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REVIEW

Immunoassays and diagnostic antibodies for *Perkinsus* spp. pathogens of marine molluscs

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ABSTRACT: *Perkinsus* sp. protozoans are parasites of a wide variety of molluscs around the world and are responsible for episodes of mass mortalities and large economic losses for aquaculture industries and fisheries. The first step towards the management of infectious episodes is the reliable detection of *Perkinsus* species. While historic methods for diagnosis of *Perkinsus* sp. infections in mollusc hosts include histological, *in vitro*, molecular-genetic, and immunoassays, antibody-based diagnostic assays may prove most practical with development of improved reagents and techniques. This paper reviews historic developments of antibodies against *Perkinsus* species, and of diagnostic immunoassays. Thirteen research papers reported the development of antibodies against *Perkinsus* sp. or their extracellular products, mainly *P. olseni* and *P. marinus*. Nine of those tested the cross-reactivity of their antibodies against different life stages or species than the one used as immunogen. While all antibodies raised against trophozoites labelled hypnospores, several antibodies raised against hypnospores did not label trophozoites, suggesting antigenic differences between those cell types. Antibody specificity studies showed that there is antigenic heterogeneity between *Perkinsus* species and *Perkinsus*-like organisms, and also that common epitopes occur among *Perkinsus* species, as well as some dinoflagellates. This review summarizes the current knowledge and aims at helping the future development of *Perkinsus* species-specific antibodies and immunoassays.

KEY WORDS: *Perkinsus* · Diagnostic · Immunology · Antibody

1. INTRODUCTION

Protozoan endoparasites of the genus *Perkinsus* are responsible for diseases that are collectively called perkinsosis, which affect a number of molluscs worldwide and cause mass mortalities in host populations, leading to large economic losses (Andrews 1988, Choi & Park 2010, Villalba et al. 2011, Waki et al. 2012). While the economic impacts of *Perkinsus* spp. infections have not been specifically calculated, 2 of the 7 accepted species of *Perkinsus*, i.e. *P. marinus* and *P. olseni* (= *P. atlanticus*), are on the list of notifiable pathogens of the World Organisation for Animal Health (OIE 2021b). Consequently, trade in animals infected with these 2 parasites is considerably hindered, and wild and farmed populations are at risk. For example, South Australian fisheries report a loss of at least $500 000 AUD ($375 860 USD) per year since the early 1980s due to *P. olseni* infections, and more recently a loss of $10 000 AUD ($7517.20 USD) per year in the currently fished areas of South Australia (Crane 2014). Following a perkinsosis outbreak in New South Wales,

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large parts of the abalone fisheries closed in 2005, and 94 t were harvested in 2011 compared to the pre-outbreak yields of 300 t (Crane 2014).

Perkinsus species infect their hosts and are directly transmitted without the need for any intermediate hosts (Ray 1954, Auzoux-Bordenave et al. 1995). They have 3 principal life stages that occur in different physical locations, as illustrated by a schematic diagram of the lifecycle of *P. olseni* in Fig. 1. Inside their host, *P. olseni* trophozoites undergo internal karyokinesis and cytokinesis to generate up to 32 daughter cells inside 1 mother cell that takes the name tomont or schizont. Once the internal daughter cells enlarge to rupture the schizont cell wall, the immature trophozoites are released into the tissue, where they enlarge with expanding vacuoles to become mature trophozoites (Goggin & Lester 1995, Perkins 1996). The cycle repeats itself inside the host until its death, when trophozoites are then released into the environment (Ragone Calvo et al. 2003). However, some of these cells are also expelled in the environment while the host is alive, through diapedesis within blood cells and in faeces (Bushek et al. 2002b). Because Ray's fluid thioglycollate medium (RFTM) has comparable chemical properties to necrotic host tissues (Perkins 1968, Valiulis & Mackin 1969) or other anaerobic environments such as faeces and benthic sediments (Dernby 1918, Pittman 1946, Regester & Whiles 2006), i.e. nutrient-rich medium and low oxygen and pH levels (Dernby 1918, Pittman 1946, Regester & Whiles 2006, Casas & La Peyre 2013), trophozoites enlarge in this medium and form a thick durable cell wall to become hypnospores or prezoosporangia as they would naturally (Azevedo 1989, Sagristà et al. 1996, Bushek et al. 2002b, Ragone Calvo et al. 2003, Park et al. 2010). When prezoosporangia are released to the environment, they may develop into zoosporangia that undergo internal proliferation to form hundreds of contained zoospores. These motile, biflagellate ellipsoidal zoospores are released through a discharge tubule to the external environment or *in vitro* media (Perkins & Menzel 1966, Lester & Davis 1981, Azevedo 1989, McLaughlin et al. 2000, Casas et al. 2002, Villalba et al. 2004, Liggins & Upston 2010).

