Foreword

The major focus of the intensive farming of crocodiles is the production of high quality skins to supply the expanding demand for high quality leather. However, any blemishes on the skin that may have resulted from diseases of the skin will substantially downgrade the economic value of the skin.

The very fact that farmed crocodiles live in an aquatic environment and at high density will favour the spread of microrganisms capable of inducing diseases of the skin. Because crocodile farming is a relatively new animal industry, there is a paucity of scientific knowledge on the causes, treatment and control of skin diseases.

This publication describes the most important of the skin diseases of farmed saltwater crocodiles in northern Australia, identifies the features of the causative agents and provides information on simple and cheap methods for the treatment and control of skin diseases.

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Abbreviations

AITVAS - Australian Institute of Tropical Veterinary and Animal Science
DMEM- Dulbecco’s Modified Eagle’s medium
DPIF- BVL – Department of Primary Industries and Fisheries – Berrimah Veterinary Laboratory
PCR – Polymerase chain reaction
PI – Post inoculation
PT – Post treatment
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Executive Summary

In crocodile farming, the major focus is the production of high quality skins to supply the expanding demand for high quality leather. In recent years, there has been an increasing demand for quality crocodile skin currently in short supply, presumably because of the occurrence of diseases affecting the skin. As a consequence, it is critical that any diseases of the skin should be controlled and damage should be kept to the minimum.

This study was conducted to determine the occurrence of skin diseases in farmed crocodiles in Australia. Six farms, three in Queensland and three in the Northern Territory were visited during the period May to August 1996. Samples of skin with lesions were obtained and data on morbidity and mortality, possible aetiological agents, and other likely predisposing factors were collected. The approximate number of crocodiles on these farms varied from 800 to 3,000. Furthermore, pathology case records of histopathological slides of crocodile skin lesions on file from 1989 to 1995 were included. All data gathered on recent (1996) cases, especially the gross and microscopic findings were combined for analysis with the data on retrospectively examined cases of skin diseases on file in the Australian Institute of Tropical Veterinary and Animal Science, James Cook University.

Two hundred and three lesions (119 were from retrospectively examined cases and 84 were recent) from 180, mostly young crocodiles were examined. The skin lesions were obtained from crocodiles on nine farms, from a group of experimental animals and from one adult found dead in the wild. Necropsies were performed either on the crocodile farms, at James Cook University, or at the Department of Primary Industries and Fisheries – Berrimah Veterinary Laboratory, Northern Territory. The histopathological and bacteriological examination of the 84 recent cases was carried out at the Australian Institute of Tropical Veterinary and Animal Science.

Five specific skin diseases in uncomplicated form including dermatophilosis, mycotic dermatitis, poxvirus infection, probable mycobacterial dermatitis and capillariasis were identified. Dermatophilosis was the most prevalent skin disease and was also frequently diagnosed, being present in 62 of 66 (94%) in mixed or dual infections. The lesions were discrete focal, 1 to 4 mm diameter 'brown spots' of variable prominence, predominantly on the ventral abdomen but also elsewhere in the body. The histopathological examination confirmed the presence of filamentous organisms mostly in debris that had accumulated on the ulcerated or eroded epidermis but also elsewhere in the subcutis, and in severe cases in the muscular layer.

Duplicate samples of skin confirmed histologically as dermatophilosis were homogenised for bacterial culture. Dermatophilus sp was positively identified initially based on the cultural characteristics. The organisms were filamentous and branching, Gram-positive, non-acid fast, catalase positive and oxidase negative. Other biochemical tests also supported the identity of the organism as closely resembling Dermatophilus congolensis.

Several transmission experiments were carried out. In a pilot study on transmission of dermatophilosis, two isolates of Dermatophilus sp (strains TVS 96-366-5A and TVS 96-490-7B), both from cases of dermatophilosis in farmed crocodiles were used. Within several days after inoculation, the hatchlings developed typical 'brown spot' lesions not only at inoculation sites but also other locations both in infected and in-contact control animals. Histopathological examination revealed changes characteristic of dermatophilosis. Dermatophilus sp confirmed as TVS 96-490-7B but not strain TVS 96-367-5A on the morphological, cultural and biochemical characterisation was isolated from 'brown spot' skin lesions.
In the second transmission experiment, the protocol was changed to evaluate possible control and treatment procedures. Although occasional minute skin lesions developed in crocodiles, the lesions quickly regressed so effective transmission was not achieved. A third transmission experiment was conducted using facilities and procedures as in the pilot study but with control and inoculated hatchlings maintained in separate tanks in separate buildings 60 metres apart. Both inoculated and in-contact controls in the principal group developed 'brown spot' lesions. Again only strain TVS 96-490-7B was isolated from both the principal and control groups. A few hatchlings in the control group however became infected with either 'brown spot' disease or poxvirus after 20 days post inoculation.

The *Dermatophilus* sp isolated from spontaneous outbreaks and recovered isolates from several transmission experiments was compared with *Dermatophilus congoensis* type strain (ATCC 14637) and *Dermatophilus chelonae* (DCH 2) by morphological, cultural and biochemical characterisation. In addition, more advanced comparison at the molecular level using 16S rDNA sequence and ribotyping were also used. The results showed that the isolate used for inoculation and the recovered isolates were identical, thus confirming its role in causing 'brown spot' disease. Overall, both the phenotypic and genotypic differences were sufficient to suggest that the crocodile isolate is a distinct species of *Dermatophilus*, thus a new species of *Dermatophilus* – *Dermatophilus crocodyli* sp nov. is proposed.

Following successful transmission of 'brown spot' disease and identification of the aetiological agent – *Dermatophilus crocodyli*, treatment and control studies were carried out. An initial *in vitro* study on the antibacterial properties of copper sulphate, salt and formalin showed that copper sulphate was effective against *Dermatophilus* sp. This was followed by a treatment trial in which some hatchlings with “brown spot” disease were placed in pen with flowing water and others placed in pen with static water. This experiment showed the flowing water had no effect on the control of 'brown spot' disease. In the second experiment, all infected animals were treated with either formalin or copper sulphate. Hatchlings treated with formalin developed severe lesions compared with those treated with copper sulphate. All infected hatchlings were treated with copper sulphate using three protocols. Of the three protocols, it was found that immersion of infected hatchlings for 15 minutes in medicated water containing 1 ppm copper sulphate was the most effective.

Studies on poxvirus were carried out to include the gross and histopathological characterisation of the disease, electron microscopy of poxvirus and transmission of poxvirus. The gross and microscopic appearance of the skin lesions infected with poxvirus was consistent in all hatchlings. Marked circumscribed grey-white lesions up to 3 mm diameter were present on the lower limbs, foot pads, tail and back, whereas the lesions on the neck, chest and abdomen were diffuse, irregular and translucent. Electron microscopy revealed the presence of 'dumb bell' shaped virus typical of poxvirus. Attempts to grow the virus in cell culture, chicken chorioallantois and crocodile embryos were unsuccessful. A transmission experiment was attempted but failed to produce lesions.

It was concluded that 'brown spot' disease is the most prevalent and probably the most important skin disease in farmed crocodiles in Australia. A newly proposed species – *Dermatophilus crocodyli* sp nov. was identified as the aetiological agent of 'brown spot' disease and copper sulphate at 1 ppm concentration was shown to be an effective treatment for 'brown spot' disease.
1. Introduction

The crocodile industry is well recognised as an animal industry and over a thousand crocodile farms have been established around the world. Although crocodile farming began in the late 1960's in Australia, the industry showed only limited development. The industry grew significantly in 1986 when exports of crocodile skin was permitted and the demand of crocodile leather had increased substantially. Two species of crocodiles, the indigenous freshwater crocodile (Crocodylus johnstoni) and saltwater crocodile (Crocodylus porosus) were originally farmed, however, the industry has concentrated more on saltwater crocodiles because of the higher economic value of their skin.

Presently, there are 16 commercial crocodile farms in Australia mostly located in Queensland and the Northern Territory. These farms produce their own stocks through captive breeding or the hatchlings are supplied by other farms or from the Parks and Wildlife Department. In the Northern Territory, the farmers are permitted to collect crocodiles or eggs from the wild and hatch the eggs on the farm. The extent of development of the industry and its economic importance is illustrated by the fact that from 1994 to 1997, the number of C porosus processed for skin and meat had increased from 3,000 to almost 8,000 in the Northern Territory (Simlesa, 1998).

Because crocodile farming has recently emerged as a new form of agriculture, the husbandry techniques used varies between farms. As there is no universal standard method to emulate a sound crocodile farm management, farmers have relied mostly on overseas concepts of animal husbandry, which were also a product of empirical methods. In 1986, the crocodile farms in Australia were constructed in such a way that the design of pens mimicked the natural habitat of crocodiles in the wild. In addition, the nutritional requirement of crocodiles is still poorly understood, and is currently being studied in an attempt to produce optimum growth (Davis, et al., 1999).

