Vesicular diseases associated with poxvirus-like infection in cultured soft shell turtles (*Pelodiscus sinensis*) in Taiwan

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Abstract

An outbreak of a poxvirus-like infection in a population of 20,500 50-day-old soft shell turtles (*Pelodiscus sinensis*) at a culture farm in Pingtung County, southern Taiwan, occurred from late November to early December 2008. A mortality rate of approximately 44% was recorded and the clinical findings included anorexia, lethargy, swollen necks and several unusual vesicles and bullas were present on the legs and shell skin. When the surviving turtles reached the age of 11 months (October 2009), another outbreak of this disease occurred in the same pond, resulting in a mortality rate of approximately 20%. In both outbreaks, histopathological examination revealed typical acute lesions, such as ballooning degeneration and acute cell swelling of keratinocytes of the epidermis. Furthermore, tiny eosinophilic cytoplasmic inclusion bodies were observed in the vacuoles. Sloughed necrotic epidermis, inflammatory cells, and a predominance of heterophils were found to have infiltrated the area of the exposed underlying dermis. In addition, patches of skin ulceration and deep layer inflammation were present, and secondary bacterial infection was suspected.

Six soft shell turtles were challenged with a 0.45 μm filtered virus suspension and showed clinical signs of anorexia and lethargy. The observed cutaneous lesions, 14 days post-challenge were similar to those reported on the farm and included medium to large size vesicles, white and circular thin-layered pustules and sloughed necrotic epidermis over the legs, head and shell skin. In addition, numerous white pock lesions on the CAM of embryonated soft shell turtle eggs which were also inoculated with the virus suspension were observed 3 days post-challenge. A transmission electron microscopy study; conducted from both farmed and experimentally infected turtles revealed enveloped, ovoid-shaped viral particles, measuring approximately 239 nm in diameter and 483 nm in length. According to its morphology and size, the virus was very similar to a poxvirus. Therefore, we provisionally named this agent, “soft shell turtle poxvirus-like virus”.

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Introduction

The poxviridae are sub-divided into insect pox viruses (Entomopoxvirinae) and vertebrate pox viruses (Chordopoxvirinae), the latter consisting of eight genera: Avipoxvirus, Capripoxvirus, Leporipoxvirus, Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, Suipoxvirus, and Yatapoxvirus. However, California harbor seal poxvirus, Nile crocodile poxvirus, Mule deer poxvirus, Dolphin poxvirus and Cotia virus are unassigned viruses in the family (Smith et al., 2008). Poxvirus infection in reptiles was first reported in three juvenile captive spectacled caimans (Caiman sclerops) with skin lesions (Jacobson et al., 1979). Since then, studies have described diseased crocodilians with different clinical lesions including non-fatal dermatitis with 1 to 3 mm diameter wart-like or scattered, gray-white, and circular skin lesions (Buenviaje et al., 1992; Horner et al., 1988). Lesions commonly develop in the stratified squamous epithelium of the skin with parakeratotic hyperkeratosis, acanthosis, edematous infiltration and moderate mononuclear cellular infiltration (Huchzermeyer et al., 1991; Penrith et al., 1991). It is also known to be a fatal disease in crocodilians with an associated high mortality rate (Vetési et al., 1981).

Poxvirus infection has been reported in the tegu lizard (Tupinambis teguixin) (Stauber and Gogolewski, 1990), circulating monocytes of a flap-necked chameleon (Chamaeleo dilepis) (Jacobson and Telford, 1990), the emerald spiny lizard (Emerald Swif; Sceloporus malachiticus) (Gál et al., 2003), and the herbivorous green iguana (Iguana iguana) (Gál et al., 2005). A xanthic California desert tortoise (Gopherus agassizi) was suspected of having a poxvirus infection due to the presence of intracytoplasmic inclusion bodies observed in epidermal cells (Frye, 1991).