The gold standard diagnostic method for routine surveillance of *Perkinsus* infections recommended by the OIE is the RFTM assay, developed in the 1950s (Ray 1952). This method takes advantage of the fact that Ray's high-salt formulation of fluid thioglycollate nutrient medium prompts a significant non-proliferative enlargement of *Perkinsus* sp. cells up to 30-fold (Stein & Mackin 1957), followed by optional staining with Lugol's iodine to allow easy visualisation and counting (Dungan & Bushek 2015). This methodology has been optimised multiple times since, by using different host tissues as inocula (Ray 1952, 1954), including the whole body of the host (Choi et al. 1989, Oliver et al. 1998, McLaughlin & Faisal 1999), solid tissue subsambles, or haemolymph (Gauthier & Fisher 1990, Nickens et al. 2002). RFTM is variably supplemented with antimicrobial and metabolite compounds to inhibit growth of microbial contaminants or to enhance enlargement of *Perkinsus* sp. cells for facile microscopic enumeration (Ray 1952, 1966, Villalba et al. 2004, Dungan & Reece 2006, Dungan & Bushek 2015).

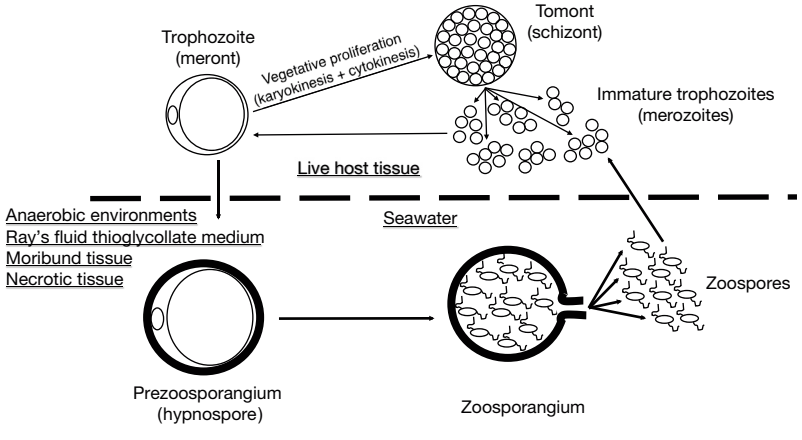

Fig. 1. *Perkinsus olseni* lifecycle, adapted from Liggins & Upston (2010; used by permission). The projected hypothetical lifecycle spans 2 biological compartments (seawater and host) and includes 5 cell types: trophozoites and schizonts in the live host tissue, hypnospores in anaerobic environments, and zoosporangia and zoospores in seawater

Historically, abundances of enlarged *Perkinsus* sp. hypnospores in assayed mollusc tissues were ranked using semi-quantitative numeric scales to estimate infection intensities (Ray 1954, Mackin 1962, Sparks 1986, Crosby & Roberts 1990). Some quantitative modern versions of RFTM assays use weighed mollusc tissue inocula and fully enumerate enlarged *Perkinsus* sp. hypnospores to yield tissue weight-normalized estimates of infection intensities (Choi et al. 1989, Rodríguez & Navas 1995, Almeida et al. 1999, Nickens et al. 2002). Depending on the aim of the measurements, different RFTM assays are then recommended for their simplicity or non-lethality (Bushek et al. 1994). To efficiently count *Perkinsus* sp. hypno-

spores following RFTM-enlargement, tissues of host molluscs may be hydrolyzed with 2 M sodium hydroxide (NaOH) before staining residual parasite hypnospores with Lugol's iodine for microscopic enumeration.