Intensive farming of crocodiles is not different from conventional farm animals, in that there are many important requirements needed to maximise production. In our previous studies (Buenviaje, et al., 1994), it was found that temperature, food and pen design were the main factors that influenced the occurrence of many diseases in farmed crocodiles. For example when the temperature of pens was maintained at 32°C and fish was excluded from diet, there was a lower incidence of bacterial, fungal and lungworm infections.

Although there are still more aspects of the husbandry of intensive crocodile farming which need to be examined scientifically, it is also equally important to define the aetiological agents of diseases and their control and treatment, especially diseases affecting the skin. At the present time, there is a paucity of information in the scientific literature on the prevention, control and treatment of diseases in crocodiles. Any outbreak of infection affecting the skin will cause heavy economic losses, considering that even a single blemish downgrades the market value of skin. It is particularly important because when one animal is sick, the infection can spread readily to other animals through the water. When optimal conditions and sound management techniques of crocodiles are applied, many diseases can be minimised if not eradicated.

The main objective of this research project was to investigate the diseases affecting the skin of farmed crocodiles in Australia. This includes a complete description of the diseases but particularly the identification by gross and microscopic pathological examinations of the most important of the skin diseases.

The specific objectives were:

• To identify the most important skin disease of farmed crocodiles in Australia.
• To identify and characterise the aetiological agent of the most important skin disease and to show by transmission studies its role in causing the disease.
• To develop strategies for the control and prevention of the most important skin disease in farmed crocodiles.

Based on these objectives, this report describes the research work conducted in six sections.

1. Pathology of skin diseases of crocodiles
2. Isolation of *Dermatophilus* sp from skin lesions in farmed saltwater crocodiles (*Crocodylus porosus*)
3. Transmission of dermatophilosis (“brown spot” disease) in saltwater crocodiles (*Crocodylus porosus*)
4. Phenotypic and genotypic characterization of *Dermatophilus* sp isolated from “brown spot” lesions in farmed crocodiles
5. Treatment and control of dermatophilosis (“brown spot” disease) in farmed crocodiles
6. Studies on poxvirus in crocodiles
2. Pathology of Skin Diseases of Crocodiles

2.1 Methodology
Skin samples were obtained from crocodiles on nine farms (four in Queensland and five in the Northern Territory), and from a group of experimental animals and one crocodile found dead in the wild. One affected crocodile in the Northern Territory had recently been introduced to a farm after capture in the wild. Six of the farms, three in Queensland and three in the Northern Territory were visited between May and August 1996. When representative samples of lesions were obtained, data on morbidity and mortality, possible aetiological agents and other likely predisposing factors, were also collected. All data gathered on recent (1996) cases, especially the gross and microscopic findings, were combined for analysis with data on retrospectively examined cases on file in the Australian Institute of Tropical Veterinary and Animal Science (AITVAS), James Cook University.

All microslides of crocodile skin lesions on file were re-examined and each lesion was classified according to the suspected or known aetiological agents. For each case the origin, type of skin sample (skin biopsy or from either dead or euthanased animal), gross and microscopic findings, results of microbiological culture, species, age, length (snout to tail tip) and sex was recorded. A total of 119 skin samples from 109 cases initially received and processed at the AITVAS during the period 1989 to 1995, was included.

In addition to the retrospective cases, 84 skin lesions from 71 recent (1996 to 1997) cases were examined. These lesions were mostly on emaciated crocodiles that died (28 cases), or were killed by barbiturate overdose for necropsy (34) to ascertain the cause of illness; nine were obtained by biopsy (the retrospective cases on file). More than one skin sample was collected from 23 crocodiles with multiple lesions and in some animals more than one diagnosis was made. Collectively (both retrospective and recent cases), 135 of the crocodiles were saltwater (Crocodylus porosus), 44 were freshwater (Crocodylus johnstoni) and one was of unrecorded species. Their mean length was 82.9 cm (range 25 to 110 cm, n=121) and ages were between two to 36 months (average 15 months, n=89). Eighty-seven were male, 32 were female and in 84 sex was not ascertained or recorded.

Necropsies were performed either on the crocodile farm, at James Cook University or at the Department of Primary Industry and Fisheries – Berrimah Veterinary Laboratories (DPIF-BVL), Northern Territory. From each crocodile, three samples of skin of at least 2 cm², and including representative lesions were fixed in 10% buffered neutral formalin and embedded in paraffin wax. For histological examination, sections were cut at 6 μm and stained with haematoxylin and eosin, or other stains such as Gram-Twort for bacteria, Gomori methenamine silver and periodic acid-Schiff for fungi, and Ziehl-Neelsen for mycobacteria.

Duplicate specimens of some suspected 'brown spot' lesions were excised and placed in sterile 5 mL plastic tubes for bacteriological examination, which was commenced at the BVL or AITVAS, then continued at the latter, where microbiological procedures used were aimed primarily at isolating the filamentous organism, which was presumed to be the aetiological agent of 'brown-spot' disease (Buenviaje, et al., 1997). No attempt was made to culture other bacteria.

2.2 Results
Table 1 compares the relative frequency of crocodile skin diseases within our collected cases, based on their aetiology and source for the period 1989 to 1997. Five specific diseases in uncomplicated form, namely dermatophilosis, mycotic dermatitis, poxvirus infection, probable mycobacterial dermatitis and capillariasis, were identified. Two other skin disease categories were mixed infections, and those of undetermined other cause, but with bacteria present superficially. Of the four major skin diseases, dermatophilosis was the most prevalent (57 cases, 28.1%), followed by mycotic dermatitis (30, 14.8%), pox (7, 3.4%), and probable mycobacterial dermatitis (5, 2.5%). Lesions with dual or multiple pathogens present, such as concurrent infections with poxvirus, fungi and dermatophilosis or other bacteria were common, being present in 66 cases. The 38 lesions classified as 'other dermatitis'...
included a single case of capillariosis (presumably *Capillaria crocodilus* infection); in the other 37 lesions a mixture of both Gram-positive and Gram-negative, but mostly Gram-positive, bacteria was present.

**Dermatophilosis**
Dermatophilosis was present in crocodiles on six farms. Most affected animals were emaciated and weak, with stunted growth. There were discrete, focal, 1 to 4 mm diameter, 'brown spots' of variable prominence on the skin, predominantly on the ventral abdomen but also elsewhere on the abdomen, tail or head. The lesions were situated mostly at the centre of scales (24, 42.1%) or along the 'hinge joint' between scale. Lesions on the skin of the lower jaws (19 cases, 33.3%) however, usually presented as linear erosions up to 5 cm in length. In severe cases lesions were large, with irregular ulceration up to 2 cm² diameter on the abdomen. A *Dermatophilus* sp that resembled *Dermatophilus congolensis* both biochemically and morphologically was isolated from five such skin lesions in crocodiles from two farms in Queensland and the Northern Territory ([Buenviaje et al.](#), 1997). Except in some cases of mixed infection, the *Dermatophilus* sp filaments were easily seen in sections stained with haematoxylin and eosin or particularly with periodic acid-Schiff stain (PAS).

**Table 1**: Frequency of occurrence of skin lesions in 203 lesions from 180 crocodiles, based on histological examination.

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<th>Poxvirus</th>
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<th>Other causes</th>
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<td>30 (14.8%)</td>
<td>43 (21.2%)</td>
<td>66 (32.5%)</td>
<td>203</td>
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</table>
3. Isolation of *Dermatophilus* Sp from Skin Lesions in Farmed Saltwater Crocodiles (*Crocodylus Porosus*)

3.1 Methodology

Thirty-three farmed saltwater crocodiles (*Crocodylus porosus*) with various skin lesions were examined clinically. Twenty were males, five were females and eight were unsexed, and their lengths ranged from 64 to 147 cm. Twenty-three crocodiles were killed by barbiturate overdose and these, and a further 10 that died naturally, were necropsied. At least three skin samples, each about 2 cm² and representative of different types of lesions, were collected from each affected crocodile, fixed in 10% buffered neutral formalin and processed for histopathological examination in the routine manner. Duplicate specimens excised for bacteriological examination were placed immediately in 5 mL sterile plastic tubes and stored at −20 °C. Blood agar was prepared with polymyxin B (1,000 unit/mL of medium). At least 24 hours after collection the skin samples were ground with a mortar and pestle and 1 mL of sterile pH 7.0 PBS was added. At least three drops of each suspension were then spread on five blood agar plates with polymyxin B, and incubated at 37 °C in an atmosphere of 5% CO₂. Cultural, morphological and the biochemical examinations were performed on the isolates.

3.2 Results

After culture for 48 hours, a few white to grey colonies, up to 3 mm in diameter and with pitting into the medium, were observed in three isolates (TVS 96-367-15A, TVS 96-490-7B and TVS 96-490-9B). After one week the colour of colonies changed to orange. The organisms were filamentous, Gram-positive, non-acid-fast, catalase positive and oxidase negative. Comparative bacteriological studies were performed using *D congolensis* type cultures obtained from the University of Queensland and one clinical isolate from the Queensland Department of Primary Industries, Oonoonba Veterinary Laboratory.

