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# Detection of DNA damage in white spot syndrome virus –infected shrimp (*Peneaus mondon*) by using comet assay

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**Abstract:** White spot syndrome virus (WSSV) is the causative agent of a disease which has recently caused high shrimp morbidity, mortality and severe damage to shrimp cultures. In this study a strain of WSSV from tiger shrimp *Penaeus monodon* was used to detect DNA damage in muscle cells by using Comet Assay. The term "comet" refers to the pattern of DNA migration through the electrophoresis gel, which often resembles a comet. The present results declare that Comet Assay is a rapid, simple, visual and sensitive technique for detecting and analyzing DNA in damaged cells. In WSSV infected shrimps, damaged cells looked like a tiny comet under a microscope. When DNA is broken in infected cells it forms a tail that moves away from the unbroken DNA. The amount of DNA damage in cells was estimated from comet tail length as the extent of migration of the genetic material. Significant increases in intensity percentage of DNA tail portion, tail length and tail moments of infected shrimps were observed in comparison with normal healthy shrimps. Furthermore, the clinical signs of white spot disease were also discussed in this study.

**Keywords:** White Spot Virus, Penaeus *Monodon*, Clinical Sign, DNA Damage, Comet Assay

## 1. Introduction

In 1992, a new virus appeared in shrimp farms in northern Taiwan causing disease and massive mortality [1]. In late 1993, the viral agent was first isolated from an outbreak in Japan [2] and within a few years this new pathogenic agent spread to several shrimp farming countries [3]. At first, it was thought that different viral agents had simultaneously appeared in different regions and each were given a specific name: hypodermal and haematopoietic necrosis baculovirus (HHNBV) [4] or white spot baculovirus [5] systemic ectodermal and mesodermal baculovirus [6], penaeid rod-shaped DNA virus [7]. Later, it was recognized that a single viral agent was responsible for these reports. Eventually an informal consensus was reached to call it white spot syndrome virus (WSSV). This pathogen is now recognized as the most serious for shrimps aquaculture worldwide [8].

WSSV has been detected in a wide range of decapod and non-decapod crustacean hosts from natural environments. In addition, all decapod crustaceans from marine and brackish or freshwater sources that have been subjected to experimental infection trials have been successfully infected. WSSV has also been detected in a number of other species [9]. Regarding, penaeid shrimp species, this virus is known to effect most commercially important species of penaeid shrimp including *Penaeus monodon*, *P. japonicus, P. indicus, P. chinensis, P. merguensis*, *P. aztecus, P. stylirostris, P. vannami, P. duorarum* and *P. setiferus* [10]. All farmed penaeid shrimp species from late post-larvae to juvenile and adult stages are highly susceptible to WSSV infection [9]. The rapid spread of WSSV within wild and cultured stocks of shrimp may be caused by unregulated processing, disposal of infected imported shrimp, or the use of contaminated broodstock [11]. The WSSV can even survive freezing and consequently survives in previously -frozen farmed shrimp sold in the market so, the scientists concluded that the virus can spread to the local natural environment, which constitutes a substantial risk [11].

WSSV infection is characterized by the gross signs of rapid reduction in food consumption and a loose cuticle with white spots on inner surface [12]. In some cases, moribund shrimp may also display reddish brown colouration [13]. Other signs of disease include swelling of branchiostegites

[14]; enlargement and yellowish discolouration of the hepatopancreas [15]; thinning and delayed clotting of haemolymph [16]. Furthermore, WSSV provoked some biochemical and hematological changes that represented by significant increase in glucose and total carbohydrate levels in the hemolymph of WSSV-infected shrimp in comparison to values for healthy shrimp and reductions in muscle and hepatopancreas of infected shrimp. The average total protein and free amino acids were significantly different between two groups in hemolymph, muscle and hepatopancreas. The fatty acid level increased significantly in the hepatopancreas of WSSV-infected shrimp, but was reduced in the hemolymph and muscle of WSSV-infected shrimp in comparison to healthy shrimp. Moreover, significant reductions in total hemocyte counts and hemocyanin contents were observed in WSSV-infected shrimps. Additionally, reductions in oxygen consumption and ammonia excretion in WSSV-infected shrimp were recorded [17]. Moreover, the white spot disease had a serious economic impact on the shrimp aquaculture industry in affected countries, i.e in outbreak shrimp ponds, cumulative mortality may reach 80 to 100% within 7 to 10 days post-infection [13, 18].

