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Parasitism by the protozoan *Perkinsus atlanticus* favours the development of opportunistic infections

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ABSTRACT: It has been suggested that opportunistic pathogens could contribute to the mortality of *Perkinsus atlanticus*-infected clams. Examination of *Tapes semidecussatus* clams from the northern Mediterranean coast of Spain revealed that while 86% of the clams heavily infected with *P. atlanticus* were co-infected by bacteria and/or viruses, neither non-infected nor lightly *P. atlanticus*-infected specimens had bacterial or viral infections. The bacteria, which had a Gram-negative cell wall, were always located in the apical pole of gill epithelial cells and enclosed within membranous compartments. Bacteria-containing cells were hypertrophied and showed dysplasia with loss of cilia and microvilli. The viruses shared ultrastructural, morphologic and cytopathic characteristics of a polyomavirus. Viral particles with icosahedral symmetry were found in both the cytoplasm and the nucleus of numerous cell types. Virus-infected cells showed severe alterations, including hypertrophy, reduction of the intracellular compartments and extrusion of the nuclear envelope. Moreover, gill epithelial cells showed disorganization and swelling of the apical region, which affected the ciliary structure. Our findings show that *P. atlanticus* parasitism favours the development of opportunistic infections which have detrimental effects in this clam population.

KEY WORDS: Bacterium · Cytopathology · Opportunistic infection · *Perkinsus atlanticus* · Polyomavirus · *Tapes semidecussatus*

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INTRODUCTION

Protozoa of the genus *Perkinsus*, *P. marinus* (Mackin et al. 1950), *P. olseni* (Lester & Davis 1981), *P. atlanticus* (Azevedo 1989) and *P. qugwadi* (Blackbourn et al. 1998) are severe pathogens with highly destructive potential in bivalves and gastropods worldwide (Perkins 1993, 1996, Bower et al. 1994). Since its discovery, the taxonomic and phylogenetic position of this genus has been controversial. Although its placement in the phylum Apicomplexa (Levine 1978) seemed appropriate, recent molecular data indicate that *Perkinsus* is closely related to dinoflagellates (Siddall et al. 1997).

On the Atlantic and Mediterranean coasts of Europe, *Perkinsus atlanticus* trophozoites have been associated with epizootic outbreaks involving heavy mortalities of commercially valuable venerid clams of the genus *Tapes* (= *Ruditapes* = *Venerupis*), such as the indigenous species *T. decussatus* (Da Ros & Canzonier 1985, Comps & Chagot 1987, Azevedo 1989, Figueras et al. 1992, Villalba et al. 1993, Santmartí et al. 1995) and the introduced species *T. semidecussatus* (= *T. philippinarum* = *T. japonica*) (Sagristà et al. 1991, Santmartí et al. 1995). Our earlier reports showed that *P. atlanticus* parasitism elicits a unique defensive response in these clams (Montes et al. 1997). The infection induces an inflammatory response involving the infiltration of granule-containing haemocytes. These recruited granulocytes synthesize a secretory product, stored in membrane-bound granules, which is released and organized as a capsule around the trophozoites (Montes et al. 1995a, 1996). The main component of this defensive product, the polypeptide p225, is not expressed in non-infected clams (Montes et al. 1995b, 1996).

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The mechanism responsible for the high mortality of *Perkinsus atlanticus*-infected clams has not been clarified. We have shown that the defensive response of *Tapes* spp. prevents trophozoite dissemination. However, the massive haemocytic response observed in the gill filaments in advanced stages of infection produces the collapse of blood sinuses, which lead to host death (Montes et al. 1995a, 1996). On the other hand, several authors have suggested that clam mortalities attributed to *P. atlanticus* or unidentified *Perkinsus* spp. could be related to stress caused by environmental factors (McLaughlin et al. 1995, Cigarría et al. 1997) and/or opportunistic pathogens (McLaughlin et al. 1995, McLaughlin & Faisal 1998). In this way, several protozoan parasites, including haplosporidians (Villalba & Navas 1988, Figueras et al. 1992, Navas et al. 1992), labyrinthulids (Azevedo & Corral 1997) and apicomplexans (Azevedo & Cachola 1992), have been reported from different populations of *Tapes* spp. that showed high prevalence with *P. atlanticus*.

The aim of the present study is 2-fold. Firstly, to determine whether parasitism by *Perkinsus atlanticus* favours the development of secondary infections. Secondly, to evaluate the possibility that opportunistic pathogens could have contributed to the *Tapes semidecussatus* mortalities reported since 1990 in the northern Mediterranean coast of Spain.

MATERIALS AND METHODS

Test animals. Market-sized *Tapes semidecussatus* were collected from cultured beds of the Alfacs Bay, delta of the River Ebro, Tarragona (NE Spain), Mediterranean Sea, an area endemic for *Perkinsus atlanticus*. Clams were taken each summer over the years 1991 to 1996.