Culturally, all the isolates were identical and were indistinguishable from the type culture. All *Dermatophilus* sp isolates and the type culture, when grown at 37°C in an atmosphere of 5% CO₂, produced grey to yellow colonies up to 3 mm in diameter and with pitting into the medium. A zone of beta haemolysis developed between one and five days and the colonies were raised and rugose to crateriform. Two of the four isolates produced occasional, white aerial hyphae similar to the type culture. Furthermore, all isolates cultured on 10% ovine serum in tryptose phosphate broth at 37°C produced after 72 hours a clear supernatant with granular clumps at the bottom of the tubes. Cellular morphology of all organisms at 48 hours was similar, namely a Gram-positive filamentous organism with hyphae branched at right angles. The width of hyphae ranged from 0.5 to 1.5µm while the zoospores were 1µm in diameter.
4. Transmission of Dermatophilosis in Saltwater Crocodiles (*Crocodylus porosus*)

4.1 Methodology

Three separate experiments were conducted. In an initial pilot study, 16 'runt' crocodiles approximately one year of age individually identified by metal tags inserted into the webbing of the feet were divided into experimental and control groups containing 12 and four animals, respectively. Animals in each group were placed together in a single oval tank made of recycled plastic (Reln Pty Ltd, Ingleburn, New South Wales), with a floor area of 2.35 × 1.1 m. The floor of the tank was slightly inclined so that approximately two thirds of the area was covered with water up to 10 cm deep while one third was dry. An epoxy-painted marine ply lid with a central hinge joint completely covered the tank except during cleaning, feeding and examining the crocodiles. An automatic immersion heater (Rena Corporation, Charlotte, NC, USA) was used to maintain the water temperature at 31° to 32 °C. All materials used were disinfected with a 1% chlorine solution and allowed to air dry for at least 12 hours before the experiment. The tank was refilled with warm tap water daily after cleaning. Crocodiles were offered minced chicken heads with a vitamin premix (22 to 25 g/crocodile/day) immediately after cleaning and refilling the tank.

Two isolates of *Dermatophilus* sp (Strains TVS 96-366-5A and TVS 96-490-7B), both from cases of dermatophilosis in farmed crocodiles in Queensland and the Northern Territory (Buenviaje et al, 1997) were used. The isolates were initially grown on blood agar plates; after four days all colonies were harvested into 10% bovine serum in tryptose broth, then incubated for a further 24 hours at 37°C in an atmosphere of 5% CO₂ in air. On the following day the inoculum was prepared by homogenizing the broth using a dounce homogenizer, then collecting the fluid into 5 mL Bijoux bottles. After a settling period of one week, infection of 12 crocodiles was attempted by directly applying broth culture of one of the two isolates of *Dermatophilus* sp to marked areas of normal skin or skin tightly scarified with a scalpel. Six crocodiles were inoculated with strain TVS 96-366-5A and six with strain TVS 96-490-7B. To prevent the inoculum being washed from the skin, hatchlings were kept out of the water for one to two hours after inoculation. Sterile broth only was similarly applied to the four in-contact control hatchlings.

Within several days of inoculation, typical 'brown spot' lesions were apparent, not only at inoculation sites, but also at other locations in both infected and in-contact control animals, indicating direct spread of infection, presumably via organisms in the tank water. Microscopic examination of the lesions in hatchlings killed at five and 20 days post inoculation (PI) revealed changes characteristic of spontaneous dermatophilosis (Buenviaje et al., 1997; Buenviaje, *et al.*, 1998a), and *Dermatophilus* sp was isolated from skin lesions on day five PI and from tank water collected at day 30 PI. Bacteriological and biochemical characterisation of the isolates indicated that on each occasion it was strain TVS 96-490-7B; strain TVS 96-366-5A was never reisolated.

Anticipating, in view of the pilot study, that transmission could be readily reproduced, the protocol for the second transmission experiment was changed to evaluate possible control and treatment procedures; therefore following inoculation with strain TVS 96-490-7B, four groups of six-week-old *C porosus* hatchlings were selected (an inoculated but untreated control group of 10 crocodiles plus three inoculated then treated groups of 19 crocodiles per group). Each group was held in adjoining but separate concrete tanks in which the water was non-medicated or contained chlorine (4 ppm), salt (500 g/100 L of water) or formalin (0.04%). Tank water was maintained at 32EC by automatic, thermostatically controlled intermittent flow of warm water into the tank. Again both dry and wet areas were available to hatchlings.
Although occasional minute skin lesions developed in crocodiles in both treatment and control groups but mostly in the non-medicated water (control) group, these quickly regressed so effective transmission was not achieved.

A third experiment was conducted using facilities and procedures as in the pilot study but with control and inoculated hatchlings maintained in separate tanks in separate buildings 60 metres apart. On this occasion only strain TVS 96-490-7B was used for inoculation. Sixteen six-month-old *C. porosus* hatchlings were divided into a sham-inoculated control group of six and an experimental group of 10, which in turn was divided into two subgroups of five animals each; the skin of crocodiles in one subgroup (A) was scarified then inoculated while those in the other subgroup (B) served as non-inoculated but in-contact controls. Sterile 10% bovine serum in, tryptose broth was used to inoculate scarified sites on the six animals in the control group. All crocodiles were caught and individually examined on days three, six, nine and 20 PI, and at termination of the experiment on day 36 PI when all remaining crocodiles were killed by barbiturate overdose.

4.2 Results

In the final transmission experiment (third experiment), on day three PI focal lesions up to 1 mm in diameter were present in eight crocodiles - five inoculated and three in-contact. Microscopic examination of skin lesions from two emaciated animals at this time (one died and one killed - both from subgroup B) revealed branching filaments in superficial debris. A *Dermatophilus* sp that was culturally and biochemically identical to isolate TVS 96-490-7B, was grown from lesions in the crocodile that was killed.

On day six PI, skin lesions were present in all crocodiles in the experimental group. Microscopically the skin lesions from one animal killed at this time were confirmed as ulcers, and a filamentous organism was found associated with hypertrophic epidermal cells that contained characteristic poxvirus inclusions. A *Dermatophilus* sp identical to strain TVS 96-4907B was again isolated from these lesions. On day nine PI, linear ulcers up to 10 mm were on present on one crocodile, but there was little change in either the number or size of lesions present and no attempt was made to reisolate *Dermatophilus* sp. On day 20 PI, however, there were frequent focal lesions of both of the above types on all crocodiles in the experimental group. Some linear ulcers were also present. Microscopic examination of skin lesions from one crocodile in subgroup B revealed both dermatophilosis and poxvirus infection. *Dermatophilus* sp was isolated from the lesions.

At the termination of the experiment on day 36 PI, lesions up to 2 mm in diameter were present on all animals but whereas some initial lesions had resolved, other new ones had developed. Microscopy confirmed the presence of both dermatophilosis and poxvirus infection and again a *Dermatophilus* sp identical to strain TVS 96-490-7B was isolated.

In control crocodiles no skin lesions were observed until day 20 PI when a few minute and slightly depressed grey circular lesions in one animal were confirmed microscopically as poxvirus infection. On day 36 PI, three focal lesions up to 1 mm in diameter and confirmed by microscopy as dermatophilosis, were detected on two crocodiles; one of these also had poxvirus lesions. Similar translucent to grey circular lesions observed in four of five control crocodiles killed on day 36 PI were confirmed microscopically as poxvirus infection. Overall, 11 animals, six in the treatment group and five in the control group, had concurrent poxvirus infection. *Dermatophilus* sp was again isolated from one animal with dermatophilosis only.
5. Phenotypic and Genotypic Characterisation of *Dermatophilus* sp Isolated from ‘Brown Spot’ Lesions in Farmed Crocodiles

5.1 Methodology

Five crocodile field isolates, a *Dermatophilus congolensis* type culture (ATCC 14637) and a strain of *Dermatophilus chelonae* (DCH 2) were compared with each other phenotypically. Six additional isolates recovered from infected animals during the transmission study and one isolated from water sampled during the pilot transmission experiment were also included for comparison. The isolates were initially grown on blood agar containing 5% sheep blood, and incubated in two different temperature settings at 37 °C in the presence of 5% CO₂ and at 28 °C in the ambient atmosphere. All isolates were inoculated in tryptose phosphate broth containing 10% bovine serum, brain heart infusion broth, Mueller-Hinton agar and Mueller-Hinton agar supplemented with 9% sheep blood, and incubated at 37 °C in an atmosphere of 5% CO₂, except *D chelonae*, which was found to grow best at 28 °C. The bacterial cultures were examined daily for two weeks.