Interestingly, the non-viral intracytoplasmic inclusions have also been found in the epithelial cells in the desert tortoise (Jacobson and Samuelson, 2007). A poxvirus-like infection was also reported in a captive Hermann’s tortoise (Testudo hermanni) with papular lesions on the lower eyelid and on the left side of the rostrum (Orós et al., 1998). To date, there have been no reports of this virus in cultured or wild soft shell turtles (Pelodiscus sinensis). The findings detailed in this study may allow researchers to gain a better understanding of the infectious diseases present in cultured soft shell turtle farms.

Materials and methods

Animals

Approximately 31,000 50-day-old soft shell turtles were raised in two ponds in Pingtung County, southern Taiwan. The juvenile soft shell turtles originated from the same hatch farm. Pond 1 (600 m², 2.5 m depth; divided into 2 areas) contained 10,500 soft shell turtles and Pond 2 (1,160 m², 2.5 m depth; divided into 3 areas) was inhabited by 20,500 soft shell turtles. The average weight/size of the population was 3 g/3 cm × 2.5 cm. The turtles cultured in Pond 2 demonstrated clinical signs of infection from late November through early December 2008. When the surviving turtles were approximately 11 months old, a recurrence of the disease was reported in Pond 2 from late October through early December 2009. The moribund diseased soft shell turtles from both the 2008 and 2009 outbreaks were put into a cold preservation bag and were sent to the Central Taiwan Aquatic Animals Disease Diagnostic Center at National Chung-Hsing University for advanced diagnosis.
**Histopathology examination**

Once the moribund soft shell turtles from the 2008 (n = 10) and 2009 (n = 14) outbreaks arrived at the diagnostic laboratory, they were humanely euthanized with Urethane® (Sigma, USA). They were sampled immediately on arrival; skin, brain, liver, intestinal tissue, kidney, spleen, trachea, and lung samples were aseptically collected and submitted for histopathological examination. Tissues were dehydrated by serial ethanol and xylene stages for automated processing to wax, and 5μm sections were examined. All of the slides were stained with hematoxylin and eosin (HE) (BBC Biochemical, USA) and observed microscopically.

**Transmission electron microscopy (TEM)**

After the histopathological examination, 1mm³ samples of skin tissue from the relevant areas of the histological sections where the inclusion bodies were observed were extracted from wax blocks. Xylene (Union Chemical Works Ltd., Taiwan) was used to dissolve the wax for 8 h, then the sample was stored in 70 % ethanol and embedded in LR white resin (ProSciTech, Australia). Ultrathin sections measuring 80 nm were mounted on 200 mesh grids, stained with 2 % uranyl acetate (BDH Chemicals Ltd., Poole, England) for 15 min, lead citrate (Sigma, USA) for 5 min and examined with a transmission electron microscope (Jeol, JEM-1400, Japan). The primary magnifications ranged from 2,500× to 300,000×.

**Experimental infections**

The experimental infections were conducted and analyzed in 2009. The skin of diseased soft shell turtles (n = 14) was collected and ground using a mortar. A solution of 0.01 M sterilized phosphate buffered saline (PBS; pH 7.2, Gibco, USA) and 5 % antibiotics (including 10,000 unit/ml penicillin and 10,000 μg/ml streptomycin; Hyclone, USA) was added to the tissue homogenate. The homogenate was then filtered by 0.45 μm filter (Millex, USA) and stored at -20 °C.