The present study is aimed to detect the damage in DNA of muscle cells of infected WSSV shrimps which in turn open the door to further studies to investigate the variation in biochemical components of infected shrimps due to DNA damage and in turn clarify their effects on human health. This study is considered the first study which investigate the effect of WSSV on DNA of edible muscles by using comet assay. Different methods have been developed for detecting DNA strand damage micronucleus and sister-chromatid exchange assays which are based on the enumeration of downstream aberrations after DNA damage. A more recent method is the Comet assay which detects the DNA strand breaks and alkali labile sites by measuring the migration of DNA from immobilized nuclear DNA [19]. A large number of studies report that Comet assay is more sensitive when compared to sister chromatid exchanges or micronucleus test [20, 21]. The main advantages of the Comet assay include the collection of data at the level of the individual cell, the need for a small number of cells per sample and the sensitivity for detecting DNA damage [20]. Furthermore, Comet assay has become one of the most popular tools for detecting DNA stand breaks in aquatic animals [21] and has been employed to assess the DNA damage in aquatic invertebrates such as oysters, mussels, clams and shrimp, cells from haemolymph, embryos, gills, muscles and digestive glands were used for Comet assay [20].

# 2. Material and Methods

### *2.1. Collection of Samples*

The study was carried out on the aquaculture tiger shrimps *Penaeus monodon* that were collected from one of the infected white spot virus aquacurium in Saudi Arabia. All the collected samples were females. Samples were washed with deionized water to remove any adhering contamination and drained using filter paper. Fifty infected shrimps were examined for clinical signs including external lesions and morphological changes compared with healthy samples.

All samples were put in crushed ice in insulated containers and brought to the laboratory for preservation prior to analysis. The samples were separated into the exoskeleton and the endoskeleton (i.e. edible muscles). Muscle samples collected in plastic tubes and weighed, and were placed in Eppendorf tubes. Suspension of cells was prepared by crushing the abdominal muscles by a homogenizer.

### *2.2. Biochemical Analysis*

The Comet Assay is a single cell gel electrophoresis assay for evaluating DNA damage in cells. The premise is that damaged DNA becomes fragmented. Increasing amounts of DNA damage results in increased number of fragments and smaller fragments based on gel electrophoresis. This technique [19] was carried out for 5 infected samples and 5 normal healthy samples according the following steps:

1 gram of crushed muscle samples were transferred to 1ml ice cold PBS. This suspension was stirred for 5 min and filtered. Cell suspension (100 µl) was mixed with 600 µl of low –melting agarose (0.80% in PBS). 100 µl of this mixture was spread on pre-coated slides were immersed in lysed buffer (0.045 M TBE, pH 8.4, containing 2.5% SDS) for 15 min. The slides were placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS. The electrophoresis conditions were 2V/ cm for 2 min and 100 m. A staining with ethidium bromide 20µg/ml at 4°C. The observations was with the samples still humid, the DNA fragment migration patterns of 100 cells for each dose level were evaluated with a fluorescent microscope (with excitation filter 420-490 nm (issue 510nm). The comets tail lengths were measured from the middle of the nucleus to the end of the tail with 40x increase for the count and measure the size of the comet. For visualization of DNA damage, observations are made of EtBr- stained DNA using a 40 X objective on fluorescent microscope. Although any image analysis system may be suitable for the quantitation of SCGE data, Comet 5 image analysis software was used that developed by Kinetic Image, Ltd (Liverpool. UK) linked to a CCD camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates the tail length which is the distance from the comet head to the last visible signal in the tail. Furthermore, the percentage of DNA in the tail is calculated from the fraction of DNA in the tail divided by the amount of DNA in the nucleus multiplied by 100, while tail moment which is the product of the amount of DNA in the tail and mean distance of migration in the tail, also the program calculates

tail DNA percent that is represented by the integrated tail intensity x 100 divided by the total integrated cell intensity for a normalized measure of the percent of total cell DNA found in the tail [19]. Generally, 50 to 100 randomly selected cells are analyzed per sample.