All isolates were grown in blood agar for antibiotic sensitivity testing. *D congolensis* isolates were harvested from blood agar after three days whereas both the crocodile isolates and *D chelonae* isolate were harvested after five days of incubation. Bacterial colonies were gently homogenised using a sterile dounce homogeniser. The homogenised suspension was diluted with a sterile pH 7.0 PBS until it matched the turbidity of a McFarland 0.5 standard. Approximately 20 μL of homogenised suspension was uniformly spread onto three Mueller-Hinton agar plates supplemented with 9% sheep blood. A disk diffusion susceptibility method was used as described by Isenberg (1992). Eight antibiotic disks (Oxoid) of ciprofloxacin (5 μg), cefotaxim (30 μg), cloxacillin (5 μg), penicillin (10 units), ampicillin (10 μg), streptomycin (10 μg), erythromycin (15 μg) and tetracycline (30 μg) were dispensed in two Petri plates, each plate containing four disks. The remaining plate was used as a control. All plates were incubated at 37 °C in the atmosphere of 5% CO₂ except *D chelonae* which requires incubation at 28 °C. The zone of inhibition obtained with the isolates was measured after 48 hours (Masters et al., 1995). Another disk diffusion susceptibility test was performed following a similar procedure to verify the initial results. *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as reference organisms for comparison on the antibiotic sensitivity of test isolates.

Gram-staining was performed to observe the morphological features of mature filaments and zoospores. Kinyoun modified Ziehl Neelsen staining method was also used to examine for acid fastness. Nigrosin methylene blue staining and a hanging drop method (Baker and Silverton, 1976) were used to determine the presence of a capsule and the motility of all *Dermatophilus* isolates, respectively. Biochemical tests used included catalase, oxidase, urease, indole production, methyl red, Voges-Proskauer, and nitrate reduction (Baron and Finegold, 1990). Acid production was recorded after inoculating each isolate into carbohydrate fermentation broth base containing 5% of either lactose, sorbitol, xylose, dulcitol, mannitol, salicin, sucrose, fructose or glucose. The proteolytic activity of the isolates was also recorded after inoculation onto Loeffler’s serum medium, casein, tyrosine and xanthine agars.

16S rDNA sequence determination and phylogenetic analysis

Four methods were used to extract the DNA from the 19 isolates. DNA extraction was initially carried out using the method described for *Aeromonas hydrophila* (Oakey, 1997). Other methods used were (1) Qiagen protocol for DNA extraction (Qiagen Pty. Ltd., Clifton Hill, Victoria); (2) caesium chloride DNA extraction; (3) modified DNA extraction protocol based upon the *A hydrophila* method.

Two PCR protocols were carried out for the amplification of 16S rDNA in *Dermatophilus* spp isolates from crocodiles. The PCR amplification protocol as described by Marchesi et al. (1998) consisted of
a forward primer (63 f) of 5’–CAG GCC TAA CAC ATG CAA GGC–3’ and a reverse primer (1387r) of 5’–GGG CGG WGT GTA CAA GGC –3’. These primers were designed to amplify approximately 1,300 base pairs of the consensus 16S rDNA genes of Gram-positive bacteria that contained a high percentage G-C base sequence. The PCR reaction described by Marchesi et al. (1998) was modified to consist of 10 ng of template DNA, 1 μM of each primer, 200 μM dNTP, 2 mM MgCl₂, 1.1 unit Taq polymerase, 5 μL 10× buffer and sterile distilled water to a final volume of 50 μL reaction mixture. The cycles were programmed and the tubes were placed in the PTC-100™ thermal cycler (Bresatec Ltd., Therbaton South Australia) preheated to 70 °C.

In the second PCR protocol, three primers were designed. One forward primer (fwd1 of 5’–AGA GTT TGA TCC TGG CTC AG–3’) and two reverse primers (rvs1 of 5’–CGC TCG TTG GAC TTA ACC–3' and rvs2 of 5’–CG CGT ACC TTG TTA CGA CTT–3') were tested for amplification abilities. The final reaction consisted of 2.5 mM each dNTP (1.75 mM total final concentration); 50 pmols of each primer; 6.25 mM (final concentration) magnesium chloride; 2.5 ng template DNA; 1.5 units Taq polymerase; 4 μL 10× reaction buffer; sterile distilled water to 40 μL. A PCR product of approximately 1,000 bp was obtained by using fwd1 and rvs1 primers. Amplicons were visualised and size estimated.

All PCR products were purified following the procedure in QIAquick PCR purification kit protocol (Qiagen Pty Ltd, Clifton Hill, Victoria). The ‘cleaned PCR’ product was used in subsequent cycle sequencing reactions and purification of extension products.

Sequence reaction products were electrophoresed with a model ABI prism 310 genetic analyser (Perkin-Elmer Corporation, USA). The 16S rDNA sequences were verified and corrected for any ambiguous sequence, and the percentage similarities of sequence between two isolates were obtained using the Sequencher 3.0 computer program. In addition to the 16S rDNA sequence generated in this study, previously published sequences of Streptomyces sp (Genbank accession number AF 012741), Frankia sp (AF 034776) and Geodermatophilus obscurus (X 92359) obtained from the Genbank sequence database were analysed. Complete multiple alignment of 16S rDNA sequences were examined by using the computer program Clustal X (Thompson, et al., 1997). The Tamura-Nei neighbour joining complete deletion method of the computer program, Molecular genetic analysis (MEGA) version 1.01, was used to obtain a phylogenetic tree. Bootstrap confidence values were obtained with 1,000 resamplings.

Ribotyping
DNA samples from 13 isolates excluding DCD, DCS1, DCS2, DCC1, Fi 4 and Fi 5 (see Table 2) were digested using Eco R1 and Sca 1. The agarose gel containing digested DNA with rulers next to the marker lanes of Hind III cut Lambda DNA and 1,000 kb DNA markers loaded at each end of the digests was visualised over UV light. The transfer of fragmented DNA from the gel to a positively charged nylon membrane was achieved through a Southern blotting technique. The 16S and 23S RNA from E coli was labelled with digoxigenin (DIG) , and the concentration was estimated. The labelled RNA was used as a probe for high stringency hybridisation to the Southern blot membrane. Hybridisation was detected with chemiluminescence. The migration (mm) of hybridised ribotype restriction fragments against known fragment sizes of DNA markers were determined and analysed

5.2 Results
Phenotypic characterisation
All crocodile isolates were Gram-positive and non-acid fast filamentous bacteria. The hyphae branched at right angles, and the width was measured from 0.5 to 1μ m. The zoospores were motile, and measured 1 μm in diameter. Except for the two field isolates and one isolate recovered from water, all isolates shared similar phenotypical characteristics. The colonies were initially a grey colour on blood agar containing 5% sheep blood. In addition, all colonies were pitting and exhibited haemolysis between two and five days after inoculation.
In tryptose phosphate broth and brain heart infusion broth, all isolates produced granular flocculent sediments. Biochemical tests showed that all isolates were catalase positive and oxidase negative. Acid was always produced from glucose but not from sorbitol, xylitol, dulcitol, mannitol or salicin. The results for methyl red and Voges-Proskauer, xanthine and tyrosine tests were negative. Two of five field isolates (Fi 4 and Fi 5) produced a clear zone around the colonies in xanthine agars and no hydrolysis on tyrosine agars except for the presence of dark pigments.

Also, the colonies of field isolates Fi 4 and Fi 5 had different cultural characteristics compared with the three field isolates (Fi 1, Fi 2 and Fi 3). The size of the colonies was much bigger (1 to 3 mm after 48 hours of incubation), and became dark in colour after five days compared with the other three field crocodile isolates. After five days incubation, the colonies were 3 to 5 mm in diameter, and white aerial hyphae were prominent on the surface of the colonies.

**DNA extraction**
Of the four DNA extraction protocols, only the modified DNA extraction protocol for *A. hydrophila* produced DNA extracts from all isolates. Approximately 250 ng/L of DNA were extracted after the protocol was modified. The modification included freezing and thawing of the pelleted bacteria at least three times, increasing the repeated lysozyme (from 1 mg/mL to 5 mg/mL) in the lysis buffer and an increase in incubation period of suspension in ice (from 5 to 15 minutes). Adding 2 mL of hexadecytrimethyl bromide (CTAB) to the mixture and incubation in a water bath at 55°C overnight was for DNA extraction. DNA was also extracted from only five isolates after the caesium chloride DNA extraction technique was modified, however, the amount extracted was very low (5 ng/L).

**PCR amplification and phylogenetic analysis of 16S rDNA sequence**
Two of 19 isolates (Fi 4 and Fi 5) did not produce PCR products. Prior to the direct sequencing of the PCR products, the reproducibility of PCR from the 15 isolates was tested at least three times. Isolates DCD and CD52 did not produce a quality sequence and were removed from the study. To confirm the fidelity of the nucleotide sequences, the 16S rDNA sequence from the PCR products using the designed primers was compared with the 16S rDNA sequence from the PCR product using the primers described by Marchesi et al. (1998).