General strategy and specimen selection. With the aim of determining an opportunistic bacterial or viral infection associated with the parasitism by *Perkinsus atlanticus*, clams, heavily, lightly and non-infected by *P. atlanticus*, were used in this study. Lightly and non-infected specimens constituted the control groups.

Individuals heavily infected with *Perkinsus atlanticus* were selected from among those that showed white abscesses in more than 2 different organic localizations. Control groups were determined after thioglycollate assay (Ray 1952) of the 2 hemibranchs from 1 side. Lightly and non-infected control groups corresponded respectively to range 1 and 0 on the Mackin scale (Mackin 1961).

Tissue processing for TEM. Small tissue samples from gill, gut and digestive gland from 36 heavily, 20 lightly and 22 non-infected clams were fixed in 2% (w/v) paraformaldehyde, 2.5% (v/v) glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) for 2 h at room temperature. Primary fixation was followed by postfixation with 1% (w/v) osmium tetroxide in 0.1 M PBS for 1 h. Embedding was performed in Spurr's resin according to standard procedures. Semi-thin sections were stained with methylene blue and ultrathin sections with uranyl acetate and lead citrate. Semi-thin sections were observed and photographed with a Reichert-Jung Polyvar 2 optical microscope and the ultrathin sections with a Hitachi H-600 AB or MT 800 transmission electron microscope.

Immunocytochemistry. For immunocytochemical techniques, gill abscesses from heavily infected clams were fixed in 4% (w/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde in 0.1 M PBS (pH 7.4) for 2 h at 4°C. Samples were processed for Lowicryl K4M resin embedding (Polysciences LTD, Northampton, UK) as described by Carlemalm et al. (1982).

Immunogold labelling for DNA was carried out as described previously (Testillano et al. 1991). Briefly, grids were incubated with 20 µg ml^{-1} anti-DNA monoclonal antibody (Boehringer Mannheim, Mannheim, Germany). After washing, sections were incubated with 10 nm colloidal gold conjugated goat anti-mouse IgM (Janssen Biotech, Olen, Belgium). Immunogold labelling for p225 was as described earlier (Montes et al. 1995b). Bound polyclonal antibody was visualized following incubation with 10 nm protein A gold (Sigma, St Louis, MO, USA). All sections were stained with uranyl acetate and lead citrate.

RESULTS

Extent of the co-infection

Eighty-six percent (31/36) of the clams heavily infected with *Perkinsus atlanticus* were concurrently infected by bacteria and/or viruses. Absolute percentages for individual infections were 8% (3/36) for bacteria and 61% (22/36) for viruses. In addition, concurrent bacterial and viral infections were observed in about 17% (6/36) of the individuals. None of the specimens of the control groups, the lightly infected or the parasite-free, were infected with bacteria or viruses.

Bacterial infection

The bacteria observed in the clams heavily infected with *Perkinsus atlanticus* were exclusively located in the ciliated epithelial cells of the gill. Light microscopy revealed regional lesions of the gill epithelium associated with the bacterial infections (Fig. 1 inset). The most prevalent histological changes were hypertrophy