Since 1952, diagnostic RFTM assays have been used to effectively evaluate *Perkinsus* spp. infections in numerous mollusc hosts worldwide. For example, RFTM assays have efficiently detected *P. chesapeaki* in the soft-shell clam *Mya arenaria* (McLaughlin & Faisal 1998, Dungan et al. 2002), *Perkinsus* sp. in the venerid clams *Ruditapes decussatus* and *R. philippinarum* (Navas et al. 1992, Maeno et al. 1999, Lee et al. 2001, Park et al. 2005, Villalba et al. 2005), and *Perkinsus* sp. in Australian blacklip abalone *Haliotis ruber* (Lester & Davis 1981, Liggins & Upston 2010). Since all known *Perkinsus* spp. except *P. qugwadi* (incertae sedis) enlarge and stain with iodine for detection by RFTM assays, such assays are genus-specific, at best (Fisher & Oliver 1996, Novoa et al. 2002). Indeed, while Novoa et al. (2002) reported enlargement of *Pseudoperkinsus tapetis*, a species closely related to the genus *Perkinsus*, this result could not be duplicated with other *Pseudoperkinsus* species. Although its reagents are relatively inexpensive and the necessary technical equipment is widely available, traditional RFTM assays require incubation times of several days, as well as multiple manipulations of analytical samples. Those characteristics yield relatively slow data outputs at elevated labour costs.

For surveillance using molecular techniques, conventional and quantitative PCR are generally recommended, as these techniques are typically faster, more sensitive, and more specific than the RFTM assays for *Perkinsus* spp. diagnosis (Aranguren & Figueras 2016, Carrasco et al. 2017). Conventional genus-specific PCRs, which were developed for *Perkinsus* spp. (Casas et al. 2002) and refined later (Audemard et al. 2004), target the internal transcribed spacer of rRNA gene clusters with a sensitivity of up to 0.01 genome equivalents, meaning the PCR is sensitive enough to detect *Perkinsus* sp. even when 1% of the total genomic DNA is present in the sample (Dungan & Reece 2020). Likewise, other PCR assays amplify target sequences of non-transcribed spacer regions of rDNAs (Murrell et al. 2002). If *Perkinsus* genus-specific PCR tests yield positive results, subsequent species-level confirmations may also include the following molecular assays: PCR-restriction fragment length polymorphism (PCR-RFLP), denaturating gradient gel electrophoresis, conventional PCR, or real-time quantitative PCR (qPCR) (Audemard et al. 2004, Abollo et al. 2006, Reece et al. 2008, de Faveri et al. 2009, Takahashi et al. 2009, Balseiro et al. 2010, Gudkovs et al. 2016, Ramilo et al. 2016, Pagenkopp Lohan et al. 2018). Direct, species-specific PCR and qPCR methods and reagents are also available for most described *Perkinsus* species (Dungan & Reece 2020), including *P. qugwadi* (Itoh et al. 2013).

Another molecular technique is the *in situ* hybridization (ISH) assay, using probes targeting sequences of ribosomal RNAs and rRNA genes of *Perkinsus* species (Elston et al. 2003, Moss et al. 2008, Reece et al. 2008, 2017, Ramilo et al. 2015, Dungan & Reece 2020). Although ISH techniques may be specific at either genus or species levels, they are generally employed to confirm or narrow results from genus-specific PCR, RFTM, and other assays because they are technically demanding and expensive. As recommended (Burreson 2008, Itoh et al. 2013), results of PCR assays should be selectively and routinely confirmed and qualified by *in situ* histological verifications.

Near infrared (NIR) reflectance spectroscopy is a relatively new method that has been applied to the detection and quantification of *P. marinus* in the eastern oyster *Crassostrea virginica* (Guévélou et al. 2021). This technology can measure multiple components of the oyster tissue at once, allowing for extensive information about the sample in one high-throughput rapid assay. The study demonstrated that *P. marinus* has a specific spectral signature that is quantifiable, and that NIR could be applied to the detection of such protozoan infections. While this technology is unlikely to replace other assays due to the amount of preparation needed in the NIR experiments, it might still be used when other methods cannot be used.

