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RESEARCH ARTICLE

MUD CRAB *SCYLLA SERRATA* (FORSKAL) AS CARRIER OF WHITE SPOT SYNDROME VIRUS (WSSV) IN CORINGA MANGROVES, ANDHRA PRADESH.

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Abstract

The prevalence of white spot syndrome virus in wild mud crabs in Coringa mangroves was studied at molecular level. 357 crab samples were tested through PCR at three sampling stations-Kakinada Bay, Coringa and Gaderu. Of them, 58 were positive (16%) and 299 negative in the first step PCR whereas 132 were positive (37%) and 225 were negative in the nested PCR. At Kakinada Bay, there was 16.4 % positivity in first step PCR and 36% in the second step, whereas in Coringa creek it was 14.4% and 37.3% in the first and second step respectively. In Gaderu creek the crabs were 17.1% positive in the first step PCR and 37.6% in the second step. 31.8% were positive to the virus in 2007 and in 2008 it was 36.09%. WSSV prevalence was more in smaller size group (0-4.0cm) *i.e.*, about 52% in 2007 and 2008 and decreased with increasing size groups (36.6% in 2007 and 31.7% in 2008 in the size group (5.0-8.0cm). In 9.0-13cm size class it was 29.69% in 2007 and 36.09% in 2008

Introduction:-

Mass mortality of commercially important shrimps has occurred in many Asian countries due to disease out breaks (Kim *et al.*, 1998) and viral pathogens cause major damage leading to serious economic loss (Flegel, 2001). In nature, a high percentage of apparently normal and healthy animals harbour potential pathogens without evidence of clinical signs or over disease (Wedemeyer, 1970). Aquatic organisms particularly fisheries are generally affected by endemic, epizootic and infectious diseases and diseases caused by microorganisms are more devastating. White Spot Syndrome Virus is known to affect the tissues of ectodermal and mesodermal origin like cuticle, gill, gut, lymphoid organs, antennal gland, hematopoietic tissues, connective tissue, ovary and the ventral nerve cord (Wongteerasupaya *et al*., 1995, Wang *et al*.,1999). This disease affects shrimps of all ages & sizes, also present in a wide range of hosts- penaeids, crabs, lobsters and other crustaceans like copepods and amphipods (OIE, 2003). WSSV infection is characterized by reduction in food intake and a loose cuticle with white spots on the inner surface (Takahashi *et al*., 1994).

Decapod crustaceans like crabs play an important role as 'carrier' of WSSV in natural environment. Several methods are available for the detection of the white spot disease virus, including PCR (Kim *et al*., 1998; Nunan *et al.*, 1998; Peng *et al*., 1998), Dot blot hybridization (Wongteerasupaya *et al*., 1996; Sahul Hameed *et al*., 1998) and ELISA (Sahul Hameed *et al.*, 1998). Molecular primers based on the probes have been developed for WSSV

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detection by Polymerase Chain Reaction (Chang *et al*., 1996, Lo *et al.,* 1996; Van Hulten *et al.,* 2000; Hossain *et al.,* 2001). These molecular techniques are highly specific and have a high degree of sensitivity. Nested PCR has been reported to increase sensitivity of detection by 10^3-10^4 times (Lo *et al.*, 1996) and sensitivity increases as the amplicon size decreases (Hossain *et al.,* 2004).

In the present study, molecular epidemiology of White Spot Syndrome Virus (WSSV) in the mangrove crab, *Scylla serrata* (Forskal) has been taken up to assess the prevalence of the virus in crabs, which serve as carriers of the virus.