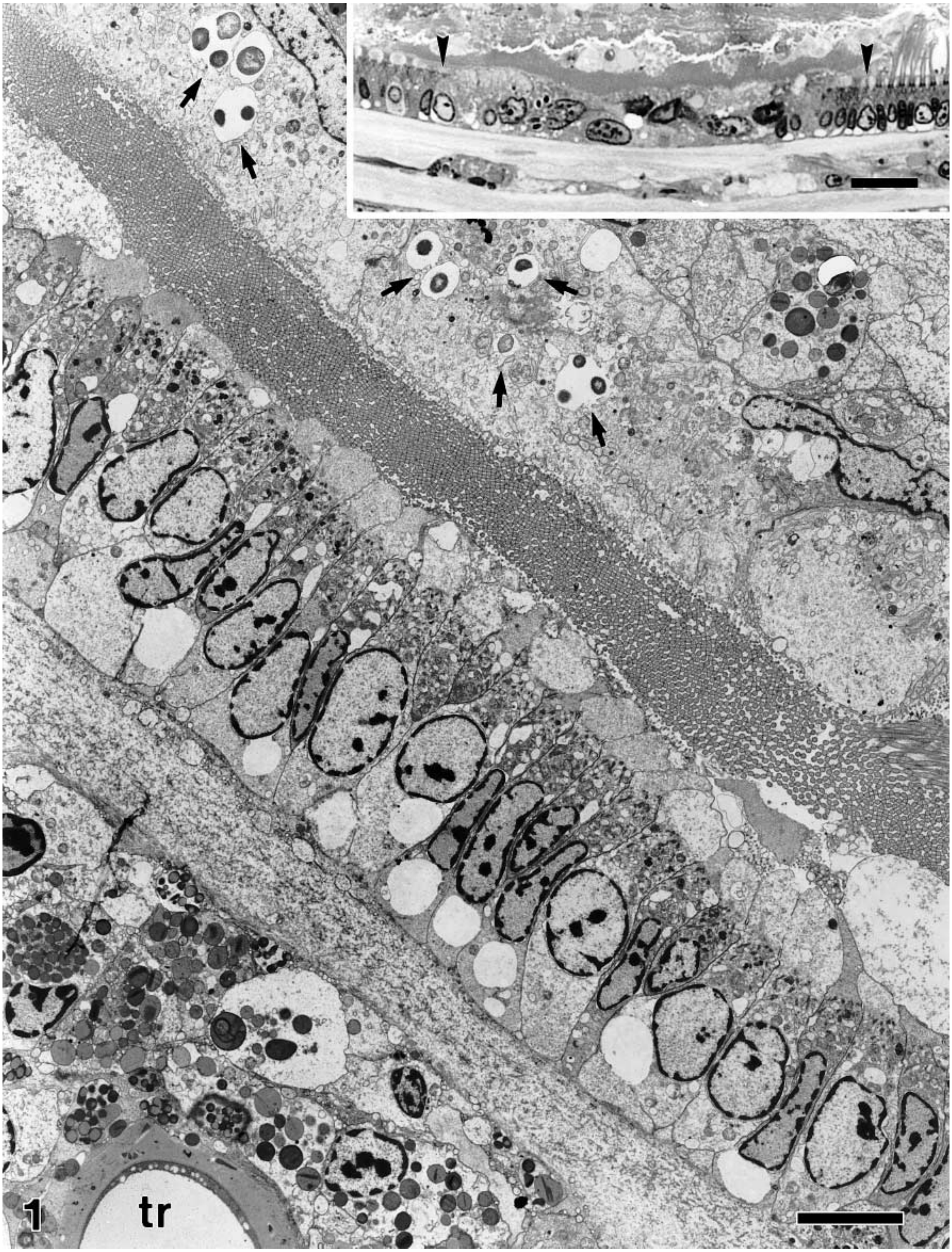
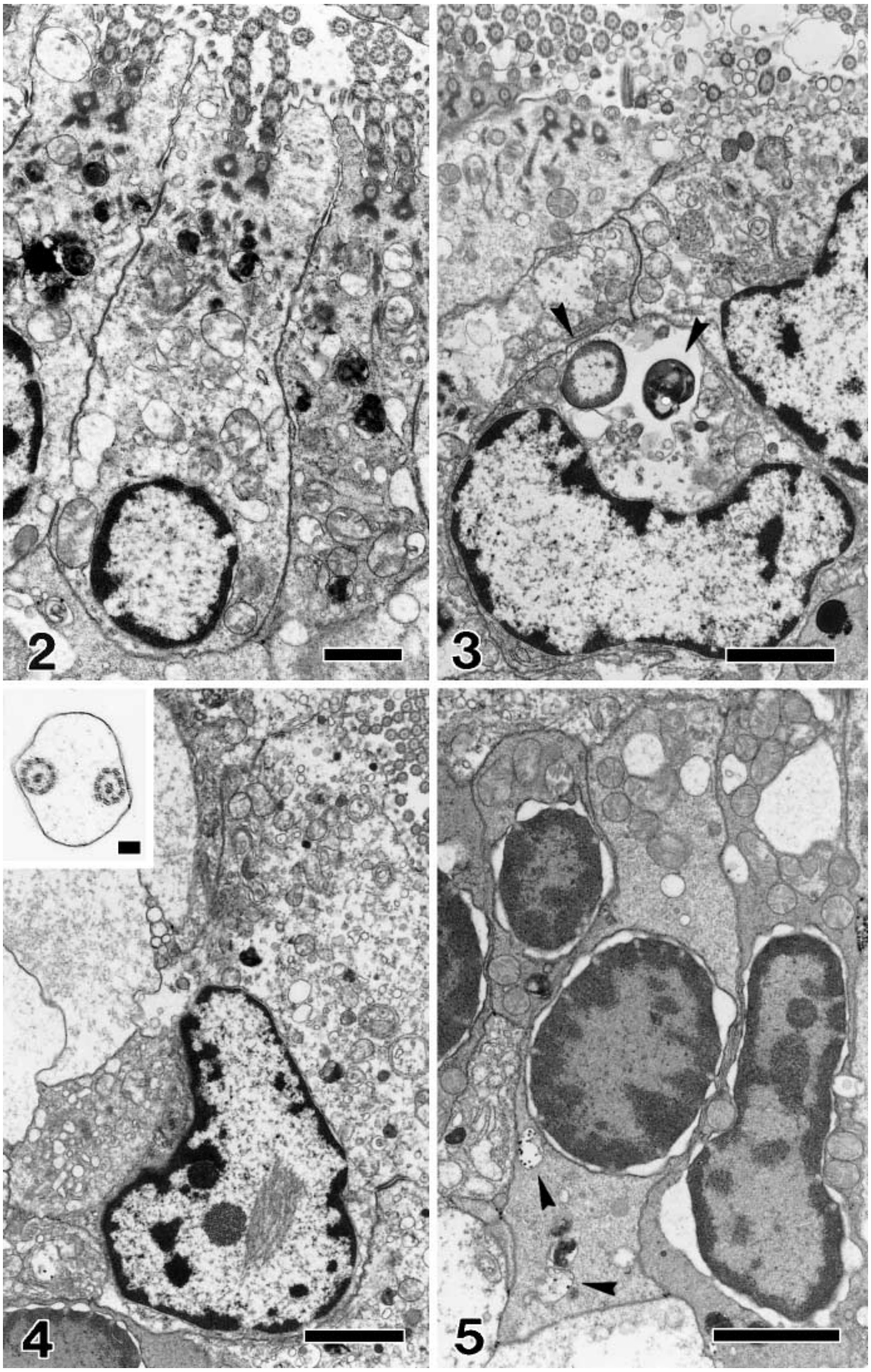