Both histological and molecular diagnostic tests currently used for *Perkinsus* sp. diagnostic procedures are time-consuming and require specialized equipment and sample preparation, such as tissue isolation, nucleic acid extraction, PCR preparation and operation, and amplicon analysis and/or sequencing. While the specificity and sensitivity of molecular-genetic methods are valuable, development of immunodiagnostic tests such as enzyme-linked immunosorbent assays (ELISA) or dipstick colorimetric assays could allow diagnostics to be performed in the field in a few hours or less, for categorical or semi-quantitative results without the need for specialized equipment. Laboratory analyses of selected positive samples could subsequently provide refined results with specialized equipment. For exam-

ple, Park et al. (2010) used both immunology and molecular techniques to isolate and identify *P. olseni* cells from sediments, faeces, and bodies of Manila clams *Ruditapes philippinarum*. While the immunological assay required only a few hours and relatively easy sample preparation, the molecular assay required sample purification, nucleic acid extraction, and PCR amplification. Additionally, the RFTM assay took 2 wk due to the required incubation.

Once an antibody against *Perkinsus* sp. has been developed, a routine immunoassay like dipstick colorimetry would require only minimal labour such as sample preparation (tissue, haemolymph, or seawater sampling), and would yield results in a matter of minutes. On the other hand, molecular assays such as PCR require more labour and specific laboratory equipment in terms of sample purification, nucleic acid extraction, and PCR operation.

To date, reliable immunoassays have not been fully developed as diagnostic tests for *Perkinsus* spp. infections in marine molluscs. As mentioned above, most current diagnostic tests of *Perkinsus* spp. infections require some form of extensive sample preparation, such as tissue isolation, cell lysis, chemical or enzymatic digestion, or nucleic acid extraction. However, the development of an immunoassay would yield a quick, inexpensive, and widely applicable genus- or species-specific diagnostic test for these parasites (Ottinger et al. 2001).

While some previous investigations produced antibodies using whole *Perkinsus* spp. cells, others targeted secreted extracellular products (ECPs) from isolates propagated *in vitro*. Various immunological tests including ELISA, immunofluorescence assays, dot blots, and Western blots have been trialled with polyclonal and monoclonal antibodies raised against whole cells, cell emulsions, or ECPs, but none has shown both the specificity and sensitivity required for diagnostic tests by the OIE (Table 1). The aim of this review is to summarise the body of work that has been accomplished over recent decades to develop antibodies and immunoassays for recognising *Perkinsus* spp., in order to facilitate and inform further immunological research.

2. SPECIFICITIES OF ANTIBODIES AGAINST DIFFERENT LIFE STAGES

To date, 13 papers have described the development of antibodies by inoculating mammals with immunogens from *Perkinsus* spp., mainly *P. marinus* or *P. olseni* (Table 1). Nine of these tested the cross-reactivity of the product antibodies against life stages of the parasite other than the one used as an immunogen (Table 1). Two studies immunised rabbits and mice with *P. marinus* hypnospores (Choi et al. 1991, Dungan & Roberson 1993) and one study used hypnospores of *P. olseni* to immunise mice (Kaewsalabnil et al. 2015). The polyclonal antibodies generated by Choi et al. (1991) and the monoclonal antibodies generated by Dungan & Roberson (1993) labelled hypnospores extracted from RFTM cultures but failed to label parasite cells within histological samples of infected host tissues, suggesting that some antigens present at the surface of the hypnospore are unique to that cell type. Polyclonal antibodies from both mice and rabbits immunised with *P. marinus* hypnospore immunogens labelled diverse *Perkinsus* spp. within histological sections (Dungan & Roberson 1993). Montes et al. (1995) immunised rabbits with hypnospores, but the serum they obtained failed to label *in vivo* trophozoites in clams, supporting the previous hypothesis. However, other studies revealed that at least some antigenic determinants are expressed in different life stages of the parasite, since the polyclonal antibodies developed by Dungan & Roberson (1993) against *P. marinus* and one monoclonal antibody produced by Kaewsalabnil et al. (2015) against *P. olseni,* both recognised zoospores. In addition, the polyclonal antibodies described by Dungan & Roberson (1993) also cross-reacted with *in vivo* trophozoites. They labelled the external surface of the cell wall, the plasma membrane, the cell cytoplasm, the nuclear membranes, and endosomes of trophozoites *in situ*. They also demonstrated that the thick hypnospore cell wall has a variable permeability to immunoglobulin molecules, and that there is a close structural and biochemical homology between hypnospores and trophozoites.

