and *Metapograpsus messor*[23] were also reported to harbour this virus. In this study, we have found that several additional wild caught shrimps and crabs are hosts for WSSV. These include shrimps such as *Heterocarpus* sp., *Aristeus* sp., *M. elegans* and crabs such as *C. hoplites* (Figure 3 *a*). It can also be observed that the prevalence is more in the west coast where Thiruvananthapuram, Mangalore and Mumbai showed 100% positivity after second-step PCR than the east coast samples where Kolkata and Chennai showed 80% and 71% positivity respectively. Reasons for higher positivity in the west coast are not clear. In fact there is not much of aquaculture activity in these areas. It raises the question whether wild animals are a source of virus for cultured shirmp or vice versa.

Among the various samples, squilla showed highest percentage positivity followed by crabs and shrimps. Hossain *et al.*[23] reported that *Squilla mantis* is one of the carriers of WSSV. None of the animals examined had white spots on their carapace or other parts of the exoskeleton. These results suggest that though samples were positive by one step PCR, they did not show signs of disease. It is possible that these animals are tolerant to the virus.

This study shows that the wild crustaceans could act as reservoirs of WSSV. They could be a source of virus to aquaculture systems. Further, the results suggest that sensitivity of detection can be improved by choosing primers that yield smaller amplicons.

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Occurrence of concurrent infections with multiple viruses in *Penaeus monodon* from culture ponds of north coastal Andhra Pradesh

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A histopathological study of *Penaeus monodon* showing external symptoms of white spot disease, collected from culture ponds at Mulakuddu and Rambilli villages (Visakhapatnam District) during May and July 2001, provided evidence for the occurrence of multiple viral infections. Each diseased shrimp carried concurrent infections with four different viruses, identified on the basis of nuclear changes, resulting in the formation of characteristic inclusion/occlusion bodies, as WSSV, YHV, MBV and IHHNV. The identification of the various viruses needs confirmation through molecular diagnostic methods. This constitutes a report recording concurrent infections with multiple viruses in a single shrimp.

VIRAL diseases have been seriously affecting the sustainability and economic success of the shrimp industry. To date over 22 different viruses are known to infect

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shrimps and several of them have been implicated in mass mortalities in cultured shrimps, such as white spot syndrome virus (WSSV), yellow-head virus (YHV), infectious hypodermal haematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV)[1]. Transmitted into the culture system either vertically from brooders or horizontally through diseased shrimps and vectors, the control of these infections has proved to be a major task confronting the industry.

As regards the situation in India, the shrimp industry which has flourished well and proved lucrative initially, was hit by viral diseases in 1993, and in due course of time, the infections have reached epizootic levels in culture ponds all along the coast causing mass mortalities and extensive damage to the system. In most cases, the associated virus has been identified as WSSV[2,3,4]. Other viruses like monodon baculovirus (MBV)[2,5,6], IHHNV[7], hepatopancreatic parvovirus (HPV)[8] and YHV[9] have also been recorded from cultured shrimps in India, but the reports are sporadic and infections have not reached epizootic levels. Consequently, WSSV has remained as the only major virus affecting shrimps in India till now.

During our investigations on viral diseases of *Penaeus monodon* from culture ponds, moribund shrimps in acute phase of infection were obtained from epidemics of WSSV infection that occurred during May–July 2001 in nursery and grow-out ponds at Mulakuddu and Rambilli areas, Visakhapatnam District, Andhra Pradesh. Clinical and histopathological studies of these shrimps revealed that although the nuclear inclusion bodies typical of WSSV were predominant, the tissues manifested cytopathological changes indicative of the co-occurrence of other viral infections. Evidence indicated that each examined shrimp carried infections with as many as four different viral infections. Although double infections with viruses have earlier been reported[9,10], multiple viral infections of this magnitude within a shrimp have not been recorded so far. Pending confirmation of the results of the histopathologcial study, by application of molecular methods like PCR, *in situ* hybridization and dot blot, we report here our observations that provide evidence for the presence of multiple viral infections.

For the histopathological study, moribund shrimps obtained from nursery and grow-out ponds at Mulakuddu and Rambilli villages and aged 30 and 60 days respectively were fixed in Davidson's fluid, sectioned (5 μm) and stained with haematoxylin and eosin.