Fig. 1. *Perkinsus atlanticus*-parasitized gills from *Tapes semidecussatus*. Bacteria and virus- infected gill epithelium adjacent to the cellular reaction against *P. atlanticus* trophozoites (tr). Transversally sectioned rod-shaped bacteria are seen within membranous compartments (arrows) in the apical pole of the infected cells. Virus-containing epithelial cells show swelling of the apical region and nuclear membrane extrusions. The underlying basal lamina is extended and disorganized. ×3300. Scale bar = 5 µm. Inset: Regional lesion of the gill epithelium (arrowheads). Bacteria-containing enlarged cells show dysplasia with loss of cilia and microvilli. ×410. Scale bar = 25 µm

and dysplasia of the infected epithelial cells, which occasionally caused disorganization of the epithelial sheet.

TEM revealed that the enlarged epithelial cells contained several rod-shaped bacteria that averaged 2.5 µm in length and about 1.5 µm in width (Fig. 1). The bacteria, which had a typical Gram-negative cell wall, were characterized by a peripheral electron-dense material composed of ribosome layers and a central light area corresponding to DNA. Neither cell division nor other developmental stages were observed. The bacteria were located in the apical pole of the host cells, always enclosed within a membranous compartment. The number of bacteria observed in each of these endocytic compartments ranged from 1 to 4. Fusion processes between the bacteria-containing compartments were observed.

Bacteria-containing epithelial cells showed strong differences with those non-infected (Fig. 2). The hypertrophied and dysplastic infected cells showed effacement of cilia and microvilli (Fig. 1 inset). The infected cells also showed a severe reduction of the endomembranes and a relaxation of the cell contacts, with an apparent loss of polarization (Figs 1 & 1 inset). Finally, nuclei of affected cells exhibited high irregularity in nuclear profile and were up to 3 times larger than nuclei of non-infected cells (Figs 1 [inset] & 3).