Two other studies respectively immunised rabbits and mice with trophozoites and trophozoite protein lysates of *P. olseni* and *P. marinus* (Romestand et al. 2001, Montes et al. 2005). Polyclonal antibodies recognised hypnospores of *P. olseni* (Montes et al. 2005), highlighting the conservation of some antigens in different life stages. Monoclonal antibodies successfully recognised *in vitro* trophozoites, hypnospores, and zoospores of *P. marinus*, without reacting with oyster tissue (Romestand et al. 2001).

In summary, all polyclonal and monoclonal sera raised against trophozoites of *P. marinus* cross-reacted with hypnospores (Gauthier & Vasta 1995, Romestand et al. 2001, Montes et al. 2005), but 27 of the 29 antibodies developed against *P. marinus* hypnospores did not cross-react with the trophozoite stage (Choi et al. 1991, Dungan & Roberson 1993, Gauthier & Vasta

Table 1. Characteristics of antibodies developed against *Perkinsus* spp., 1991–2015

Reference	Animals immunised	Antigen used	Cell type labelled	*Perkinsus* sp. labelled	Other cross-reactivity	Antibody type
Choi et al. (1991)	Rabbits	*Perkinsus marinus* hypnospore protein extract	Hypnospore	*P. marinus*	*Crassostrea virginica* host	Polyclonal
Dungan & Roberson (1993)	Rabbits	*P. marinus* hypnospore emulsion	Hypnospore, trophozoite, zoospore	*P. marinus, P. olseni, Perkinsus* sp.	*C. virginica* host	Monoclonal and polyclonal
Montes et al. (1995)	Rabbits	*P. olseni* infiltrated granulocyte secretion products, emulsion of zoospores	Hypnospore, trophozoite	Not known	Not tested	Polyclonal
Gauthier & Vasta (1995)	Rabbits	*P. marinus* trophozoite emulsion	Hypnospore, trophozoite	*P. marinus*	Not tested	Polyclonal
Blackbourn et al. (1998)	Rabbits[a]	*P. marinus* hypnospore emulsion	Not described	*P. marinus, P. qugwadi*	Not tested	Polyclonal
Romestand et al. (2001)	Mice	*P. olseni* trophozoite and protein lysate	Hypnospore, trophozoite, zoospore	*P. marinus, P. olseni*	Did not react with host tissue	Monoclonal
Ottinger et al. (2001)	Rabbits	*P. marinus* extracellular products	Not tested	Not applicable	Did not react with host tissue	Polyclonal
Montes et al. (2002)	Rabbits	*P. olseni* high molecular weight peptide from hypnospore lysates	Hypnospore, trophozoite	*P. olseni, P. marinus*	Did not react with host tissue	Polyclonal
Bushek et al. (2002a)	Rabbits[a]	*P. marinus* hypnospore emulsion	Not tested	Not tested	Dinoflagellates	Polyclonal
Montes et al. (2005)	Rabbits	*P. olseni* trophozoites	Hypnospore, trophozoite	*P. olseni*	Not tested	Polyclonal
Earnhart et al. (2005)	Mice	*P. marinus* extracellular products	Trophozoite, schizont	*P. marinus*	Tested negative against *P. olseni*	Monoclonal
Park et al. (2010)	Rabbits	*P. olseni* hypnospore protein extract	Hypnospore, trophozoite, zoospore	*P. olseni*	Did not react with host tissue	Polyclonal
Kaewsalabnil et al. (2015)	Mice	*P. olseni* zoospores	Hypnospore, zoospore	*P. olseni*	Not tested	Monoclonal

[a] Serum used was from Dungan & Roberson (1993)

1995). This suggests that some components of the pathogen protein pool undergo a strong differentiation between the trophozoite and the hypnospore stages. The granular component of the nucleolus, chromatin, cell wall, plasmalemma, lomasomes, and vacuolar membranes are the main subcellular locations presenting immunodominant epitopes consistent within these 2 cell types (Montes et al. 1995).

