertson *et al.* 2009). The formation of subsequent generations of secondary zoospores is thought to be the result of non-specific stimuli (i.e., mechanical or physical) and has been reported to occur for up to six generations, a process known as repeated zoospore emergence (RZE), or polyplanetism (Dieguez-Uribeondo *et al.* 1994; Torto-Alalibo *et al.* 2005; van West 2006; Robertson *et al.* 2009). In fish eggs, saprolegniasis is characterised by abundant mycelial growth on cells, resulting in death, whereas in adult fish, *S. parasitica* invades epidermal tissues beginning with the head or fins and spreading over the entire surface of the body (van West 2006) (Fig. 1).

Figure 1: Juvenile salmon infected with *S. parasitica*. The inflamed area beneath the pectoral fin indicates the area of infection.

While the parasitic lifecycle of *S. parasitica* has been well described, little is known about the molecular pathways underlying parasitism (van West 2006). Functional genomics and proteomic approaches for studying saprolegniasis in *S. parasitica* are anticipated to aid in the discovery of control strategies for the early detection of saprolegniasis and development of intervention strategies (van West 2006; van West *et al.* 2010; Secombes 2011). The complementary identification of genes and proteins involved in the immune response to diseased host fish infected with *S. parasitica* may provide an understanding of how to prevent saprolegniasis and ultimately control the spread of this pathogen, increasing fish health and reducing disease losses in aquaculture and natural fresh water populations (Torto-Alalibo *et al.* 2005; van West 2006; Fregeneda-Grandes *et al.* 2007; Secombes 2011).

Genomic Approaches to Understanding Saprolegniasis Provides a Framework for Developing Controls for *S. parasitica*

Profiling the expression of genes associated with the infective stages of *S. parasitica* will provide a framework for the development of new control strategies. Torto-Alalibo *et al.* (2005) identified a series of expressed sequence tags (ESTs) in *S. parasitica*. A total of 1510 ESTs were identified consisting of 1279 unique sequences. Approximately half of the consensus sequences showed similarity to known protein and protein motifs, providing a genetic "snap-shot" of the biology and pathology of *S. parasitica*. Torto-Alalibo *et al.* (2005) found a total of 70 cDNA-encoded proteins potentially secreted into the extracellular matrix, an essential mechanism for the delivery of virulence factors by eukaryotic pathogens such as *S. parasitica*. These proteins are known as effector proteins (van West *et al.* 2010), the characterisation of which can aid in the development of vaccines targeting key regulatory pathways during the infectious stages of the *S. parasitica* – host fish interaction. Effector proteins are secreted by pathogens during host-pathogen interactions, enabling the infection and suppression of host defences; however, little is understood of how these effector proteins are translocated into host cells (Grouffaud *et al.* 2010; van West *et al.* 2010). Van West *et al.* (2010) identified the open reading frame (ORF) *Sphtp1* (*S. parasitica* host targeting protein 1) gene, which encodes the putative R×LR (Arginine – × – Leucine – Arginine where × represents any amino acid) effector protein SpHtp1 that is translocated into fish cells from *S. parasitica*. The SpHtp1 protein is expressed during the pre-infection and early infection stages of *S. parasitica*, indicating a role in saprolegniasis (van West *et al.* 2010). In oomycetes, translocation depends on the N-terminal region having the core-conserved motif R×LR, which in some cases is followed by a less well-conserved EER (Glutamic acid – Glutamic acid – Arginine) sequence within 30 amino acids of the C terminus (Grouffaud *et al.* 2010). The R×LR motif described by van West *et al.* (2010) in *S. parasitica* is conserved across many oomycetes including *Phytophthora infestans*, an oomycete pathogen responsible for late blight potato disease (Birch *et al.* 2006; Grouffaud *et al.* 2010; van West *et al.* 2010). The conserved R×LR motif also resembles the host-cell targeting signal found in virulence proteins in the malaria parasite *Plasmodium falciparum* (R×L×E/D/Q) (Grouffaud *et al.* 2010; van West *et al.* 2010), maintaining the significance of effector protein translocation during host-pathogen interactions for enabling the infection and suppression of host defences in *S. parasitica*. The identification of conserved R×LR motifs in effector proteins suggested to be involved in the pathology of the *S. parasitica* – host fish interaction can confirm the nature of the translocation of these proteins into host fish cells, and their subsequent characterisation would allow for a better understanding of their function during saprolegniasis.