Material and methods:-

Crabs were collected from three stations- Kakinada Bay (Station I, $16^0$54'20.59''N, $82^0$15'01.52''E), Coringa creek (Station II, $16^0$52'31.88''N, $82^0$16'11.71''E) and Gaderu creek (Station III, $16^0$51'07.02''N, $82^0$18'43.51''E) in the Coringa mangrove forest area of Andhra Pradesh, India (Fig.1) where shrimp culture was carried out in the vicinity. A total of 357 crab samples were collected from three field stations and were categorized into size groups of 0-4 cm, 5-8cm and 9-13cm. The prevalence of WSSV was assayed every month from the gills and was grouped into 1st half of the year and 2nd half in 2007 and 2008. Polymerase Chain Reaction (PCR) technique was employed to detect the presence of virus in the crabs. Test kits for conducting the PCR were procured from M/S Mangalore Biotech Ltd, Mangalore, Karnataka, India and were used for DNA extraction, DNA amplification and DNA detection following the methods of Sambrook and Russel (2001).

Extraction of DNA:-

Crab samples were brought to the laboratory in live condition. In the laboratory, the gills were dissected out and collected in sterile plastic sachets. Digestion Buffer (Guanidium Chloride) was added (800µl) to the plastic sachet containing the gills of the live crabs and the material was macerated in the sachet and incubated at room temperature for about 10-15 minutes. The homogenate was transferred to 1.5 ml micro centrifuge tube and centrifuged (Remi, C-24) at 4000 rpm for 5 minutes. 150 µl of supernatant was transferred to a fresh micro centrifuge tube and to it DNA precipitation Buffer (Absolute Ethyl Alcohol) was added (450 µl). The tube was centrifuged at 10,000-12,000 rpm for 10 minutes at 4^0C. Then the supernatant was discarded. DNA washing Buffer (70% Ethyl Alcohol) (450 µl) was added and centrifuged at 10,000-12,000 rpm for 5 minutes at 4^0C. The supernatant was then discarded and the pellet was dried at room temperature. After drying the pellet, it was dissolved in 100 µl of double distilled water, which will contain the DNA of White Spot Syndrome Virus, if present, in the sample.

DNA Amplification:-

2 µl of template DNA was taken into the master mix tubes (reagent mixture tubes that contain amplification buffer, dNTPs, amplification grade water, Mg^{++} and Taq polymerase). Nested PCR amplification was carried out in a Thermocycler (Eppendorf) for WSSV following a temperature profile, with an initial denaturation for 5 min. at 94^0C followed by 30 cycles at 94^0C for 30 seconds; 55° C for 30 seconds; 72^0C for 30 seconds and a final extension at 72^0C for 5 min. PCR was carried out using species-specific primers namely 486 bp fragment in first step and 310 bp in the second step.

Termocycler was programmed as: I step- initial denaturation at 94^0C for 5 min followed by each cycle of denaturation at 94^0C for 30 sec, annealing at 42^0C for 30 sec, extension at 72^0C for 40 sec and a final delay at 72^0 C for 7 min, the same conditions were followed in the second step also. In every batch of reaction, a positive control and negative control was included.

DNA Detection:-

PCR products, after DNA amplification in the thermocycler were separated by agarose gel (1%) containing 0.5 µg per ml ethidium bromide and observed in a DNA transilluminator (Genei). Depending on the size of the electrophoresis tank, the quantity of agarose gel (1%) required was prepared. The agarose in 1x TAE (Tris Acetate EDTA) buffer was heated till the agarose was completely melted and cooled to room temperature. Before pouring the gel on to the already set gel platform, 5-µl ethidium bromide per 100 ml was added.

After the gel was set, it was placed in the electrophoresis chamber. 20 µl of product was taken after DNA amplification and it was mixed with 4-µl gel loading buffer (Bromophenol Blue) and loaded the gel. 8-µl molecular weight marker (100 bp DNA size marker) was loaded in a separate lane of the gel. The gel was run at 80 volts for 10

minutes and then at 120 volt till the tracking dye moves about 2 cm from the edge of gel. Finally the gel was viewed under the UV transilluminator and the results were documented with a UV documentation unit (Uvidoc).

Results:-

A total of 357 crab samples were tested for WSSV with PCR in the study area. Out of these, 58 are positive (16%) and 299 are negative in the first step PCR whereas 132 are positive (37%) and 225 are negative in the nested PCR (Fig.2).