Other studies immunised rabbits with ECPs or specific peptides (Montes et al. 1995, 2002). Montes et al. (1995) isolated a 225 kDa peptide, which is a secretion product of infiltrating defensive granulocytes of host clams against infectious trophozoites of *P. olseni*. It is stored within granules of granulocytes pending local secretion into infected clam tissues in apparent support of defensive encapsulation of proliferative, colonizing *P. olseni* trophozoites, as well as the capsule surrounding the trophozoites, the trophozoite wall, the invagination of the trophozoite plasma membrane, and the remnants of the capsule on isolated hypnospores. Later, Montes et al. (2002) isolated high molecular weight polypeptides from *P. olseni* hypnospore lysates, and immunised animals with SDS-PAGE slices containing the selected peptides following the protocol designed by Boulard & Lecroisey (1982). One serum against a polypeptide of an apparent molecular weight of 233 kDa named PWP-1 was selected for further analysis. This peptide was present in homogenate samples from *P. olseni* hypnospores, *Tapes semidecussatus* gills containing *P. olseni* trophozoites, but also in a *P. marinus* isolate. PWP-1 was immunolocalised exclusively in the cell

wall of the trophozoites and hypnospores, with no regional dominance. An ordered distribution parallel to the plasmalemma was observed in trophozoites, but in hypnospores, there was a dominance in the outer half of the cell walls. PWP-1 was present in all wall life stages of *P. olseni*, and also in *P. marinus*. The investigators showed that a considerable modification in the organisation of the serological and cytochemical characteristics of the cell wall occurred during the differentiation process from trophozoite to hypnospore (Montes et al. 2002). The antibodies produced in those 2 studies labelled peptide epitopes present on both hypnospores and trophozoites of the parasite.

3. SPECIFICITIES OF ANTIBODIES AGAINST OTHER *PERKINSUS* SPECIES

Studies that have tested cross-reactivities of antibodies against both their immunogen and other *Perkinsus* species, have all demonstrated cross-reactions (Table 1) (Choi et al. 1991, Dungan & Roberson 1993, Blackbourn et al. 1998, Bushek et al. 2002a). For example, the polyclonal antibodies that Dungan & Roberson (1993) produced against *P. marinus* hypnospore immunogen labelled *P. olseni* infecting *Haliotis laevigata* or *Ruditapes decussatus*, and diverse *Perkinsus* sp. infecting other molluscs worldwide. In addition, those polyclonal antibodies also labelled *P. qugwadi* infecting scallops (*Patinopecten yessoensis*) in British Columbia, Canada (Blackbourn et al. 1998). The monoclonal antibodies developed by Romestand et al. (2001) against *P. marinus* trophozoites and trophozoite protein lysate also labelled *P. olseni* trophozoites, indicating some shared epitopes between the trophozoites of the 2 species. The other studies that have developed antibodies did not test for cross-reactivities with other *Perkinus* species. Dungan & Roberson (1993) hypothesized that significant antigenic heterogeneity occurs among *Perkinsus*-like organisms infecting aquatic molluscs, and that a degree of serological homogeneity exists among the tested *Perkinsus* species. Overall, existing results demonstrate that different *Perkinsus* species share some common antigenic determinants.

4. SPECIFICITIES OF ANTIBODIES AGAINST NON-*PERKINSUS* SPECIES

To investigate the specificity of *Perkinsus* spp. antibodies against other marine microorganisms or host tissues, some investigations evaluated cross-reactivities of the antibodies they produced. For example, the antiserum Choi et al. (1991) raised against *P. marinus* hypnospores slightly cross-reacted with the host tissues (*Crassostrea virginica*). In studies that tested for the cross-reactivity of antibodies with mollusc host species, some cross-reactivity was observed (Choi et al. 1991, Dungan & Roberson 1993), while other antibodies did not show cross-reactivity with the host (Ottinger et al. 2001, Romestand et al. 2001, Montes et al. 2002, Park et al. 2010). However, most studies did not test the cross-reactivity of their antibodies against host tissues or other non-*Perkinsus* species (Gauthier & Vasta 1995, Montes et al. 1995, 2005, Blackbourn et al. 1998, Earnhart et al. 2005, Kaewsalabnil et al. 2015).