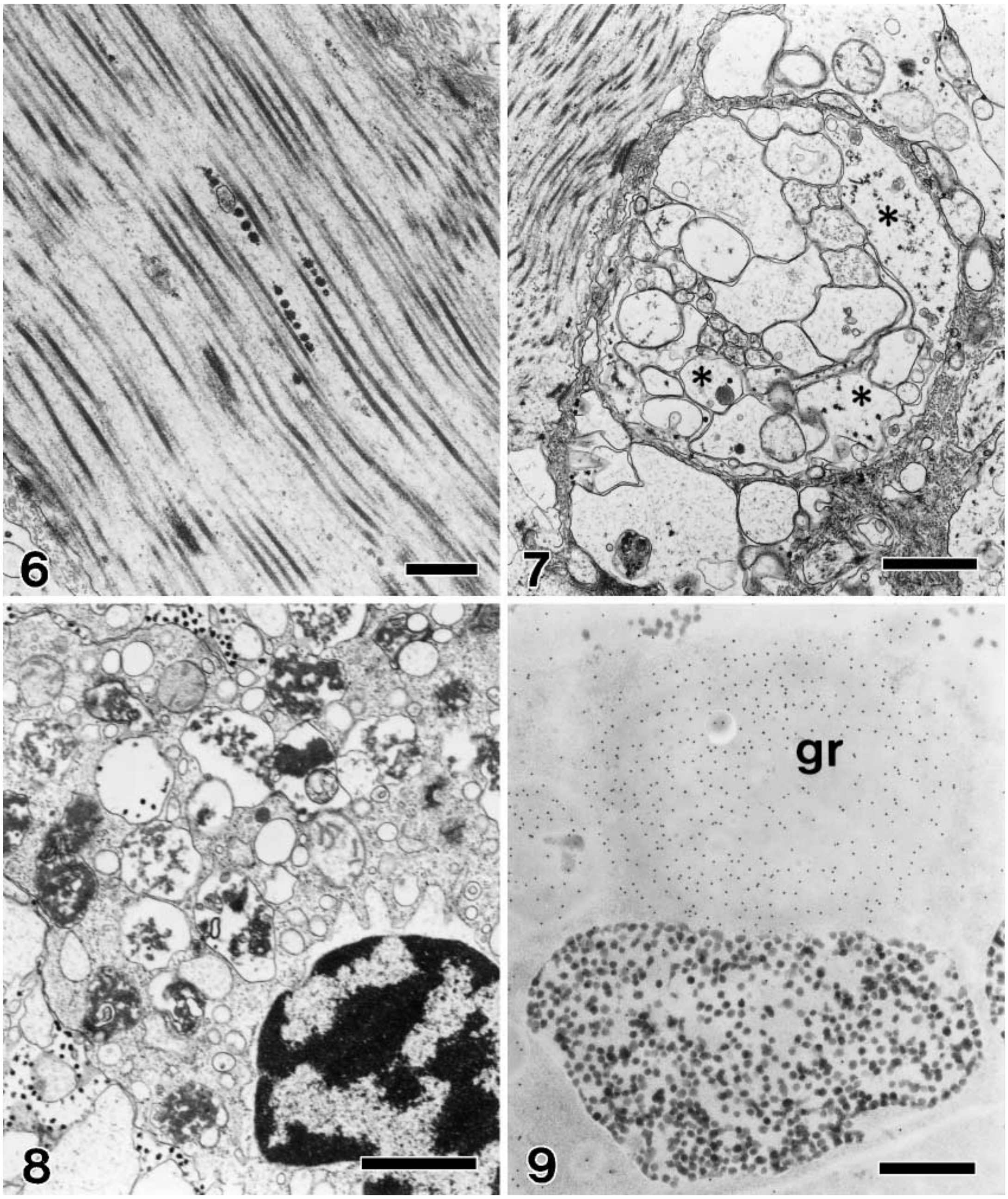


Figs 2 to 5. Alterations of infected gill epithelial cells. Fig. 2. Structure of a healthy ciliated epithelial cell. ×10 400. Scale bar = 1 µm. Fig. 3. An epithelial cell containing 2 bacteria (arrowheads) showing an enlarged host cell nucleus. ×7200. Scale bar = 2 µm. Fig. 4. Nuclear inclusion bodies and autophagosomes are seen in a virus-infected cell. ×6500. Scale bar = 2 µm. Fig. 4 inset. Compound cilia containing 2 axial microtubule complexes from a virus-infected epithelial cell. ×23 000. Scale bar = 0.1 µm. Fig. 5. Epithelial cells in advanced stages of the viral infection showing pycnotic nuclei with numerous nuclear membrane extrusions, mitochondria with dense matrix and virions within membranous compartments (arrowheads). Note the absence of cilia and microvilli. ×8250. Scale bar = 2 µm

Viral infection

Electron microscopy revealed that the viral infection observed in the clams heavily infected with *Perkinsus atlanticus* was systemic, being massive in gills and gut. The viral particles, averaging 46 nm in diameter, were homogeneously electron-dense and non-enveloped, with profiles indicative of icosahedral symmetry. They were found in the gill epithelial cells (Figs 1 & 5), striated muscular fibres (Fig. 6), neurons (Fig. 7), infiltrated granulocytes (Fig. 8), redifferentiated granulocytes of the cellular reaction against *P. atlanticus* trophozoites (Fig. 9), endothelial cells (Fig. 12) and connective tissue (Figs 13 & 14).