The shrimps appeared lethargic and showed external symptoms of WSSV, like white spots dispersed all over the exoskeleton and red discolouration of the body. Additionally, appendage rot and tail rot were also exhibited. Gills appeared pale and gill-fowling with ciliates was profuse. Histopathological screening of tissue sections revealed each shrimp to be affected by the following viral diseases, recognized by the specific nuclear changes as indicated.

WSSV: Cowdry-type inclusion bodies characterized by nuclear hypertrophy, chromatin margination (basiphilic) and eosinophilic (acidophilic) central mass (Figure 1 *a*) typical of WSSV infections, occurred distributed in abundance in the subcuticular connective tissue, gills and haematopoietic tissue.

YHV: Nuclear inclusion bodies appearing as perfectly spherical, deeply staining basiphilic bodies (Figure 1 *b*) of different sizes with a clear area around and some of them exhibiting karyorrhexis, were found distributed in the connective tissue, gills, lymphoid organ, haematopoietic and nervous tissues, being most numerous in the lymphoid organ.

MBV: Infections occurred in cells of hepatopancreas, detected by the presence of clusters of polyhedral eosinophilic occlusion bodies (Figure 1 *c*). The level of infection ranged from moderate to heavy in different shrimps.

IHHNV: Inclusion bodies indicative of IHHNV infection were present as condensed nuclei with eosinophilic centres and marginated chromatin (Figure 1 *d*). They co-occurred with WSSV inclusion bodies in subcuticular connective tissue and gills but could be distinguished by their smaller size, and the presence of a clear unstained space between the eosinophilic central mass and marginated chromatin basiphilic ring. This identification, however, needs confirmation by molecular diagnostic tools.

Apart from the above, the diseased shrimps also showed few other histopathological changes. Several cells in subcuticular epithelium exhibited nuclear pycnosis followed by karyorrhexis, resulting in development of numerous eosinophilic or basiphilic spherical bodies that give the cells a peppered appearance (Figure 1 *e*). In addition, frequently prominent cells having a characteristic appearance were found amidst cells of hepatopancreas and in the adjacent connective tissue. The cells appeared prominent because of the presence of a large deeply staining eosinophilic body occupying the major part of the cell, shifting the compressed nucleus to one side (Figure 1 *f*). The aetiological agent causing these variations is yet to be identified.

The histopathological observations provided unequivocal evidence for the presence of WSSV and MBV. Nuclear changes such as pycnosis and karyorrhexis considered as diagnostic of YHV infection may also be produced by bacterial infections. Hence positive identification of YHV depends on the detection through application of molecular diagnostic methods as well as on isolation, purification and analysis of nucleic acids. WSSV was found to be the predominant viral infection in shrimps from both the localities, which probably overshadowed expression of all other viral infections. The intensity of infection with other viral agents as determined by the numerical abundance of the various inclusion bodies varied considerably in the shrimps from the two localities. While IHHNV bodies were more abundant

in juvenile shrimps from nursery ponds at Mulakuddu, YHV infections were more predominant in shrimps from Rambilli area. MBV occurred with equal intensity in both.

Examples of co-occurrence of two or more viruses have been reported in several crustaceans[10]. Only one instance of double infection with WSSV and YHV was recorded from India[9]. However, the present study reports multiple infections involving invasion by four viruses in an individual shrimp. It is difficult to determine at this stage the status of these infections and their relative role in disease expression. The answer should come through bioassay studies. Possibility exists that infections with YHV and IHHNV are in a latent stage and appeared secondarily to stress brought on by the highly pathogenic WSSV.

Hitherto, WSSV has been the major viral disease adversely affecting our shrimp industry. There are indi-