Bushek et al. (2002a) exhaustively tested the possibilities of shared antibody-binding epitopes of a variety of free-living and parasitic dinoflagellate cells with the polyclonal rabbit anti-*P. marinus* antibodies produced by Dungan & Roberson (1993). Three of 28 (10.7%) tested free-living dinoflagellates showed similar immunofluorescence signal intensities to *P. marinus* hypnospores: *Gymnodinium catenatum*, *Gyrodinium galatheanum*, and *G. uncatenum*. Seven of 8 (87.5%) dinoflagellate-like parasites of marine crustaceans and fish were also labelled: *Hematodinium* sp. (infecting Alaska tanner crabs *Chionoecetes bairdi*), *Hematodinium* sp. (infecting Scottish Norway lobster *Nephrops norvegicus*), *Hematodinium* sp. (infecting Chesapeake Bay blue crabs *Callinectes sapidus*), *H. australis* (infecting Australian sand crabs *Portunus pelagicus*), *Hematodinium* sp. (infecting French velvet swimming crabs *Necora puber*), *Hematodinium* sp. (infecting an unidentified gammaridean amphipod from Maryland), and *Amyloodinium occelatum* (infesting red drum *Sciaenops ocellatus* in the Gulf of Mexico). Bushek et al. (2002a) tested for reciprocal immunostaining with anti-*Hematodinum* sp. polyclonal rabbit antiserum (Field & Appleton 1996), which labelled *P. marinus* and the same 7 parasitic dinoflagellates previously labelled by the anti-*P. marinus* antiserum. They concluded that *P. marinus* shares common antibody-binding epitopes with several parasitic and free-living dinoflagellates.

5. SPECIFICITIES OF ANTIBODIES TARGETING ECPS

Rather than using whole cells, cell emulsions, or cell lysates to immunise mice or rabbits in order to produce antisera, some researchers have used ECPs

secreted by *Perkinsus* spp. isolates *in vitro*. Indeed, ECPs play an important role in the virulence and pathogenicity of the parasite that secretes them. For example, ECPs interfere with the reactive oxygen intermediate generation defensive system in oyster haemocytes (Volety & Chu 1995, Anderson 1999), increase *P. marinus* infection levels (La Peyre et al. 1996), and reduce lysozyme activity, plasma agglutinin titres, and oyster haemocyte migration (Garreis et al. 1996). Furthermore, *P. marinus* ECPs are a strong immunosuppressant in mice (Earnhart & Kaattari 2003), making it possible that they also have some effect on components of innate immunity in mollusc hosts.

In the first study using ECP antigens to produce antibodies, Ottinger et al. (2001) immunised rabbits with ECPs of *P. marinus* to investigate the possible involvement of those secretion products in the pathogenesis of infected oysters. They showed that some of the ECPs from *in vitro* pathogen cultures were also found within infected oysters. While oyster tissue homogenate had a slight inhibitory effect on the detection of ECPs, oyster tissue had only low level background reactivity, showing good specificity. Moreover, the sensitivity of their antibodies was greater than the traditional RFTM assay, and the immunoassay they developed is quicker to implement than the RFTM assay.

In another study, Earnhart et al. (2005) developed 4 monoclonal antibodies against different *P. marinus* ECPs to investigate their formation and roles. The researchers hypothesised that multiple ECPs might come from a restricted number of parental molecules. The natural functions of the labelled ECPs are unknown, but the authors postulated that they can be important in early infection mechanisms, and/or stimulate cell division and nutrient transport, as well as being involved in constitutive components of different cell compartments, or they might be present outside the cell membrane, similarly to the PWP-1 protein described by Montes et al. (2002). From results of both immunohistochemical and immunogold transmission electron microscopy, the monoclonal antibody 5A2 was shown to label *P. marinus* trophozoites, schizonts, and hypnospores, but not uninfected oyster host tissue, *P. olseni* in infected clam tissues, or the parasitic dinoflagellate *Hematodinium perezi* in tissues of an infected crab (Earnhart et al. 2005). Through immunofluorescence assays, the monoclonal antibodies 1B3, 9E12, and 5A2 labelled *P. marinus* trophozoites, schizonts, and, more weakly, hypnospores; their cross-reactivities with other organisms or the host tissue were not tested.