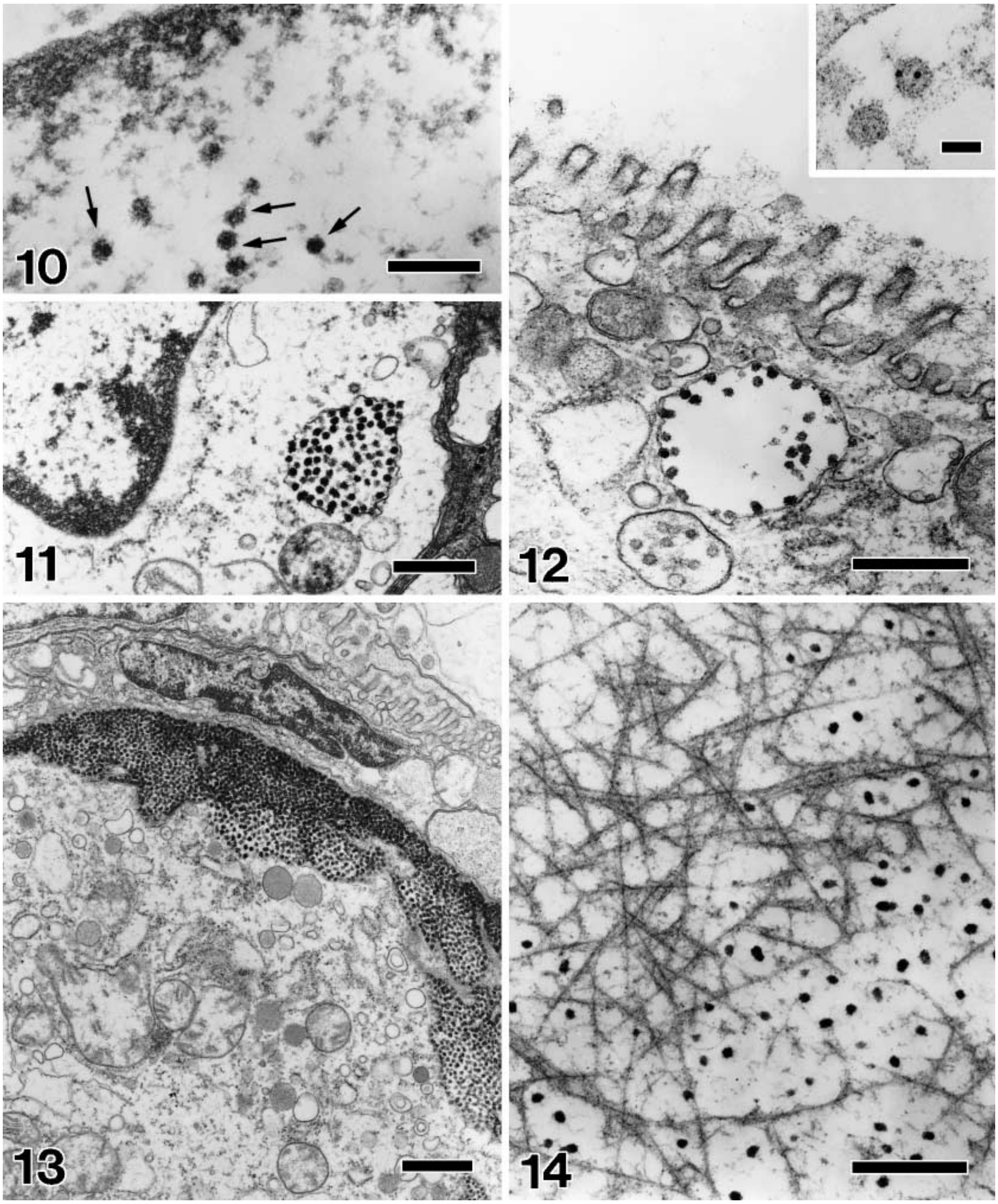
Intracellular viral particles were seen both in the cytoplasm and nucleus. The cytoplasmic virions were located within membranous compartments (Figs 5, 8, 9, 11 & 12), usually attached to the membrane of these vesicles (Fig. 12). Free cytoplasmic virions were observed in striated muscle fibres (Fig. 6), neurons



Figs 6 to 9. Some virus-infected cell types. Fig. 6. Striated muscular fibre from gut showing free cytoplasmic virions. ×20 000. Scale bar = 0.5 µm. Fig. 7. Neuromuscular junction from gut. Free cytoplasmic viral particles are seen in some neurons of the nerve (asterisks). ×13 000. Scale bar = 1 µm. Fig. 8. Infiltrated granulocyte from digestive gland. Several viral particles within membranous compartments, swollen mitochondria and nuclear membrane extrusions are noticed. ×16 000. Scale bar = 1 µm. Fig. 9. p225 immunolocalization in a redifferentiated granulocyte of the cellular reaction against *P. atlanticus* trophozoites from gill. A strongly labelled granule (gr) is close to an endocytic vesicle containing numerous virions. ×27 000. Scale bar = 0.5 µm

(Fig. 7) and cells undergoing lysis. Viral inclusion bodies (Fig. 4) and clusters of viral particles (Fig. 10) were also observed in the nucleoplasm of some infected cells. Immunogold labelling of the virions with a monoclonal antibody against DNA corroborated that the viral genome was constituted by DNA (Fig. 12 inset).