WSSV Prevalence:-

In the 1st half of the year (2007) at Kakinada bay, the incidence of WSSV was 10.71% in the first step and 32.14% in the second step. In the 2nd half 22.22% were positive and 44.44% in the second step. In Coringa creek, the incidence was 8% in the 1st step and 32% in the 2nd step in the 1st half. In the 2nd half 16.22% were positive in first step and 37.84% in second step. In the 1st half of the year in Gaderu creek, 18.52% in the 1st step and 40.74% in the 2nd step. In the 2nd half 20% were positive in first step and 37.14% in second steps (Fig. 3).

In the 1st half of the year (2008) at Kakinada Bay, the incidence was 20% in the 1st step and 44% in the 2nd step. In 2nd half 12.12% were positive in first step and 24.24% were positive in second step. In Coringa creek in the 1st half, 19.23% were positive in first step and 50% in second step. In 2nd half 13.33% were positive in first step and 30% were positive in second step. At Gaderu creek in the 1st half 17.86% were positive in first step and 42.86% were positive in second step. In the 2nd half 14.81% were positive in first step and 29.63% were in second step (Fig. 4).

Out of the crab samples tested 16% were positive in the first step whereas 37% positive in the nested PCR. At Kakinada Bay, 16.4% in first step and 36% were positive in the second step. In Coringa Creek, 14.4% and 37.3% in first step and second step respectively were positive to the disease. In Gaderu, the percentage incidence was 17.1 in first step and 37.6 in the second step (Fig.5).

Size groups:-

In 2007 at Kakinada bay, the intensity of positivity in the three different size classes *viz.*, 0-4cm, 5-8cm and 9-13cm was 41.67%, 25% and 25% respectively. In Coringa creek, the intensity of positivity was 62.5%, 44.44% and 30.77% in the three different size classes 0-4cm, 5-8cm and 9-13cm respectively for the year. In Gaderu, the intensity of positivity in 0-4cm size was 50%, in 5-8cm it was 43.48% and it was 31.82% in 9-13 size group (Fig.6).

At Kakinada Bay region, the percentage occurrence of the disease in the three different size classes 0-4cm, 5-8cm and 9-13cm was 40%, 35% and 25% respectively in 2008. At Coringa it was 57.14%, 32.26% and 25% in size groups of 0-4cm, 5-8cm and 9-13cm respectively. In Gaderu region the intensity of positivity was 57.14% in 0-4cm size group, 28.57% in 5-8cm size group and 30% in the size group of 9-13cm (Fig.7).

The overall showed that at Kakinada Bay, the intensity of positivity in the three different size classes viz., 0-4 cm, 5-8 cm and 9-13 cm was 40.84%, 30% and 30% respectively. In Coringa creek, it was 59.82%, 38.35% and 32.88% in the three different size classes. In Gaderu, the intensity of positivity in 0-4cm size was 53.37%, in 5-8cm it was 36.02% and it was 30.91% in 9-13 size group (Fig.8).

Fig1:- Study area.