The 16S rDNA sequences of isolates produced by the two different sets of primers were matched and carefully reviewed for possible sequencing errors. The size of nucleotide sequences of the 16S rDNA genes of the isolates in this study ranged from 965 to 1,345 total base pairs. Since the sizes of the nucleotide sequences of all isolates varied, only 965 bases were used for comparison to avoid biased results. The 16S rDNA sequences of crocodile isolates *D. congolensis*, *D. chelonae*, *Deodermatophilus obscurus*, *Frankia* sp and *Streptomyces* sp were used for comparison. The 16S rDNA gene sequences of the six recovered isolates from the experimental transmission studies were identical to the 16S rDNA sequence of the original challenged organism (Fi 1).

Interestingly, the recovered isolate (Ri 6) from one infected crocodile in the control group of the transmission experiment had a 100% sequence similarity with the other recovered isolates. Except for F 2, the sequences of two field isolates Fi 1 and Fi 3 from the spontaneous outbreaks of 'brown spot' disease in Queensland and in the Northern Territory respectively, exhibited a 100% similarity despite the geographical distance between the sources (Table 2). The 16S rDNA sequence of field isolate 2 (Fi 2) had 87.1% and 91.3% sequence similarity to Fi 1 and Fi 3, respectively.

**Table 2:** Levels of 16S rDNA sequence similarity between the *Dermatophilus* crocodiles and other *Dermatophilus* species.

<table>
<thead>
<tr>
<th>% Sequence similarities</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
</tr>
<tr>
<td>87.1%</td>
</tr>
<tr>
<td>91.3%</td>
</tr>
<tr>
<td>Species</td>
</tr>
<tr>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Fi 3</td>
</tr>
<tr>
<td>Fi 1</td>
</tr>
<tr>
<td>Recovered isolates</td>
</tr>
<tr>
<td>Fi 2</td>
</tr>
<tr>
<td>WS</td>
</tr>
<tr>
<td><em>Dermatophilus chelonae</em> (DCH2)</td>
</tr>
<tr>
<td><em>Dermatophilus congolensis</em> (DCD)</td>
</tr>
<tr>
<td><em>Dermatophilus congolensis</em> (DCS1)</td>
</tr>
<tr>
<td><em>Dermatophilus congolensis</em> (ATCC)</td>
</tr>
<tr>
<td><em>Dermatophilus congolensis</em> (DCC1)</td>
</tr>
</tbody>
</table>

A phylogenetic tree was determined using the Tamura-Nei neighbour joining complete deletion method (Figure 1). The crocodile isolates composed of two field isolates (F 1 and F 3) and all six recovered isolates (Ri 1 to Ri 6) were in one group separate from *D. chelonae* (DCH 2) and the group of *D. congolensis*. Crocodile isolates and *D. chelonae* had a 12 base difference or 99% sequence similarity based upon 16S rDNA sequence. The isolate WS from water sample TV 97-124 WS collected at 30 days post inoculation during the pilot transmission experiment was closely related to *Streptomyces* sp. All the major clusters in the phylogenetic tree were supported with 100% bootstrap values.

**Figure 1.** Phylogenetic tree for genus *Dermatophilus* and closest relative based on a sequence analysis of 16S rDNA gene. The tree was constructed using the Tamura-Nei neighbour joining complete deletion method. The number at the branch points are bootstrap values based on 1,000 replicates. The scale bar indicates a genetic distance of 0.01.

**Ribotype identification**
Thirteen DNA extracts were digested with restriction enzymes Eco R1 and Sca 1, and this was followed by Southern blotting and hybridisation with labelled RNA probe containing 16S and 23S E coli RNA (Boehringer Mannheim). Both restriction enzymes *Eco R1* and *Sca 1* were found to provide ribotype fragments from nine isolates. As the ribotype bands were sometimes difficult to read, the X-
Ray films were read and interpreted independently by two researchers. This was done to help eliminate subjectivity.

Four isolates, of which two were recovered (Ri 1 and Ri 3) and two strains of *D chelonae* (DCH 1 and DCH 2) did not produce ribotype bands following digestion of chromosomal DNA with Eco R1. Likewise no ribotype bands were produced from one field isolate (Fi 3), two recovered isolates (Ri 1 and Ri 3) and one strain of *D chelonae* (DCH 1) with restriction enzyme Sca 1. Eco R1 restriction enzyme revealed similar ribotype patterns of the recovered isolates (Ri 2, Ri 4, Ri 5 and Ri 6) and the original challenged isolate (Fi 1) used in transmission studies. The ribotype bands with fragment size 7,097 kb, 2,644 kb and 2,249 kb were present in all isolates tested. Isolate WS was different from the crocodile isolates by the presence of a ribotype band with fragment size of 1,514 kb.

Only a single ribotype fragment was produced by restriction enzyme *ScaI* of which six isolates (Fi 1, Fi 2, Ri 2, Ri 4, Ri 5 and Ri 6) had a fragment size of 5,385 kb. The ribotype fragment of isolates WS and *D chelonae* (DCH2) have the same ribotype fragment size of 4,352 kb.
6. Treatment and Control of *Dermatophilosis* (*“Brown Spot”*) Disease in Farmed Crocodiles

6.1 Methodology

*Evaluation on the effects of flowing and static water on dermatophilosis.*

Forty-two *Crocodylus porosus* hatchlings from the Berrimah Farm, Department of Primary Industry and Fisheries, Darwin, Northern Territory were used in this study. The hatchlings were seven-week-old with an average length of 35 cm from the snout to the tip of the tail. The hatchlings were kept in a concrete pen with a sloping floor filled with approximately 200 L of water, while two-thirds of the area was dry. Randomly selected hatchlings individually identified by a marked cut on the scutes were allocated to three groups consisting of 15 in each group A and group B, and a further 12 hatchlings in group C (sham-inoculated control group). All animals in groups A and B were inoculated with a homogenised six-day-old broth culture of *Dermatophilus* sp (TVS 96-490-7B) by dipping a cotton swab in the inoculum and gently rubbing it on at least 10 sites on the skin of the abdomen, chest, neck, jaws and tail. Animals in the sham-inoculated control group C were rubbed with sterile distilled water. Immediately after inoculation, the animals were kept in plastic crates for an hour before being returned to their respective pens, to avoid the inoculum being washed off.

**Animal management and maintenance**

The animals were housed in two enclosed sheds approximately one metre apart, and each shed has two adjoining pens. The two inoculated groups (A and B), were placed in two separate pens in the first shed whereas the sham-inoculated control (group C) was in the second shed. Group A was placed in a pen with static water (fixed volume of water for 24 hours) heated with automatic immersion heaters (Rena Corporation, Charlotte, NC, USA) maintained at 32 °C. Group B and the sham-inoculated control (group C) were in flowing water, which was supplied with intermittent inflow of warm water from a hot water tank whenever the water temperature dropped below 32 °C. In pens provided with flowing water, the water is totally replenished approximately every three hours during the daytime. The development of ‘brown spot’ lesions was recorded to compare the effect of flowing water against static water.

The pens were emptied and cleaned each morning, and the crocodiles were fed approximately 27 g/crocodile/day of minced red meat with a vitamin supplement each afternoon. To avoid possible cross-infection, each shed was provided with a set of cleaning instruments. In addition, the pen of the control group was cleaned first and all materials used in cleaning were scrubbed thoroughly and disinfected with 1% chlorine solution. Surgical gloves were discarded after handling the experimental animals in each group. The feet and hands were washed with 2% Savlon solution (Pharmedica Laboratories Pty Ltd, London) before and after working with the animals. There was only one handler in charge in the management of the experimental animals throughout the duration of the study.

The challenged animals were restrained and examined clinically on days 1, 3, 7 and 10 post-inoculation (P1). The ‘brown spot’ lesions in each animal were counted and measured. Two animals with the most lesions were euthanased by barbiturate overdose injected directly into the heart. A post-mortem examination was performed, and two skin samples (2 cm²) with ‘brown spot’ lesions were fixed in 10% buffered formalin. The samples were processed for histopathological examination to confirm if effective transmission of ‘brown spot’ disease was achieved.

*In vitro antibacterial sensitivity testing of* Dermatophilus *sp to copper sulphate,*
formalin and salt

Prior to the clinical treatment trial of ‘brown spot’ disease, an in vitro sensitivity testing using copper sulphate, salt and formalin was undertaken. The desired colony forming units (CFU) were determined initially before the in vitro study was carded out. Dermatophilus sp isolate with accession number TVS 96-470-7B used previously in several transmission experiments (Buenviaje et al., 1998b) was tested for its sensitivity to antibacterial agents. Two loopsful of a six-day-old bacterial culture from a blood agar plate were placed into a dounce homogeniser tube and 1.5 mL of sterile distilled water was added. The clumps of bacterial colonies were homogenised until the mixture became uniformly cloudy. Serial ten-fold dilutions were performed in six Eppendorf tubes from neat to 10⁶. Approximately 0.5 mL from each tube containing 1.5 mL of homogenised bacterial suspension was dispensed and uniformly spread onto three blood agar plates containing 5% sheep blood. The plates were incubated at 37°C in the presence of 5% CO₂ in air, and the colonies counted after five days. The 10⁻⁵ dilution provided 150 to 250 CFU, and was selected as the standard dilution for the sensitivity testing.