The animals were screened for one week prior to the infectious challenge. Six, one- to three-month-old juvenile soft shell turtles (average weight/size 5.1 g / 4.5 cm × 3 cm) each received a subcutaneous injection containing 1 ml virus suspension and were kept in containers at a constant water temperature of 28 °C. When the infected turtles died, they were collected for necropsy and histopathological examination. Six 15-day-old embryonated soft shell turtle eggs were also injected with 1 ml viral suspension into the air cavity. When plaques had formed, the chorioallantoic membranes (CAM) were examined with an inverted microscope (Leica, DMI 3B, Germany) at 100 × to 400 × magnifications. Six tilapias, approximately 8-cm in length, and six 10-day-old chickens, also received a 1 ml subcutaneous injection of the virus suspension. Six 10-day-old SPF embryonated chicken eggs were injected with a 1 ml viral suspension by the allantoic cavity route. The same number of animals or embryonated eggs were injected with 0.01 M PBS and kept under the same culture conditions to serve as negative controls.

**Results**

All of the moribund soft shell turtles (n=24) that were examined from the infected areas (n=3) of pond 2 on the farm showed anorexia and were lethargic. In 2008, the most remarkable gross lesions were vesicular changes scattered on the skin, such as swollen neck and papillomatous, grayish white pustules on the skin of 50-day-
old soft shell turtles (Figure 1A and B). In 2009, cutaneous ulceration, stomatitis and glossitis were also noted in some 11-month-old soft shell turtles (Figure 1C) and the infected turtles’ growth became gradually stunted. Furthermore, erythema, bleeding, and several vesicles on the abdominal shell skin, legs, neck and tail were noted (Figure 1D). Histopathological examination of tissue sections of the farmed 11-month-old soft shell turtles collected in 2009 revealed ballooning degeneration, acute cell swelling of epithelia, necrotic sloughed epidermis, inflammatory cells, predominance of heterophils infiltrating or covering the ulcerated dermis as well as patches of skin ulceration and deep layer inflammation (Figure 2A). In addition, some parts of the ballooned cells were full of small eosinophilic cytoplasmic inclusion bodies present in the vacuoles (Figure 2B).

Transmission electron microscopy (TEM) of skin tissue from both farmed and experimentally infected soft shell turtles containing inclusion bodies revealed numerous large, enveloped, ovoid shaped poxvirus-like particles (Figure 3A). Furthermore, the surface of the intracellular

Figure 1. In 2008, moribund, farmed soft shell turtles presenting lesions including swollen neck, papillomatous, grayish white pustules on the skin (white arrows) of 50-day-old soft shell turtles (A and B). In 2009, skin ulceration, stomatitis, and glossitis (white arrow) (C) and erythema, bleeding, and several vesicles on the abdominal shell skin, legs, neck and tail (white arrows) (D) were found in some moribund 11 months old soft shell turtles.
mature virus particle had an irregular appearance comprised of surface tubules (Figure 3 B). Based on the TEM images, the average size of the poxvirus-like virions (n = 30) was calculated to be 200 to 295 nm (mean = 239 nm, S.E.= 2.8) in diameter and 455 to 500 nm (mean = 483, S.E.= 3.9) in length.

All 6 soft shell turtles that were challenged with the filter prepared virus suspension showed clinical signs of anorexia and lethargy, and died 11 to 14 days post-challenge. The cutaneous lesions were similar to those observed on the farm and included medium to large size vesicles (even bulla formation), white and circular thin-layered pustules and sloughed necrotic epidermis over the legs, head and shell skin. No clinical signs were observed in the negative control turtles. Clinical lesions were not detected in the experimentally infected tilapia, nor in chickens 45 days post-inoculation, and plaque formation was not observed in the embryonated chicken eggs 7 days post-inoculation. However, numerous white pock lesions on the CAM of the embryonated soft shell turtle eggs were observed 3 days post-challenge. No clinical signs were observed in the negative control tilapia and chickens. Plaque formation was not
observed in the negative control embryonated chicken and soft shell turtle eggs.