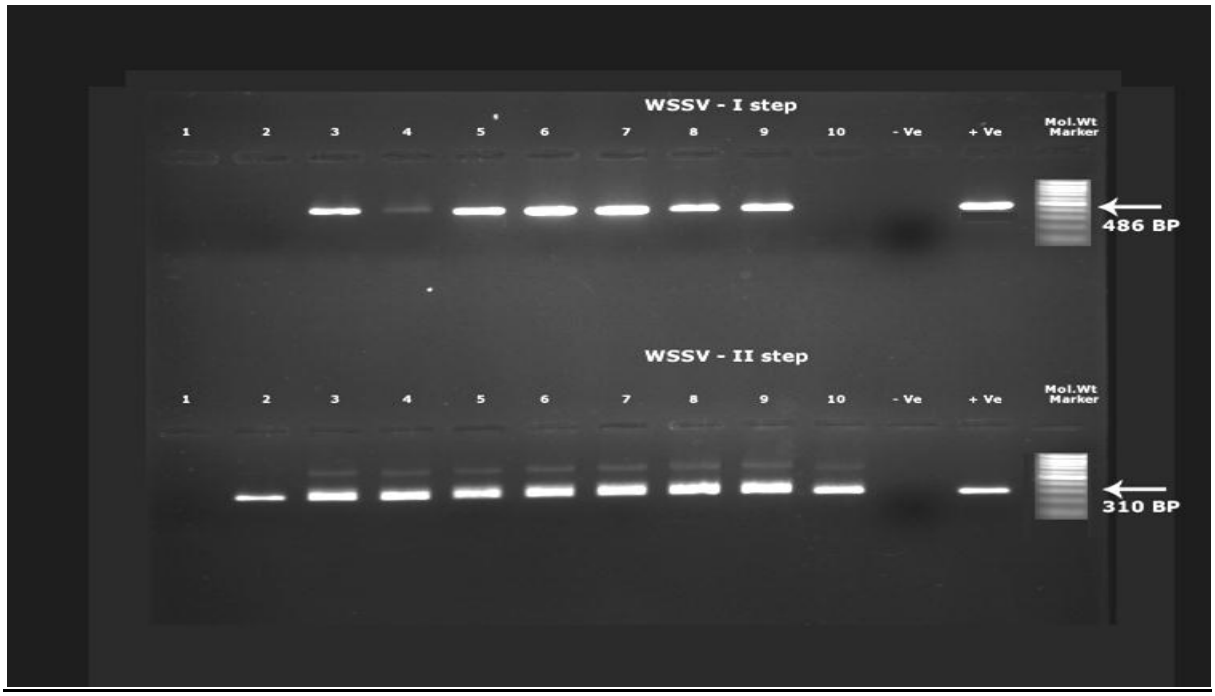

Fig2:- Electropherogram of WSSV in *S. serrata* of Coringa mangroves**.**

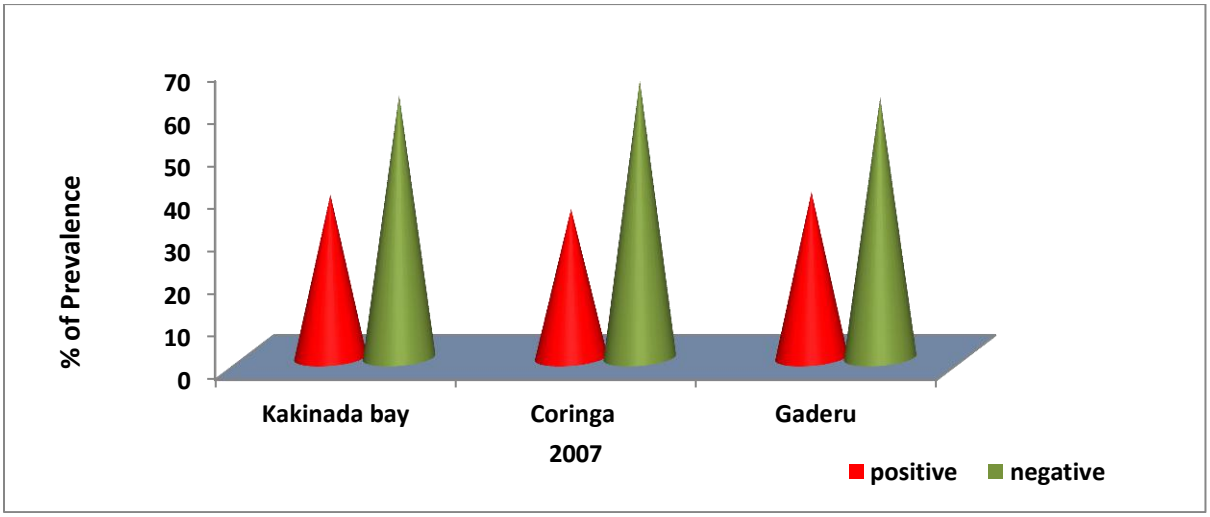

Fig. 3:-Prevalence of WSSV in *S. serrata* at three stations of Coringa mangroves in 2007.