Preparation of antibacterial agents

Copper sulphate was prepared at concentrations of 0.1, 0.25, 0.5 and 1 ppm, salt at 1 mg/mL, 2, 3 and 5 mg/mL and formalin at 0.02, 0.03, 0.05 and 0.10%. Each antibacterial solution was prepared in aliquots of 1.35 mL in Eppendorf tubes. To each tube, 150 µL of homogenised bacterial suspension at 10⁻⁶ dilution was added to achieve a final concentration of 150 to 250 CFU. The bacteria were exposed to different concentrations of the antibacterial solutions for 30 minutes, one hour, three hours, and six hours. Untreated control tubes containing sterile distilled water was prepared for every group of antibacterial solution and the same protocol was carried out as described above. Each antibacterial solution containing the homogenised Dermatophilus sp suspension, and the untreated controls were plated onto three blood agar plates after exposure times mentioned above. The number of CFU in each plate was counted after five days of incubation.

Comparative study on the efficacy of copper sulphate and formalin on dermatophilosis

Twenty-nine hatchlings with ‘brown spot’ lesions,(22 hatchlings from the study on the evaluation of flowing water and seven hatchlings from the pilot experiment on poxvirus) were randomly allocated to three groups. Group 1 (12 hatchlings) was treated with 1 ppm copper sulphate, group 2 (seven hatchlings) with 0.1% formalin and 10 hatchlings were used as the untreated control group. Copper sulphate and formalin were used as therapeutic agents based upon the results of the in vitro antibacterial sensitivity testing. The copper sulphate solution was prepared by dissolving 100 mg of copper sulphate in approximately 4 L of water, and 100 mL of formalin were added to 4 L of water. The solutions were mixed thoroughly in approximately 96L of water in each pen to achieve the desired final concentration of 1 ppm and 0.1% for copper sulphate and formalin, respectively. The solutions were prepared each day, and added to the pen immediately after cleaning. To maintain the desired concentrations of the antibacterial solutions, both the copper sulphate- and formalin-treated groups were kept in pens provided with static water. The untreated control group was placed in a pen provided with flowing water. Individual animals in the copper sulphate-treated group, formalin-treated group and the untreated control group, were restrained on clinical examination days 4, 7, and 11 post - treatment (PT). Data on the number of recovered animals and the number and size of ‘brown spot’ lesions in remaining infected animals were recorded.

6.2 Results

Gross and microscopic findings

Gross examination of the skin from the two hatchlings (each from a group provided with static water and the sham-inoculated control group) revealed the presence of ‘brown spot’ lesions up to 1 mm in diameter scattered mainly on the belly, chest and neck. ‘brown spot’ lesions were present in both inoculated and non-inoculated sites of the skin. Samples of skin sectioned and examined microscopically revealed histopathological changes typical of dermatophilosis. Changes included the presence of indentations or ulcerations of the epidermis and accumulations of debris composed of dead epidermal cells, keratin and inflammatory cells, sometimes associated with laminated keratin. The
layer of epidermis on the indented portion of the skin was thinner compared with the adjacent epidermis of the unaffected skin. Numerous ‘filamentous bacteria infiltrated the debris in deeply ulcerated sections of the skin. These filamentous bacteria were similar in morphology, and consistently present in skin samples infected with ‘brown spot’ disease in previous experiments (Buenviaje et al, 1998b).

**Effect of flowing water on the development of ‘brown spot’ lesions**

Sixty percent (9 out of 15) of the hatchlings in static water (group A) developed one to two ‘brown spot’ lesions less than a millimetre in size on day one PI (Table 3). All hatchlings were infected with dermatophilosis during the 10-day-period after inoculation, and the number of ‘brown spot’ lesions had increased up to 10 lesions. The ‘brown spot’ lesions were up to 2 mm in diameter but mostly less than a millimetre in size, located predominantly on the belly, neck and chest except a few spots on the tail. On the skin of the jaws in three animals were either ‘brown spot’ or linear lesions up to 1 cm in length. After clinical examination on day 10 PI, the lesions on the jaws and neck in three animals had resolved. In addition, the hatchlings from the two groups (group B and the sham-inoculated control group) in flowing water started eating on the 3rd day after inoculation, and ate approximately 40 of 405 grams of food per day until the 5th day, whereas, the hatchlings in static water (group B) commenced to eat approximately 20 to 30 grams of food per day on the 5th day. All animals resumed normal feeding two weeks after inoculation, however the animals in all groups stopped feeding for at least a day every after clinical examination.

*Table 3* Comparison of static water and flowing water on the development of ‘brown spot’ lesions.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Number of Animals</th>
<th>Number of animals with ‘brown spot’ lesions at PI days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PI day 1</td>
</tr>
<tr>
<td>Group A (static water)</td>
<td>15</td>
<td>9(60%)</td>
</tr>
<tr>
<td>Group B (flowing water)</td>
<td>15</td>
<td>3/20%</td>
</tr>
<tr>
<td>Group C (control)</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

Three (20%) hatchlings from a group in flowing water developed ‘brown spot’ lesions on day one PI. Affected hatchlings had one or two lesions located either on the belly or tail. The lesions from two infected hatchlings had resolved after clinical examination on day three PI. However, new lesions developed at other sites of the skin on day 10 PI. A total of four animals from the group provided with flowing water developed ‘brown spot’ lesions during the 10-day-period after inoculation. The sham-inoculated control group of 12 hatchlings had not developed any ‘brown spot’ lesions on day one PI. However, small ‘brown spot’ lesions up to 1 mm were found in four animals on clinical examination day three Pt. Likewise, some original lesions had resolved, but reappeared at other sites on day seven and 10 PI. A total of nine hatchlings developed ‘brown spot’ lesions during the 10-day-period after inoculation. Two hatchlings, one each from static water (group A) and flowing water (group B) had pale and gelatinous discolouration on the skin at the right lateral side of the abdomen, typical of superficial fungal infection (Buenviaje et al., 1994).

**In-vitro antibacterial sensitivity.**

The average number of CFU from the three blood agar plates inoculated with *Dermatophilus* sp (96-490-7B) after exposure to each concentration of antibacterial solution at a given time are presented in Table 4. No colonies were present in any of the blood agar plates inoculated with *Dermatophilus* sp exposed to different concentrations of copper sulphate at 30 minutes, one hour, three hours and six hours after five days incubation. Further incubation of plates for another week or so yielded the same results with no colonies present in any blood agar plates. In contrast, an average range of 117 to 248 CFU was present in all control blood agar plates after five days incubation. The cultural and morphological characteristics of these colonies were consistent in all plates. The colonies were initially
grey in colour, haemolytic and slightly pitting into the blood agar after three days incubation and became white and more rugose, sometimes crateriform after five days.

**Table 4. In vitro sensitivity test.** Number of colony forming units (CFU) of *Dermatophilus* sp (TVS 96-490-713) after exposure for various times to different concentrations of antibacterial solutions.

<table>
<thead>
<tr>
<th>Antibacterial solutions</th>
<th>Number of CFU at exposure times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 minutes</td>
</tr>
<tr>
<td><strong>CuSO₄ (ppm)</strong></td>
<td></td>
</tr>
<tr>
<td>0 (control)</td>
<td>248</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>NaCl2 (mg/mL)</strong></td>
<td></td>
</tr>
<tr>
<td>0 (control)</td>
<td>220</td>
</tr>
<tr>
<td>1</td>
<td>208</td>
</tr>
<tr>
<td>2</td>
<td>206</td>
</tr>
<tr>
<td>3</td>
<td>215</td>
</tr>
<tr>
<td>5</td>
<td>216</td>
</tr>
<tr>
<td><strong>Formalin (%)</strong></td>
<td></td>
</tr>
<tr>
<td>0 (control)</td>
<td>208</td>
</tr>
<tr>
<td>0.02</td>
<td>210</td>
</tr>
<tr>
<td>0.03</td>
<td>153</td>
</tr>
<tr>
<td>0.05</td>
<td>171</td>
</tr>
<tr>
<td>0.1</td>
<td>188</td>
</tr>
</tbody>
</table>

The effects of time and the different concentrations of salt and formalin on the number of CFU were analysed using the Tukey HSD statistical method. No significant difference in the number of CFU was found between concentrations of salt including the control. However, there was a reduction of the number of CFU after exposure of *Dermatophilus* sp at one hour, three hours and six hours (P<0.05). A reduction in the number of WU was observed between zero concentration (control) and 0.1% formalin (P<0.05). All other concentrations of formalin (0.02, 0.03 and 0.05%) did not differ significantly (P>0.05) on the mean number of CFU. In addition, there was no significant effect (P>0.05) of the exposure times (30 minutes, one hour, three hours and six hours) on the number of CFU.