### *2.3. Statistical Analysis*

The results were statistically analyzed using the Statistical Package for the Social Science (SPSS, for Windows, Version 15.0). The obtained data were used for descriptive statistical analysis consisting of means± standard deviation of five separated determinations. In order to test the significance of the differences among the mean values of the normal and infected white spot virus samples T- sample test was applied. The significance level was set at $P < 0.05$.

## 3. Results

### *3.1. Clinical Signs and Pathology*

Figure 1 shows the principle clinical sign of WSSV infection in shrimps which included the presence of obvious white spots or patches embedded in the exoskeleton of infected shrimps (Fig.1b & c). Other signs of disease include white to reddish-brown / reddish / pinkish / to discolouration of body and appendages. Furthermore, the infected shrimps had loose cuticle. It was observed also that the diseased samples were smaller in size than normal healthy shrimps.

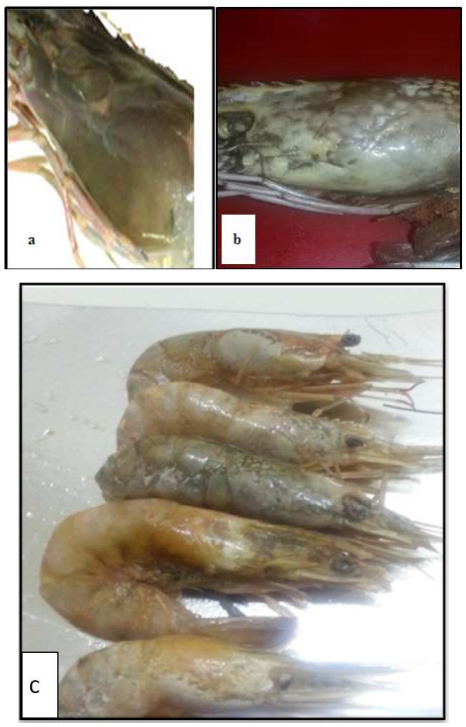


***Fig. 1.*** *Presence of white spots in the carapace and body of farmed. P. monodon infected with white spot syndrome virus, a: healthy shrimp, b) infected showing white patches, c) infected showing variation in degree of white patches*

### *3.2. DNA damage in Muscles of Infected WSSV Shrimps*

The Comet Assay is a rapid, quantitative technique in which visual evidence of DNA damage in eukaryotic cells can be measured [Plate 1]. It is based on the quantification of denaturized DNA fragments that migrate out of the cell nucleus during electrophoresis. Infection with WSSV causes DNA damage in infected samples, as shown in Plate 1, compared to healthy normal shrimps (1-5), damaged cells in infected shrimps (6-10) looked like tiny comets under a microscope, when DNA is broken in infected cells, it forms a tail that moves away from the unbroken DNA (the head). The amount of DNA damage in cells was estimated from comet tail length as the extent of migration of the genetic material. Moreover, the level of DNA damage was assessed using an image analysis package and expressed as % tail DNA. As shown in Figures 2, 3 and 4, the present results recorded that in normal healthy shrimps, the intensity percentage of DNA head portion was ranged from 86 to 90.%. While in infected WSSV, the intensity percentage of DNA head portion was from 80 to 85% (Fig. 2) where a significant decrease was detected ($p < 0.01$). On the other hand, intensity percentage of DNA tail portion in normal shrimps had lower mean value (3.02% ± 0.14) than in infected specimens (4.29% ± 0.52). Statistically, this variation is significant ($p < 0.001$). Furthermore, the recorded data in Fig. 3 showed, significant decrease ( $p < 0.000$) in tail length of healthy shrimps (mean value 2.79 µm ± 0.55) compared with diseased samples (4.21µm ± 0.22) (Fig. 3). Similarly, as illustrated in Fig.4, tail moments of normal shrimps had less mean value ( 10.14µm± 1.34) than that of infected samples (17.95 µm± 1.88). Statistically , this variation was also significant ( $p < 0.00$).