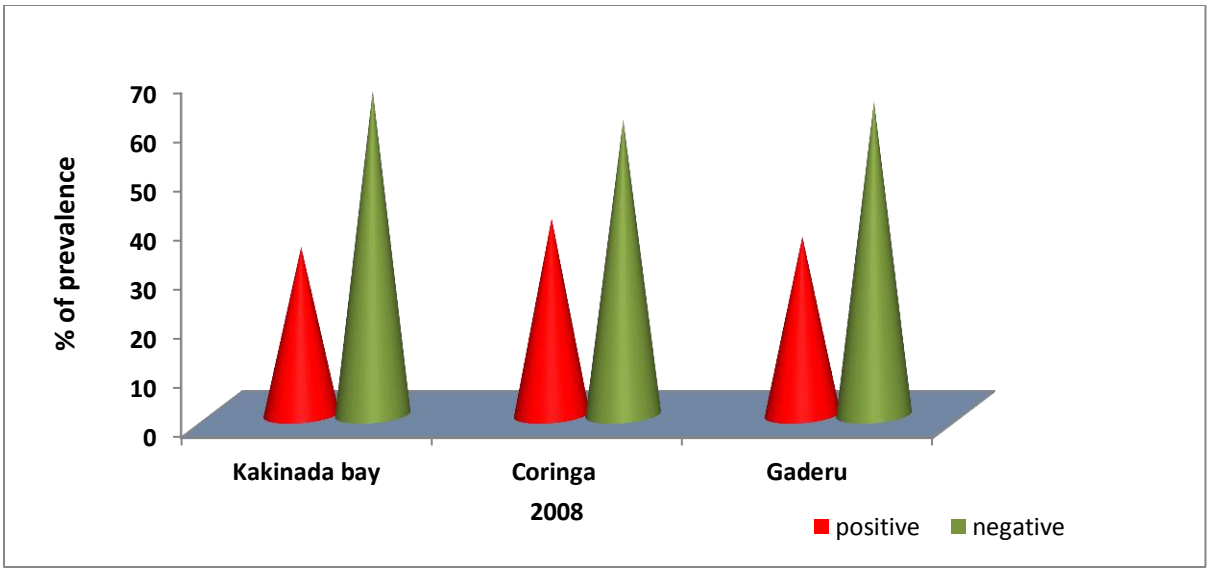

Fig. 4:-Prevalence of WSSV in *S. serrata* at three stations of Coringa mangroves in 2008.