**Treatment of dermatophilosis**

As a result of husbandry problems, the treatment of dermatophilosis was modified to four protocols. The comparative assessment on the efficacy of copper sulphate and formalin on ‘brown spot’ lesions was terminated on day 11 PT due to the increased severity of ‘brown spot’ lesions.

**Copper sulphate and formalin treatment**

The ‘brown spot’ lesions resolved in eight of 12 (67%) hatchlings from the copper sulphate- treated group on day four PT. However, there was a recurrence of ‘brown spot’ lesions in five of eight hatchlings on clinical examination day 11 PT. The ‘brown spot’ lesions present in all infected hatchlings from the copper sulphate-treated group were very small (<0.5 mm), and were apparently resolving. One of seven hatchlings in the formalin-treated group had lesions resolved on day four PT, but became reinfected after day seven PT. More lesions were present in infected hatchlings from the formalin-treated group compared with the hatchlings from the copper sulphate-treated group on day four PT. In addition, one animal from the formalin-treated group had 16 lesions (up to 2 mm in diameter) scattered mostly on the belly. On clinical examinations days 7 and 11 PT, all animals developed severe lesions with an average of 19 lesions per animal. One severely affected hatchling had 30 ‘brown spot’ lesions scattered on the belly, neck, chest and tail, but mostly on the belly. The
severity of ‘brown spot’ lesions in the control group was similar to the infected animals in the formalin-treated group.

Copper sulphate treatment in pen provided with static water
Due to the increased severity of ‘brown spot’ lesions on hatchlings both in the formalin-treated group and the control group, the formalin treatment was discontinued. The total number of animals with skin lesions included 22 hatchlings with ‘brown spot’ lesions and two hatchlings with dual skin infections (‘brown spot’ and superficial fungal skin lesions) were from the treatment groups. An additional 11 infected hatchlings from the pilot study on poxvirus (five hatchlings with ‘brown spot’ lesions, four superficial fungal skin infections, one with dual infection and one hatchling with an abscess on the right axilla) were also included. Overall, 27 hatchlings had ‘brown spot’ lesions, three with dual skin infections, four with a superficial fungal infection and a single case of an abscess.

All infected animals were placed in two separate pens in static water treated with copper sulphate. A group of non-infected and recovered hatchlings in a pen provided with flowing water also received copper sulphate medication. On the eighth day PT, the immersion heaters provided in static water failed, thus the flowing water was restored. The treatment of copper sulphate was continued for two weeks. On clinical examination after 14 days PT, 21 of the 27 (77.8%) hatchlings with ‘brown spot’ lesions, three of four hatchlings with superficial fungal infection and one hatchling with the abscess were resolved. Two of the three hatchlings with dual infections recovered from both skin infections, and the fungal lesions in one hatchling had also resolved.

Copper sulphate treatment in pen provided with flowing water
Copper sulphate medication was continued, but because of the problem of maintaining the desired concentration (1 ppm) in flowing water, the animals were treated twice. The first treatment was given in the morning after cleaning and the second treatment was given in the afternoon. All 12 infected animals including those recovered and non-infected animals were treated twice daily for 10 days. Clinical examination after ten days of copper sulphate medication showed no therapeutic response from all 12 infected hatchlings. Instead, there were additional four new cases and three recurrent cases of superficial fungal infection. Another two hatchlings (one new case from a non-infected group and one recurrent case) developed ‘brown spot’ lesions. A total of 21 infected animals included 14 with ‘brown spot’ lesions and seven with superficial fungal infection were recorded.

Immersion of animals in copper sulphate-medicated water for at least 15 minutes
The copper sulphate treatment was continued, but all animals were immersed in copper sulphate medicated water for at least 15 minutes twice daily, one in the morning after cleaning and another one in the afternoon after the animals were offered food. After six weeks of medication, 50% (7 of 14) of hatchlings with ‘brown spot’ lesions had recovered. The remaining seven infected hatchlings had one to four lesions, which were apparently resolving. All seven hatchlings infected with superficial fungal infection had also recovered. A final clinical examination after a further three weeks revealed all infected animals had completely recovered from ‘brown spot’ lesions. No recurrence of lesions resembling either ‘brown spot’ or superficial fungal infection was noted. All animals were perfectly healthy and not one scar from previous lesions was found.
7. Studies on Poxvirus in Crocodiles

7.1 Methodology
Eleven of 16 six-month-old *C. porosus* hatchlings showing lesions typical of poxvirus during the transmission study of *Dermatophilus* sp. were used in this study. All infected animals with pox lesions were euthanased by barbiturate overdose (1 mL) injected directly into the heart. On clinical examination, the gross appearance and size of the poxvirus lesions on the skin and their location were recorded. Skin lesions from infected animals were collected into sterile plastic bags at post mortem examination, and stored at –80 °C. At least three representative skin samples from each animal were fixed in 10% buffered formalin for histopathological examination. The lesions on two skin samples were excised from the adjacent normal skin with a sterile scalpel blade, cut into 1 mm², fixed in either 10% glutaraldehyde or formaldehyde and examined by transmission electron microscopy.

*Preparation of tissue culture from freshwater crocodile (Crocodylus johnstoni) embryo*
Three freshwater crocodiles (*Crocodylus johnstoni*) eggs (15 days old) were used to culture tissue from the embryo. The whole embryo was separated from the remainder of the egg, placed in a sterile Petri plate and washed twice with Dulbecco’s Modified Eagle’s Medium (DMEM) to remove some mucus and blood. The cleaned embryo was transferred to another Petri plate, cut into small pieces (<1 mm²), and 10 mL DMEM added. The embryo suspension was mixed gently, transferred to a 100 mL conical flask and allowed to stand for few minutes to settle down large particles. The supernatant was decanted into a 25 mL tissue culture flask, 1.5 mL of bovine serum was added, and labelled as primary culture. The remaining large tissue particles or sediments were added to 10 mL DMEM and 1 mL Antibiotic Versene and Trypsin (ATV) solution, and mixed gently. The suspension was decanted into a plastic, sterile centrifuge, and centrifuged at 1,000 × g for 15 minutes. The supernatant was collected into a 25 mL tissue culture flask marked "second collection", and 1.5 mL of foetal calf serum was added. Both the first and second collections were incubated at 28°C. The tissue culture was examined under the microscope every three days, and DMEM was changed after two to three weeks if necessary.

*In vitro culture of poxvirus*
The pox lesions were carefully excised from five skin samples using a sterile scalpel blade, and ground with a mortar and pestle with DMEM containing antibiotics. The suspension was transferred to Eppendorf tubes, centrifuged for 5,000 × g for two minutes to remove tissue particles, the supernatant decanted, and the remaining pellet were stored at –80 °C. At least 200 μL of the poxvirus suspension was inoculated into each of two tissue culture flasks containing baby hamster kidney cells (BHK) and another two flasks of *vero* cells, and incubated at 28 °C. After one week, the initial culture was subcultured each into at least two flasks, and subsequent four passages were carried out. The inoculated cell cultures and the controls were examined under the inverted microscope for any changes in the cells every after three days for two weeks.

*Inoculation into chicken chorioallantois and crocodile embryos*
The protocol for preparing the homogenised poxvirus inoculum was modified by using pH 7.0 PBS containing 5 x concentration of antibiotic instead of DMEM to avoid possible bacterial contamination particularly from *Dermatophilus* sp. The suspension was held at room temperature for an hour before inoculation (Tripathy and Hanson, 1980) to prevent possible temperature shock of the embryo. Inoculation with presumed poxvirus infective tissue was performed by injecting 100 μL of homogenised suspension through the chorioallantoic membrane (CAM) in ten chicken eggs and 100 μL of sterile pH 7.0 PBS in two control eggs following the procedure described by Mitchell-Hoskins (1967). After inoculation, the eggs were incubated at approximately 37 °C. Poxvirus inoculation was repeated in five batches of a dozen (11 days old) chicken eggs to have more specimens for histology and electron microscopy examination. Another poxvirus inoculation was performed in two 16-day-old *C. porosus* eggs. Approximately 100 μL of poxvirus suspension was injected through the chorioallantoic cavity (Webb, Manolis, Dempsey and Whitehead, 1987), and incubated at 28 °C.
Both chicken and crocodile eggs were examined six to eight days and one to two weeks, respectively, after inoculation.

Pilot transmission experiment of poxvirus
The Berrimah Farm, Department of Primary Industries and Fisheries, Darwin, Northern Territory supplied 17 *C porosus* hatchlings used in this study. The hatchlings were seven weeks old with an average length of 35 cm from snout to the tip of the tail, and were kept in a concrete pen with a sloping floor (filled with approximately 200 L of water) and a dry area. Prior to animal inoculation, approximately 2 mL of homogenised poxvirus inoculum kept for at least a year in –80 °C was transferred immediately into a bucket of ice, and thawed gently to prevent possible temperature shock. At least ten different sites on the skin of the abdomen, chest, neck, jaws, tail and footpads in nine animals were slightly scarified using a sterile scalpel blade. A sterile cotton swab dipped in the homogenised poxvirus inoculum was gently and repeatedly rubbed on the scarified area of the skin. The inoculated animals were kept in plastic crates for an hour before being returned to the pen containing eight non-inoculated in-contact control animals. All inoculated animals included the non-inoculated in-contact control were restrained and examined individually at post-inoculation days 1, 3, 7 and 10.