## 4. Discussion

Aquaculture is one of the fastest growing food production sectors in the world [22]. According to FAO statistics, over 80% of fish produced by aquaculture comes from Asia, with the production valued at $38.855 billion. However, disease outbreaks have caused serious economic losses in several countries. According to a World Bank Report, global losses due to prawn disease are around $3,000 million [23]. One of the most damaging viral diseases affecting the shrimp aquaculture industry is white spot disease that caused by white spot virus syndrome (WSSV). WSSV is a rod shaped, double-stranded DNA non-occluded virus that belongs to a new family of viruses (Nimaviridae) infecting crustaceans [24]. White spot disease is considered perhaps the most economically important disease of farmed warm water shrimp because it causes high morbidity and mortality rates in penaeid shrimp and other crustaceans [25].

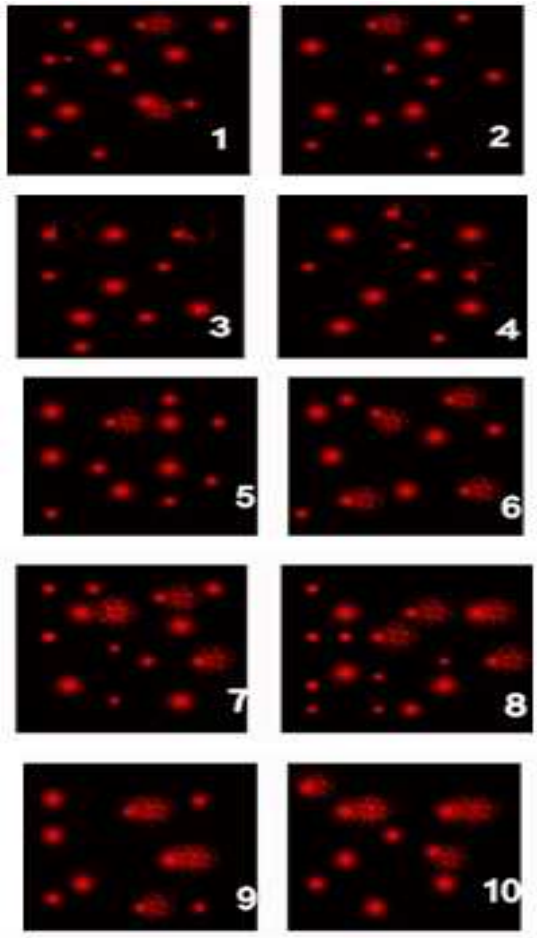


***Plate 1.*** *Photomicrographs representative DNA (comet assay): muscle DNA (1-5 normal healthy shrimps), (6-10 infected WSSVS shrimps showing DNA damage). When DNA is broken, it forms a "tail that moves away from the unbroken DNA (the "head").*