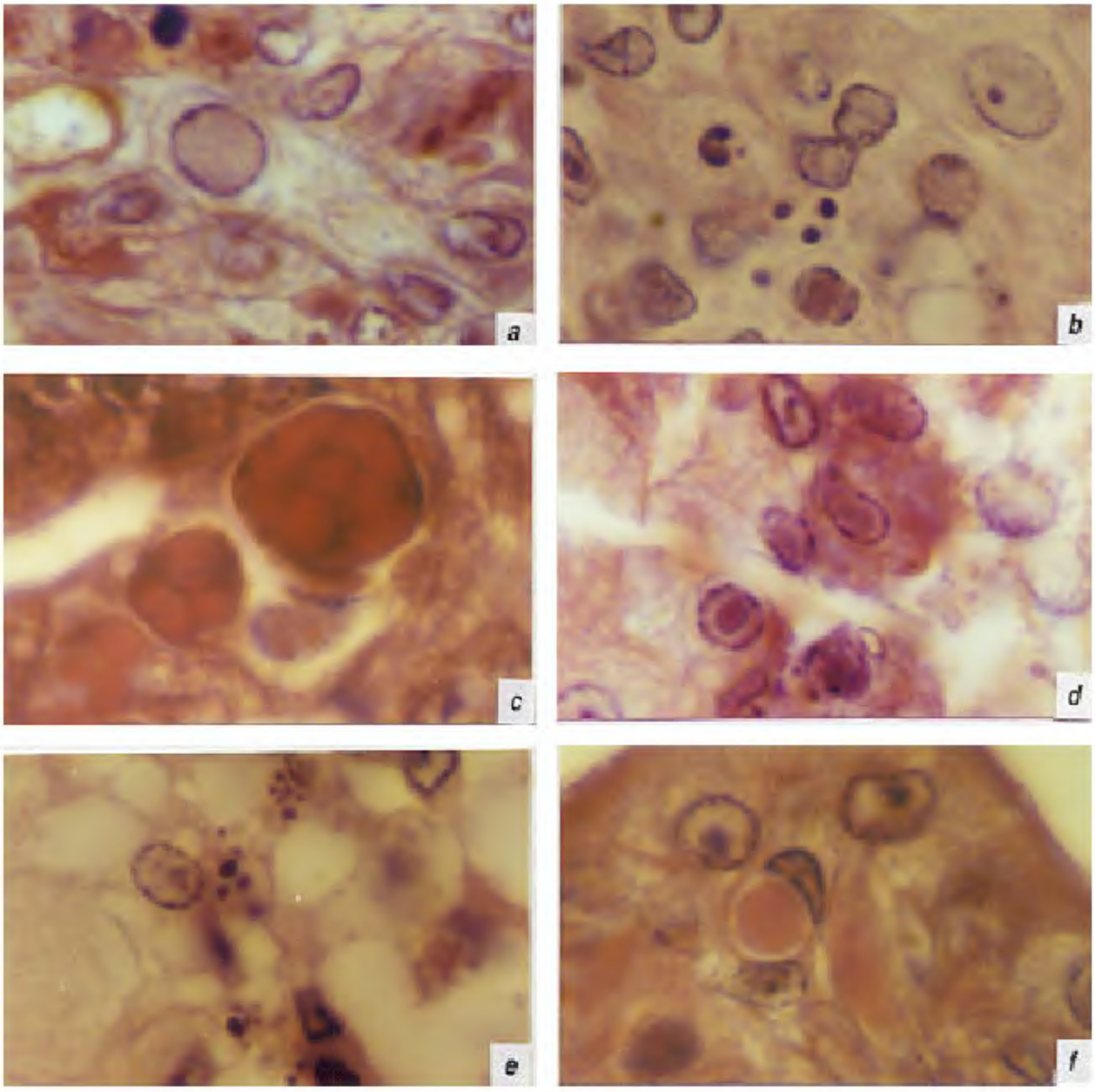

Figure 1. Sections of diseased *P. monodon* stained with haematoxylin and eosin, magnification × 2000; ***a***, Cowdry-type inclusion body typical of WSSV in the connective tissue showing nuclear hypertrophy, chromatin margination and central eosinophilic mass; ***b***, Connective tissue showing deeply stained spherical inclusion bodies typical of YHV infection; ***c***, Section of HP showing characteristic clusters of eosinophilic occlusion bodies characteristic of MBV infection; ***d***, Connective tissue showing cellular changes typical of IHHNV infection, i.e. eosinophilic central mass surrounded by clear zone and a marginated chromatin basiphilic ring; ***e***, Epithelial cells showing numerous eosinophilic to basiphilic spherical granules giving a peppered appearance; and ***f***, Subcuticular connective tissue showing prominent cells, enclosing a large eosinophilic body occupying a major part of the cytoplasm inside the cell, with the nucleus shifted to one side.

cations that in near future the industry is bound to pose problems due to emerging viral infections if proper precautionary measures are not implemented.

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