Although secreted ECPs of *in vitro*-propagated *Perkinsus* sp. isolates have potential use in developing immunological diagnostic tools, they have not been used by subsequent investigations that instead have used whole cells or cell lysates as immunogens.

6. CONCLUSIONS

While changes in antigenic properties of the cell walls of *Perkinsus* species are observed between different life stages, and especially during the transition from trophozoites to hypnospores, some epitopes are maintained, and have a promising potential for immunology and diagnostic purposes. However, they must be carefully chosen to avoid some reported shared serological structures of some *Perkinsus* species with related organisms, such as dinoflagellates or other *Perkinsus* species. In fact, the cross-reactivity of antibodies developed against *Perkinsus* sp. needs to be tested extensively against the tissues of the potential hosts, as well as with other closely related species, to ensure a rigorous specificity. Nine out of the 13 studies mentioned in this review tested for the cross-reactivity of their produced antibodies against host tissues or other *Perkinsus* species. Recommendation for further studies would be to consistently test newly produced antibodies against other species of *Perkinsus*, host tissues, and closely related organisms found in the vicinity of the tested species such as dinoflagellates. Moreover, such antibodies must be tested against the different infective life stages of targeted *Perkinsus* species. Indeed, changes of epitopes between life stages have been highlighted and could be an explanation for the difficulty of obtaining such reliable antibodies. Additionally, parasites are known to modulate their surface antigen expression depending on their environmental conditions (Schmid-Hempel 2009). Consequently, the production in mammals and ability of antibodies to recognise a specific epitope from the outer membrane of the parasites might be hindered. Additionally, some parasites have been shown to evade their host immune system by retracting key surface components when they are opsonised by host cells, here in mammals used to produce antibodies (Sacks & Sher 2002, Schmid-Hempel 2009).

An option to develop *Perkinsus*-specific antibodies would be to isolate and target *Perkinsus* spp. surface proteins common to all life stages through proteomics and genetics experiments. Once one or a few target proteins have been identified, production of antibodies against these peptides could begin and

yield extremely specific antibodies. Alternatively, techniques other than hybridomas are now available to produce specific monoclonal antibodies. Antibody phage display, for example, has been developed and optimised and uses filamentous phage display (Smith 1985) to produce monoclonal antibodies strictly *in vitro*, independently from animal immune systems (Schirrmann et al. 2011). Nucleic acid aptamers are also appearing as an interesting alternative to antibodies in diagnostic applications (Jayasena 1999, Brody & Gold 2000, Bouchard et al. 2010, Keefe et al. 2010, Hong et al. 2011). These small oligonucleotide molecules mimic the antibodies, and can show high binding affinity and selectivity towards their targets (Ellington & Szostak 1990, Eaton et al. 1995, Bridonneau et al. 1998, Brody & Gold 2000, Bouchard et al. 2010). They are produced independently from animals, in a simple and inexpensive process.

The characterisation of ECPs secreted by each species has been promising for their use as immunogens to develop diagnostic assays. To date, no immunoassay has been successfully developed for the detection and discrimination of *Perkinsus* species on a large scale. Such an assay would need to go through extensive validation procedures to meet the expectations of specificity and sensitivity of the OIE (OIE 2021a). Once an assay has been validated, it would then be possible to implement it widely, and train people on site for consistent use. The development of specific monoclonal antibodies against specific species of *Perkinsus* such as *P. olseni* or *P. marinus* would allow for the elaboration of more cost-effective diagnostic tests. An example of such an application in the field would be the integration of such antibodies into dipstick colorimetric tests, as mentioned in Section 1. In addition to being inexpensive, these immunoassays would need less sample preparation than the traditional RFTM and molecular assays, and would yield results in a matter of hours rather than days, allowing for quick management of stocks on farms during *P. olseni* or *P. marinus* outbreaks.

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