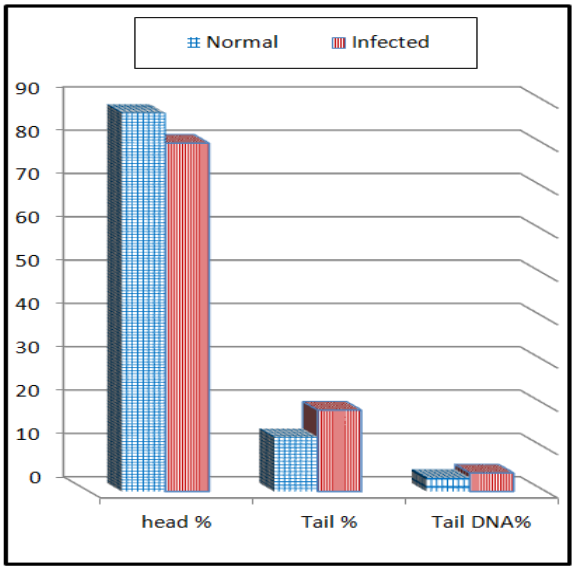


***Fig. 2.*** *Intensity percentages of head, tail and tail DNA in normal and WSSV infected shrimps.*

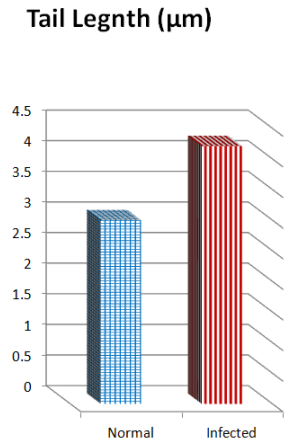


***Fig. 3.*** *Mean value of tail length (µm ) in normal and WSSV infected shrimps.*

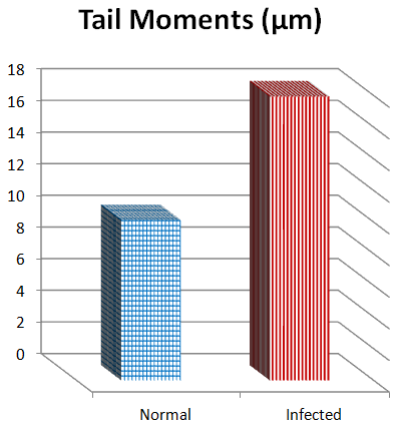


***Fig. 4.*** *Mean value of tail moments (µm) (product of distance and normalized intensity integrated over the tail length) of normal and infected shrimps.*

Furthermore, the presence of WSSV in the Mediterranean may hamper the development of shrimp aquaculture, especially in North African countries. The introduction of WSSV-infected organisms to the areas where the pathogen was previously unknown may be possible through ballast water from cargo ships [26], or even frozen commodities [27]. So, the present study is an attempt to investigate the effect of virus on DNA of muscle cells and to open the door to other studies to investigate the assessment of the potential impact of eating infected shrimps on public health of man.

In the present study, WSSV infected shrimps displayed obvious white spots or patches embedded in their exoskeleton. Other signs of disease include a reddish discolouration of body and appendages. Previously, these clinical signs were observed by other studies in crustaceans species infected with WSSV [28 - 30]. The exact mechanism of white spot formation is not known. It is possible that a WSSV infection may induce the dysfunction of the integument resulting in the accumulation of calcium salts within the cuticle and giving rise to white spots [31]. As regarding, reddish discolouration of body and

appendages of infected shrimps, [32-33] attributed this discolouration due to the expansion of chromatophores. Furthermore, the infected shrimps in the present results had loose cuticle and smaller size These characters were also recorded by [34]. Smaller shrimp at harvest could have been a reflection of decreased growth rate but the possibility that during a white spot disease outbreak bigger shrimp die first cannot be excluded. So this fact cannot be explained entirely by the occurrence of early harvest because of the lack of association between the length of the production cycle and the outcome variable [35]. Other clinical symptoms included swelling of branchiostegites because of accumulation of fluid [37] enlargement and yellowish discolouration of the hepatopancreas [37]; thinning and delayed clotting of haemolymph were recorded [38- 39]. In addition to, reduction in feed uptake [40- 41]; preening and response to stimulus [42- 43]. White spot syndrome virus targets are cells of organs of ectodermal and mesodermal origin, including those of the epidermis, gills, foregut, hindgut [44], antennal gland, lymphoid organ [45], muscle, eye-stalk, heart [46], gonads [34]. haematopoietic cells and cells associated with the nervous system [47]. In the late stages of infection the epithelia of the stomach, gills and integument may become severely damaged. This may cause multiple organ dysfunctions and probably leads to death [45].