A potential gene interference target for the control of *Saprolegnia* may include cellulose binding domain (CBD) proteins. CBD proteins may have an endogenous function in cell wall biogenesis as cellulose is a major component of the cell wall in oomycetes. Suppression of CBD proteins could offer a point of control for *S. parasitica*. Torto-Alalibo *et al.* (2005) identified the fungal-type I CBD protein as being highly diverse amongst *S. parasitica* and other oomycetes.

Type I CBD was found to contain a core of four conserved cysteines and aromatic residues known to bind the cellulose substrate, supporting its role in oomycete cell wall biogenesis (Torto-Alalibo *et al.* 2005). Targets for gene interference must, by definition, be highly specific to pathogens and not hosts. Fugelstad *et al.* (2009) identified and characterised putative cellulose synthase genes (*CesA*) *in S. monoica* (*SmCesA*), which was likely to be involved in the cellulose biosynthesis of the cell wall. *SmCesA* is the first of the *CesA* genes to be described in *Saprolegnia*, and it was found to be orthologous to the *CesA* gene in *Phytophthora* species by Southern blot analysis (Fugelstad *et al.* 2009). The conservation of the *CesA* genes across the *S. monoica* and *Phytophthora* species suggests the presence of *SmCesA* in other *Saprolegnia* species including *S. parasitica*. Furthermore, Fugelstad *et al.* (2009) found that in the presence of cellulose synthesis, the inhibitors 2,6-dichlorobenzonitrile (DCB) and Congo Red (CR) affect the cellulose biosynthesis process of *S. monoica*, inhibiting mycelial growth and leading to a compensation mechanism with increased expression of the *CesA* genes. Similar studies of the presence of *CesA* genes in *S. parasitica* and their involvement in cellulose biosynthesis and subsequent mycelial growth may lead to an understanding of the role cellulose biosynthesis plays in *S. parasitica* – host fish saprolegniasis. The development of small molecules that interfere with the function of *CesA* genes could provide a potential alternative for the control of saprolegniasis.

Because sporulation and the formation of subsequent generations of secondary zoospores are important to the infection process, the analysis of the molecular mechanisms underlying the sporulation, encystment and germination of *S. parasitica* zoospores can provide a framework for the development of controls for saprolegniasis. *S. parasitica Puf*1 is homologous to a family of RNA binding proteins named the Pumilio (Puf) family (Andersson & Cerenius 2002), and proteins of the Puf family play an important role in developmental regulation. The expression of *Puf*1 was discovered to be induced upon encystment and during the late stages of sporulation; however, it is lost when *S. parasitica* undergoes germination. Andersson and Cerenius (2002) identified *puf*1 as a cyst-specific transcript that is initiated immediately after the signal to encyst is received and lost when the cyst is preparing to release a new zoospore or germinate. The authors argue the possible role of *puf*1 as a posttranscriptional regulator that maintains the undetermined cyst stage or regulates mRNA turnover upon germination or zoospore release. *Puf*1 makes an interesting target for future developmental studies.

The Immunoregulatory Response of *S. parasitica* Infected Host Fish

In any host pathogen system, it is advantageous to consider not only the pathogen but also the host response. Studies focusing on the immunoregulatory response of host fish to infection by *S. parasitica* can be used to identify protective mechanisms needed for saprolegniasis resistance, and pathways in the host fish immune response that must be triggered to allow for effective vaccination. Roberge *et al.* (2007) conducted a genome-wide survey of the gene expression response in particularly vulnerable juvenile Atlantic salmon (*Salmo salar*) exposed to *Saprolegnia*. By using a 16,006-gene salmonid cDNA

microarray, Roberge *et al.* (2007) identified 430 cDNA genes with modified transcription levels in *S. salar* exposed to *Saprolegnia*. From the 430 cDNA genes observed, 25 for which the transcription levels were the highest were identified, and 24 were over-expressed genes encoding several acute phase proteins. Thus, it appears that salmon infected with *Saprolegnia* undergo an acute phase response. The other genes found to be over-expressed suggest the expression of proteins involved in facilitating the transmigration of leucocytes. It is interesting to note that *Tob-1* and *B-cell translocation gene 1* were both under-expressed, enabling T cell proliferation and the release of cytokines involved in the immunoregulatory response of infected salmon, contradicting previous studies (Roberge *et al.* 2007). Further studies need to focus on specific genes with modified transcription levels during saprolegniasis to identify and characterise their role in the immune response.