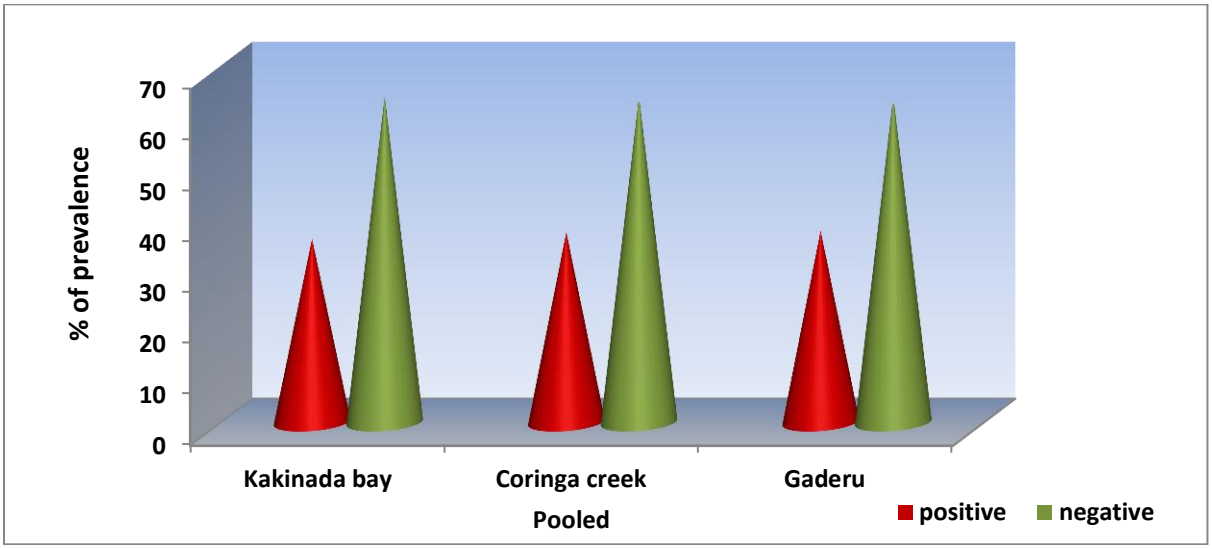

Fig. 5:-Prevalence of WSSV (Overall) in *S. serrata* at three stations of Coringa mangroves.

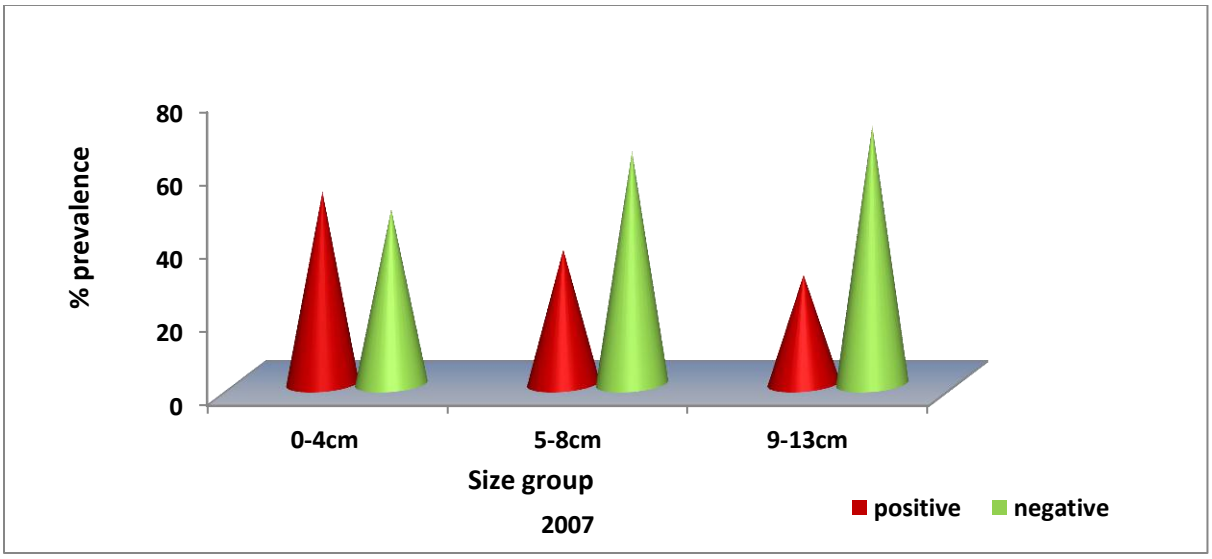

Fig. 6:-Prevalence of WSSV in different size groups of mud crab *S. serrata* of Coringa Mangroves in 2007.