To investigate the DNA damage induced by WSSV in the present study, it was used the comet assay which is also known as the single cell gel electrophoresis (SCGE) assay. It is a rapid, simple, visual, uncomplicated and sensitive technique for detecting and analyzing DNA strand breakage in a variety of organs and various cells . It allows any viable eukaryote cells to be analyzed [20]. For these reasons, the comet assay is now widely used in researches of bio-monitoring and DNA damage processes to routine assessments of genotoxicity. The resulting images were subsequently named “Comet” because of their appearance and their total length was considered directly related to the DNA damage. From that tail moment a range of applications of the comet assay have been used in investigations of the physiochemical behavior of DNA, through studies of cellular responses of DNA damage, to bio- monitoring of human population [48]. According to comet assay which measure DNA damage in muscle cells, the present data declare that WSSV infection induced a damage in DNA ; under a microscope the damaged cells appeared as tiny comets with a tails, these tails resulted from DNA broken. Furthermore, the amount of DNA damage in cells was estimated from comet tail length as the extent of migration of the genetic material. The present results show that the intensity percentage of DNA head portion of infected muscle cells had less mean value than of normal healthy shrimps where significant decrease was recorded. On the other hand, significant increases in intensity percentage of DNA tail portion, tail length and tail moments of infected shrimps were observed in comparison with normal healthy shrimps. Following electrophoresis, the presence of DNA strand breaks allows fragments of DNA to move from the nucleoid core towards the anode, thus resulting in (Comet) formation [19]. The amount of DNA breakage in a cell in the comet assay was estimated from the migration extent (tail length) of the genetic material in the direction of anode[19]. Furthermore, the percentage of DNA in the tail (tail intensity) has been shown to be proportional to the frequency of DNA strand breaks [48]. Tail moment is a simple descriptor calculated by the computerized image analysis system considering both the migration tail length as well as the fraction of DNA migrated in the tail [49].

DNA is a molecule that contains all the necessary information for the survival and perpetuation of an organism. DNA is indirectly responsible for protein production. Therefore, DNA alterations can lead to damage in proteins [and the resulting enzymes]. These alterations can be quantified through biochemical biomarkers [50]. The damage of DNA in infected WSSV muscle cells resulted in alternation in transcription and translation processes and these changes in turn may be explained the variations of biochemical parameters such as total carbohydrates, glucose, total protein, amino acids, and fatty acids provoked by white spot syndrome virus in muscles of infected *Penaeus indicus* as well as changes in hematological changes which were measured in study by [51].

# 5. Conclusion

One of the most damaging viral diseases affecting the shrimp aquaculture industry is white spot disease caused by white spot virus (WSSV), which causes high morbidity and mortality rates in penaeid shrimp and other crustaceans. WSSV has caused serious epizootics in cultured shrimp in several parts of the world. This virus is known to effect most commercially important species penaeid shrimp including *Penaeus monodon*. In conclusion, the present work revealed that *Penaeus monodon* is highly susceptible to infection with WSSV. Symptoms of WSSV include smaller size of infected shrimps compared to healthy ones; also the skeletons of infected shrimps had obvious white spots or patches embedded and loose cuticle. Additionally, white to reddish-brown / reddish / pinkish / to discolouration of body and appendages were observed. Furthermore, the present study declares that the comet assay had adequate sensitivity to detect the differences in the levels of DNA damage among infected shrimp. The present data show that WSSV induced DNA damage in muscle cells. So, therefore, further studies on the mode of action and characterization of the active components in WSSV infected shrimps should be carried out to evaluate the variations in such components which resulted from DNA damage that in turn illustrate and understand more about the effects their consumption. Furthermore, the consumer health risks associated with eating imported farmed shrimp will be clarified.

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