Saprolegniasis leads to the epidermal destruction and macrophage recruitment of infected host fish. Kales *et al.* (2007) studied the cellular response of the rainbow trout monocyte/macrophage cell line RTS11 exposed to *S. parasitica* as macrophages play a significant role in the initial immune response of fish during saprolegniasis (Kales *et al.* 2007). Within the first 48 hours of exposure to *S. parasitica*, host macrophages displayed chemotaxis, adherence and homotypic aggregation (HA) toward live and heat-killed spores and mycelium. Because the spore size of *S. parasitica* ranges from ~10–20 μm and trout macrophages generally measure between 7 and 15 μm, there will be a certain proportion of spores that cannot be physically engulfed by macrophages during phagocytosis. In addition, Kales *et al.* (2007) observed changes in the gene expression profile of the RTS11 cell line exposed to *S. parasitica* by utilising reverse transcriptase (RT)-PCR. The class I major histocompatibility (MH) II receptor and its chaperone, the invariant chain, was down regulated, while genes encoding inducible cyclooxygenase (COX-2), interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNFα) were strongly up regulated. Down regulation of the MH II receptor and the invariant chain indicates a role in immunosuppression during infection, a form of immune system evasion for the pathogen, as the MH II receptor is critical for the recognition of exogenous antigens including *S. parasitica* (Kales *et al.* 2007). Furthermore, *S. parasitica* produces arachidonic acid, the direct precursor of eicosanoids, which down regulates the macrophage activity in fish, providing evidence of MH II down regulation (Kales *et al.* 2007). COX-2 converts arachidonic acid into prostaglandin, an eicosanoid; therefore, the authors suggest that the up-regulation of COX-2 may be a response to excess arachidonic acid. Future studies directing attention to the expression of specific genes in RTS11 and other cells involved in immune response are required to better understand the immunoregulatory pathways in fish.

Studies concerning the production of specific antibodies involved in the immunoregulatory response of host fish infected with saprolegniasis can aid in vaccine development and the early detection of *S. parasitica*. Fregeneda-Grandes *et al.* (2007) injected brown trout (*Salmo trutta*) with antigen extracts from pathogenic *S. parasitica* and detected specific serum antibodies produced in response to saprolegniasis. Enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and Western blotting (WB) analyses were used to

analyse the presence of serum antibodies, and antibodies were detected in 66.7%, 54.5% and 48.5% of the serum samples, respectively, for each of the three techniques. The production of specific antibodies in *S. trutta* in response to antigen extracts from *S. parasitica* can therefore be detected by standard immunological techniques. Recently, Fregeneda-Grandes *et al.* (2009) further analysed the prevalence of serum antibodies directed against *S. parasitica* in wild and farmed *S. trutta* using ELISA. *S. trutta* samples were taken over a two-year period in the months of January, April and August. Though there was no significant difference found in the prevalence of serum antibodies detected based on the time of year, these authors found a positive correlation between the level of serum antibodies produced and larger (older) fish. This finding indicates a positive correlation with age and an increased immune response in fish exposed to *S. parasitica*. *S. trutta* in both natural and wild populations were able to produce specific serum antibodies in response to exposure to *Saprolegnia*; however, the authors commented that the low number of serum antibodies produced may be indicative of immune suppression by *S. parasitica* (Kales *et al.* 2007; Fregeneda-Grandes *et al.* 2009). Future studies characterising antigen production are required to better understand the specific immune response in *Saprolegnia* infected fish.

CONCLUSION

Since the international ban of Malachite green in 2002, the need to develop alternative methods for the control of saprolegniasis has become increasingly urgent. Genomic and proteomic studies of *S. parasitica* and other pathogenic oomycetes have provided an excellent resource for studying the molecular processes underlying saprolegniasis. Furthermore, the complementary identification of genes and proteins involved in the immune response of diseased host fish infected with saprolegniasis will provide an understanding of how to prevent saprolegniasis and ultimately control the spread of *S. parasitica*. Future studies of the molecular processes underlying saprolegniasis in *S. parasitica* – host fish interactions will undoubtedly increase our knowledge and understanding of the pathology of *S. parasitica*, enabling the development of effective fish vaccines and early detection of *S. parasitica* creating an alternative method for controlling saprolegniasis. Controlling saprolegniasis is necessary to ensure continued growth in the aquaculture industry, notably in Asian tropical aquaculture systems where over 80% of fish produced by aquaculture come from the area. For an industry that accounts for approximately 30% of the global production of fish for consumption, it is important to continue studying the underlying molecular processes of saprolegniasis in *S. parasitica* – host fish interactions.

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