Virus-infected cells showed significant morphological and ultrastructural changes, the gill epithelial cells showing the most marked alterations. The infected epithelial cells of the gill were hypertrophied, showing a characteristic swelling of the apical region, which affected the ciliary structure (Fig. 1). The cilium mem-



Figs 10 to 14. Intra- and extracellular location of the viral particles. Fig. 10. Several viral particles (arrows) in the nucleus of a gill epithelial cell. ×66 000. Scale bar = 0.2 µm. Fig. 11. Endocytic vesicle containing numerous virions in a connective tissue cell from gut. ×23 000. Scale bar: 0.5 µm. Fig. 12. Viral particles attached to the membrane of an endocytic vesicle of a gill endothelial cell. ×33 000. Scale bar = 0.5 µm. Fig. 12 inset. DNA immunolocalization on Lowicryl sections from gills. Virions contained in a membranous compartment are labelled. ×100 000. Scale bar = 50 nm. Fig. 13. Large cluster of densely packed virions located between connective tissue cells of the gill. ×10 200. Scale bar = 1 µm. Fig. 14. Damaged basal lamina of the gill epithelium. The matrix areas that contain viral particles show disruption of the fibrilar elements. ×33 000. Scale bar = 0.5 µm brane was usually detached from the axoneme, and compound cilia containing multiple axial microtubule complexes within a single ensheathing ciliary membrane were frequently observed (Fig. 4 inset). Nuclei of all infected cell types were slightly enlarged and occasionally pycnotic. Furthermore, in some infected cells, the outer nuclear membrane was detached and enlarged forming a nuclear extrusion (Figs 1 & 8). This cytopathic effect was constant in gill epithelial cells, in which the nuclear extrusion was characteristically located in the basal pole (Fig. 1). In pycnotic nuclei-containing cells, the nuclear membranes were held together only by the pore structures (Fig. 5).

Alterations of the endomembranous compartment was another common feature of the virus-infected cells. The primary change was a reduction in the number of organelles and the appearance of autophagic membranous rings. Moreover, large swollen mitochondria were observed in granulocytes (Fig. 8) and connective tissue cells (Fig. 13). Mitochondria with a dense matrix were also observed in pycnotic nuclei-containing gill epithelial cells (Fig. 5).

Numerous viral particles were also found in the extracellular matrix. They were preferentially found in the connective matrix and in the basal lamina of the gill epithelium. The distribution of these extracellular virions varied according to their location. Thus, whereas clusters of densely packed virions were often seen between connective tissue cells (Fig. 13), individualized viral particles were distinguished between the fibrilar elements of the basal lamina (Fig. 14). Gill epithelium basal lamina containing virions appeared swollen and disorganized, with many short individual matrix fibres (Fig. 1). The disruption of the fibrilar elements was apparently provoked by the virions, since the fibres close to the virions were shorter than those that were free of them (Fig. 14).

DISCUSSION

The present study is the first description of a concomitant *Perkinsus atlanticus* and bacterial and/or viral infection. In addition, we report the first characterization of a virus infecting the Manila clam *Tapes semidecussatus*.

Although a wide variety of prokaryotic agents have been described infecting different bivalve molluscan species (Bower et al. 1994, Fryer & Lannan 1994), the intracellular prokaryotic organism observed in the gill epithelial cells of *Perkinsus atlanticus*-infected clams *Tapes semidecussatus* and the associated pathology have not been reported. In the venerid clams, *T. semidecussatus* and *T. decussatus*, most of the infections involving intracellular prokaryotic agents have been associated with rickettsia or chlamydia-like organisms (Elston 1986, Mialhe et al. 1987, Bower et al. 1992, Navas et al. 1992, Villalba et al. 1993, Figueras et al. 1996). However, they differ from the Gram-negative bacterium described in the present report with respect to the pleomorphism, generation of microcolonies, organic distribution and benignancy. Further studies are needed to identify it.

Many pathogenic bacteria can induce their own internalization into eukaryotic cells that are non-phagocytic (Ireton & Cossart 1998). To prevent or resist the exposure to lysosomal antimicrobial activities, these intracellular pathogens have developed several strategies either to disrupt the endosome, to block endosome-lysosome fusion or to survive in the lysosome (Finlay & Falkow 1997). In this respect, the *Tapes semidecussatus* bacterium was exclusively located within endomembranous compartments of a non-phagocytic cell type, the gill epithelial cell. The apical location and the appearance of these bacteria-containing compartments strongly suggest that they are related to endocytic pathways and, therefore, this bacteria might impede the fusion of the endosome with the lysosome. *Mycobacterium* spp., *Salmonella typhimurium* and *Legionella pneumophila* are some of the bacterial pathogens of mammals which can elude endosome-lysosome union (Finlay & Falkow 1997).