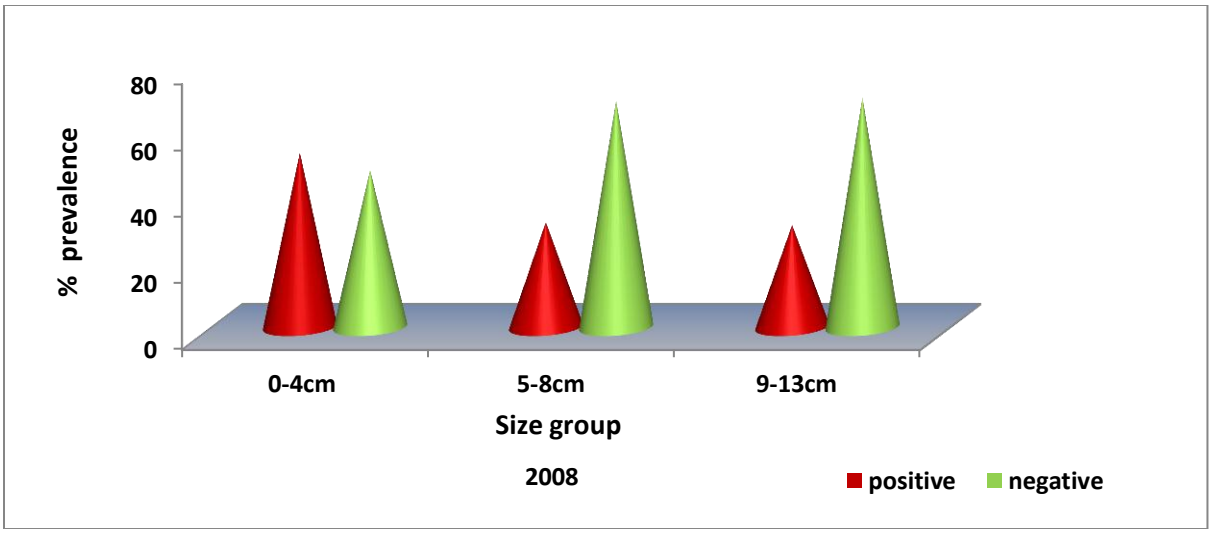

Fig. 7:-Prevalence of WSSV in different size groups of mud crab *S. serrata* of Coringa Mangroves in 2008.

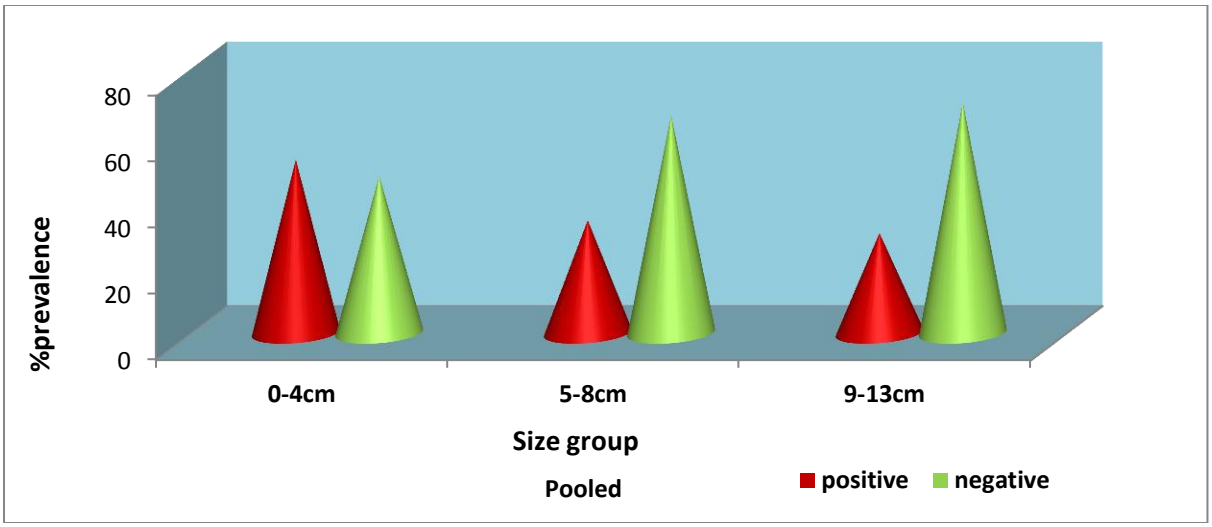

Fig. 8:-Prevalence of WSSV (Overall) in three size groups of mud crab *S. serrata* of Coringa mangroves.

Discussion:-

WSSV has become a major threat in shrimp culture causing overnight mass mortalities and there are no reports of incidence of WSSV in India before 1994. Karunasagar *et al* (1997) have reported severe mortalities of cultured shrimp *Penaeus monodon* due to WSSV. The white spot virus is believed to have been transmitted through seed and brooders brought to India from Southeast Asian countries, where the virus has been very much prevalent (Shankar and Mohan, 1998). A list of susceptible species of WSSV has been presented by Lo *et al* (1996). Crustaceans other than shrimps may also serve as carriers of the disease though they do not exhibit any clinical signs of WSSV externally and are also active and healthy. The mud crab *S. serrata* has also been found to be the same.