**Discussion**

This is the first study to describe a vesicular disease associated with a poxvirus-like infection in soft shell turtles bred on a farm. Poxvirus infections have previously been identified in crocodilians (Buenviaje et al., 1992), lizards (Gál et al., 2005) and a captive Hermann’s tortoise (Orós et al., 1998). Most cases were associated with proliferative skin lesions ranging from mild, non-fatal papular lesions to nodular dermatitis. The morbidity caused by poxvirus in *Crocodylus niloticus* was almost 100%, while the mortality depends on factors influencing the resistance of the host (Gál et al., 2005). The only reported cases of poxvirus-like infections causing high mortality rates have been reported in the caiman (Vetési et al., 1981) and herbivorous green iguana (Gál et al., 2005).

Poxviruses are usually associated with eosinophilic intracytoplasmic inclusion bodies (Buenviaje et al., 1998; Horner, 1988). However, herpesvirus infection in tortoises is often characterized by the development of respiratory symptoms (Hervás et al., 2002). Ranavirus is a genera of the family Iridoviridae and is increasingly being recognized as an important pathogen in chelonians. Iridoviridae infection was observed in soft shell turtles with red neck and cervical edema (Chen et al., 1999). In this study, the main characteristic of the poxvirus-like infection was that only the epidermis was affected.

Poxviruses are large, slightly pleomorphic, enveloped, with double-stranded linear DNA and are the most complex kinds of viruses. The virions vary in their shape depending upon the species but are generally shaped like an ovoid or have a brick-shaped form (Gál et al., 2005). The surface of the intracellular mature virus particle contains surface tubules that have either a basket-weave symmetry or irregular appearance (Smith et al., 2008). In this study, the enveloped poxvirus-like virions were large and ovoid-shaped. The virion size was approximately 239 nm in diameter and 483 nm in length. The surface of the intracellular mature virus particle had an irregular appearance comprised of surface tubules. However, it has been reported that non-viral intracytoplasmic inclusion bodies when present in low concentrations may not be distinguishable from mature viral particles (Jacobson and Samuelson, 2007). Interestingly, the TEM results revealed that the intracytoplasmic inclusion bodies were large, enveloped, and ovoid shaped viral particles. The size and shape of those viral particles were quite different from those of herpesvirus and ranavirus. The viral suspension was also studied and observed by the negative stain technique. The vesicular liquid was cultured for bacterial isolation. Moreover, no pathogenic bacterium were isolated. Herpesvirus or ranavirus infection in tortoises is often characterized by the development of respiratory symptoms. However, the main characteristic of a poxvirus-like infection is that it only affects the epidermis. To rule out the presence of a herpesvirus, samples were screened using a PCR method and primer set detail by Origgi et al. (2004). The results obtained from the PCR analysis suggest that the virus was not a herpesvirus (data were not shown). According to the results of the clinical signs, gross lesions, histological examination, and virion ultrastructural analysis, we established that the disease affecting a
population of soft shell turtles at a breeding farm was a poxvirus-like infection. Therefore, we provisionally named this virus, “soft shell turtle poxvirus-like virus”.

Our experimental infection results revealed that all of the viral-inoculated soft shell turtles showed severe clinical signs and died within two weeks. The cutaneous lesions were similar to those of the field cases. Numerous tiny white pocks were observed on the CAM of soft shell turtle embryos but no plaque was observed in embryonated chicken eggs after virus inoculation. No clinical lesions were observed in experimentally infected tilapia or chickens. These findings demonstrate that “so/g286 shell turtle poxvirus-like virus” is a lethal pathogen to soft shell turtles, but it might not be pathogenic to either tilapia or chickens.

At present, there are no serological or PCR diagnostic assays available to identify reptile poxviruses. Further studies, such as replication of the virus in soft shell turtle embryo primary cell culture, negative stain, development of specific primers to assist viral sequencing, and development of a PCR diagnostic technique are required in order to better understand the structure and mechanism of this virus.

Acknowledgements
The study was supported by the Council of Agriculture, Executive Yuan, Taiwan (98AS-9.2.4-BQ-B1(21)). The authors would like to thank Miss Pei-Chi Chao for her technical assistance with the transmission electron microscope.

References