Internalization of bacterial pathogens is frequently accompanied by morphological changes in the host cell membrane and underlying cytoskeleton (Ireton & Cossart 1998). In agreement with these observations, the presence of the *Tapes semidecussatus* bacterium was associated with severe alterations of infected gill epithelial cells, including dysplasia with loss of microvilli and cilia. Disorganization of microvilli and ciliary structures has also been described in gill epithelial cells of the oyster *Crassostrea gigas* concurrently infected by unidentified bacterium and rickettsia (Azevedo & Villalba 1991). Likewise, disruption of microvilli is a common feature in the intestinal epithelial cells of mammals during adhesion or internalization of different enteropathogenic bacteria (Rikihisa et al. 1992, Babakhani et al. 1993, Finlay & Falkow 1997).

This bacterium also caused cell and nuclear hypertrophy, reduction of endomembranes and apparent relaxation of the cell contacts. Although hypertrophy and lysis of cellular organelles is a common sign of bacterial-infected host cells (Bower et al. 1994), the loss of polarization observed in the bacteria-containing gill epithelial cells of *Tapes semidecussatus* is an unusual bacterial-induced pathology.

Numerous viral diseases, primarily due to iridioviruses, herpesviruses, picornaviruses and papovaviruses, occur in marine molluscs worldwide (Farley 1978, Bower et al. 1994, Elston 1997). The *Tapes semi-*

decussatus virus described in the present study closely resembles members of the Polyomavirinae subfamily of the Papovaviridae in morphological characteristics, cellular locations and particle diameter (Cole 1996, Shah 1996). Although viruses belonging to the Papovaviridae family are associated with various diseases in oysters (Farley 1976a, Norton et al. 1993, Comps et al. 1999) and clams (Farley 1976b, Harshbarger et al. 1979), the *T. semidecussatus* virus is only the second record of a polyomavirus in molluscs. Previously, a polyomavirus was found in the soft-shell clam *Mya arenaria* (Farley 1976b).

The *Tapes semidecussatus* virus was the same shape and size as that of *Mya arenaria* polyomavirus (Farley 1976b) and shared several similarities in cell specificity. Thus, the *T. semidecussatus* virus was primarily found in the gill epithelium, haemocytes and connective tissue. We also observed it in endothelial cells, neurons and striated muscle fibres. Our findings are consistent with the present *T. semidecussatus* virus classification, since some mammalian polyomaviruses have also been reported infecting these cell types (Shah 1996).

The most common signs of polyomavirus-induced cytopathology are cell and nucleus hypertrophy, formation of intranuclear inclusions, and reduction of the endomembranous compartment (Shah 1996). These alterations, together with the extrusion of the nuclear envelope, were the main cytopathic effects observed in *Tapes semidecussatus*, and are in line with the previous polyomavirus description in molluscs (Farley 1976b). The ciliated epithelial cell of the gill was the infected cell type that showed the most marked alterations, showing up as a disorganization and swelling of the apical region, which affected the ciliary structure. As described previously (Durfort et al. 1994), compound cilia containing 2 or more axial microtubule complexes were frequently observed in these infected cells. Similar ciliary alterations also occur in virus-containing ciliated cells of mammals (Ghadially 1997). These morphological changes could represent disorganization of certain components of the cytoskeleton, which would increase the intracellular osmotic pressure with subsequent cell swelling. Previous studies have demonstrated that simian polyomavirus 40 (SV40) induces both disruption of the actin containing microfilaments (Graessmann et al. 1980) and disorganization of the intermediate filaments (Ben-Ze'ev 1984).

Interestingly, many matrix fibres of the gill epithelium basal lamina were shortened, thus altering the structural integrity of the basal lamina. Although intrinsic proteolytic activity of virions is an uncommon finding, our observations strongly suggest that the disruption of the matrix fibres is the direct consequence of proteolytic viral activity, since the ends of the shortened fibrilar elements were topographically associated with the virions. Similarly, Lepore et al. (1996) have reported that enhancin, the protein found in the viral occlusion bodies of granulosis viruses (Baculoviridae) and responsible for the degradation of the peritrophic membrane of lepidopteran insects (Derksen & Granados 1988, Wang et al. 1994), is a metalloprotease.

Bacteria and viruses infecting adult molluscs are considered secondary invaders or stress parasites rather that primary pathogens, since they are often seen only in molluscs that are suffering from another disease or from environmental stress (Lauckner 1983, Fryer & Lannan 1994). Our data are consistent with these observations, since while 86% of heavily *Perkinsus atlanticus*-infected clams *Tapes semidecussatus* were concurrently infected by bacteria and/or virus, none of the lightly infected nor of the *P. atlanticus*-free specimens had bacterial or viral infections. Overall, these findings suggest that *P. atlanticus* parasitism could reduce the efficiency of the defensive mechanisms of the Manila clam *T. semidecussatus*, allowing the development of bacterial and viral opportunistic infections. Furthermore, our observations show that these secondary infections could have contributed to the *P. atlanticus*-infected *T. semidecussatus* mortalities reported since 1990 in the northern Mediterranean coast of Spain.

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