Mud crabs are known as carriers and vectors of WSSV (Lo *et al*., 1996). Natural WSSV infections have been found in captured and cultured specimens of the mud crab *S. serrata* in many countries of Asia (Lo *et al.,* 1996; Kanchanaphum*et al.,* 1998). WSSV has been detected in around 60% of the benthic larvae of mud crab, *S. Serrata* both under natural and experimental conditions (Chen *et al.*, 2000). Although mud crab is known to be a carrier of WSSV in India, studies are limited. In the present study, nested PCR has been used for better sensitivity. It has also been suggested by Lo *et al* (1996) that the two-step PCR 10^3 to 10^4 times more sensitive than 1-step PCR. Kou *et al* (1998) have opined that the sensitivity of the 2-step amplification is 10^3 to 10^4 times greater than that of the 1-step amplification.

Otta *et al* (1999) have carried out a study to evaluate the crustaceans as potential carriers of WSSV using PCR. After the WSSV outbreaks in India during late 1994, in the shrimp culture ponds, several instances of White spots on the exoskeleton of mud crab, *S. serrata* have been observed. Mud crab has been shown to be a carrier of WSSV in Taiwan (Lo *et al.*, 1996) and Thailand (Kanchanaphum *et al.*, 1998, Supamattaya *et al.*, 1998). Otta *et al* (1999) have tested one mud crab with external symptoms and 20 healthy samples without white spots and reported that the one with external symptoms is positive in the 1st step PCR whereas 3 out of 20 healthy specimens are positive in the 2nd step PCR indicating that *S. serrata* is also a carrier of WSSV. In India, it has been noticed that the natural prevalence of WSSV in crab is about 5.06% while in shrimp culture ponds it is about 30% (CIBA, 2009). In the present study the prevalence of WSSV has been found to be 37.8% for the year 2007 and 36.09% for 2008.

It is evident from the present study that the samples collected from Kakinada bay region have shown that only 16.4% positive in 1st step PCR whereas 36% are positive in 2nd step. Coringa creek region have shown that 14.41% samples are positive PCR whereas 37.29% in 2nd step PCR reaction. In case of Gaderu creek region, 17.1% samples are positive in 1st step PCR and 37.61% in 2nd step PCR. Thus this study has confirmed that nested PCR is very sensitive to viral disease diagnosis than 2nd step PCR.

The wild populations of mud crabs harbouring WSSV as carriers may adversely affect the shrimp production in the culture ponds, since shrimp culture ponds has been located in the vicinity of crab fishery. According to Rajendran *et al* (1999) *S. serrata* which is orally infected with WSSV has shown 10-20% mortality in 30 days. The infected crabs have shown marked histopathological changes similar to those reported earlier for WSSV in naturally infected shrimp (Wongteerasupaya *et al*., 1995). Similar results have been observed by Hossain *et al* (2001). Chakraborty *et al* (2002) have reported that all the specimens of *S. serrata* collected from different localities are positive to WSSV.

The prevalence of the disease is more during wet season (June-Sep) than the dry season (Jan-June) in both years. Of the three regions, the prevalence is 36% at Kakinada Bay, 37.3% in Coringa and 37.6 % in Gaderu. It may be due to the discharge of waters from shrimp ponds, containing pathogen into the creeks and drainage canals, since the shrimp culture is very high in the region (about 675 ha near Coringa and 1050 ha near Gaderu). Much difference in the percentage of positivity has also been observed in samples of different size classes. It is evident that among the three size classes 0-4 cm, 5-8 cm, 9-13 cm, the smallest size class showed higher percentage of positivity *i.e.* 52.38% in 2007 and 52.63% in 2008.The same is noticed in all three areas of study. The lowest of positivity was noticed in the size class 9-13 cm confirming that the smaller ones are more susceptible to this virus.

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