7.2 Results
The gross and microscopic appearance of skin lesions infected with poxvirus was consistent in all 11 hatchlings. The poxvirus lesions were located on the skin of the limbs, abdomen, neck and chest, but mostly on the skin of the tail and footpads. Marked circumscribed grey-white lesions up to 3 mm dia. were present on the pigmented skin particularly on the lateral sides of the tail, back, lower limbs and footpads. The lesions on the tail were slightly pitted onto the skin and located between scales whereas the lesions on the abdomen, neck and chest were diffused irregular and translucent. Many of the skin lesions were ulcerated and accompanied by reddening of adjacent skin.

Microscopic examination on the affected skin revealed the presence of an ulcer, and the epidermal layer extending from a relatively normal skin into the ulcerated part had hypertrophic cells of varying sizes. Affected cells of the epidermis were enlarged and contained either a single large intracytoplasmic inclusion body or multiple small intracytoplasmic inclusion bodies of different sizes. The nucleus was distorted and displaced to the edge of the cell as a result of the accumulation of the inclusion bodies. The intracytoplasmic inclusion bodies were eosinophilic and bigger as the surface cells were approached. Degenerating or dead epidermal cells containing the inclusions were intensely eosinophilic and flattened, which filled the concave portion of the affected epidermis. There was not a marked inflammatory reaction present in the dermis beneath the pox lesions except for cases where the pox lesions were adjacent to 'brown spot' lesions.

Transmission electron microscopy
Transmission electron microscope examination of the skin lesions confirmed the presence of poxvirus particles. The inclusion bodies consisted mainly of numerous oval or brick shape virions that were at least 200 nm long. Inside the virions there were different forms of nucleocapsid such as rounded dense material, 'sausage' shape but mostly elongated biconcave bodies with rounded ends forming a 'dumb bell' shape. An outer thin membrane enclosed some virions present inside the inclusion bodies. In the cytoplasm outside the inclusion bodies there were different structures such as circular bodies and a crescent shape with granular materials adjacent to the concave side. Inside the circular bodies varied from homogeneous to localised aggregation of materials sometimes forming a 'sausage' shape inner core. All circular bodies were enclosed with an outer membrane.

Attempted inoculation of poxvirus in cell culture, chicken chorioallantois, crocodile embryo and *C porosus* hatchlings
Several attempts to grow the poxvirus in *vero* and BHK cell cultures failed to produce any cytopathic effect. Initial growth of fibroblast and epithelial cells from both the primary and secondary cultures of
crocodile embryo were satisfactory. However, the cells gradually began to degenerate and died after four passages, hence the inoculation of poxvirus into crocodile cell culture was not carried out.

One to two focal nodular lesions (2 to 5 mm in diameter) were produced on the chicken chorioallantoic membranes. The lesions were white to opaque colour, and varied from slightly raised to rounded foci. Histologically, the white to opaque focal lesions contained foci of hyperplastic and hypertrophic cells. A small number of cells contained empty vacuoles or eosinophilic material that resembled the inclusion body of poxvirus. However, poxvirus particles were not present from several samples of infected chorioallantoic membranes examined with the transmission electron microscope.

In the pilot experiment, no gross lesions characteristic of poxvirus were seen on clinical examination. Instead seven animals developed 'brown spot' lesions on clinical examination at day 10 post-inoculation.
8. Discussion

Although considerable research work on crocodiles has been published, the topics have been mainly limited to the behaviour and population demography and enforcement guidelines for the crocodile industry. There has been a limited published information on the aetiology, pathogenesis, treatment and control of crocodile diseases particularly affecting the skin. The lack of information on skin diseases has precluded the development of strategies that will minimise, if not arrest, the spread of infection.

The results extrapolated from the survey of skin diseases of farmed crocodiles in this study serve as benchmark information, which are important in many ways for improved husbandry management. In 1992, superficial fungal infection was the most prevalent skin disease in farmed crocodiles especially during winter months (May to August) (Buenviaje et al., 1994). Superficial fungal infection in this study was not considered a serious problem under systems of improved husbandry management, especially if the water temperature was maintained at 32 °C.

Although four major diseases of skin in farmed crocodiles were identified in the epidemiological survey, dermatophilosis or 'brown spot' disease was the most frequently diagnosed. It was considered the most important disease from an economic point of view, being present on six of nine crocodile farms in this study. The expeditious spread of 'brown spot' disease to the non-inoculated control group in transmission studies strongly suggests that the disease is highly infectious. 'Brown spot' disease can develop rapidly to cause damage even to healthy animals, probably because the aquatic environment facilitates the spread of infection. Direct contact or mechanical transfer of Dermatophilus by insects was confirmed in the experimental transmission study of D congolensis using both Stomoxys calcitrans and Musca domestica in rabbits (Richard and Pier, 1966). However, the transmission studies of dermatophilosis reported here revealed that scarification of the skin was not necessary to effectively produce 'brown spot' lesions. Therefore it was presumed that this highly pathogenic organism required no predisposing factors such as stress or minor scratches on the skin to enable it to establish infection.

Based on the experimental transmission studies, the organism was capable of causing 'brown spot' lesions in a short period (three days after inoculation). The 'brown spot' lesions pitted the scales to various depths especially in the abdomen, in a manner similar to 'brown spot' in alligators (Newton, 1992). It has been reported that D congolensis produced significant amounts of keratinase during a 12-day incubation period, and was considered to cause extracellular proteolytic activity responsible for keratinised tissues being the initial target of infection (Hanel, et al., 1991). It is presumed that the Dermatophilus sp used to inoculate the crocodiles in the transmission studies also produced a keratolytic enzyme similar to D congolensis (Hermoso et al., 1993). Of particular interest in cases involving dual or multiple skin infections in farmed crocodiles, Dermatophilus sp was the most prevalent microorganism which clearly suggests its role as the primary pathogen inducing initial damage to the superficial epidermis. Skin is the first line of defence for the body and once damaged it becomes the portal of entry of other opportunistic pathogens such as fungi, bacteria and viruses, which in turn may invade the underlying tissues causing severe damage.

The Dermatophilus sp isolated from a spontaneous outbreak of 'brown spot' disease in farmed crocodiles was used as the challenge isolate in transmission studies. Both the isolate used for inoculation and the recovered isolates were identical based upon standard microbiological procedures and 16S rDNA sequence analysis, thus confirming its role in causing 'brown spot' disease.

Although the initial microbiological examinations revealed a close resemblance of the crocodile isolate to Dermatophilus congolensis type strain (ATCC 14637), there were a number of morphological, cultural and biochemical characteristics that differentiated the crocodile isolate from D congolensis. Comparison between Dermatophilus sp from D chelonae (Masters et al., 1995), the aetiological agent of dermatophilosis in turtles, also revealed substantial differences in morphological, cultural and
biochemical characteristics. The transverse and longitudinal septation considered typical of *D congolensis* in domestic animals (Roberts, 1981) and lizards (Montali *et al*., 1975) was not a feature of the *Dermatophilus* sp isolated from crocodiles. The rate of growth and the colonial morphology of the crocodile isolates differed significantly from *D chelonae* and *D congolensis*. The latter grew faster, and the colonies were more rugose and crateriform and golden yellow colour whereas the former grew more slowly and the colonies were white umbonate with a smooth surface. Besides, the *Dermatophilus* sp isolated from crocodiles was nutritionally more fastidious than *D congolensis* with a requirement for blood to grow on Mueller-Hinton agar.

A 16S rDNA analysis clearly discriminated the *Dermatophilus* sp crocodile isolate from the *D congolensis* type strain. The differences in the number of nucleotide bases between the *Dermatophilus* sp isolated from crocodiles and the *D congolensis* type strain strongly enforces the conclusion that the crocodile isolates are distinct from *D congolensis* both genotypically and phenotypically. Although there was greater homology of 16S rDNA sequence between *Dermatophilus* sp and *D chelonae*, the phenotypic differences were sufficient to conclude that the crocodile *Dermatophilus* sp is different from *D chelonae*. Overall, both the phenotypic and genotypic differences were sufficient to suggest that the crocodile isolate is a distinct species of *Dermatophilus*, thus a new species of *Dermatophilus – Dermatophilus crocodyli* sp nov is proposed.

In the control and treatment of 'brown spot' disease, copper sulphate was found to be the most effective antibacterial agent based upon both *in vitro* and *in vivo* studies. Copper sulphate prevented the further development of 'brown spot' to more severe lesions in crocodiles experimentally infected with *Dermatophilus* sp. It has also been shown that copper sulphate was effective against superficial fungal infection and abscesses. Following copper sulphate treatment, all animals recovered and importantly without evidence of scarring of the affected skin. Although initially there were recurrences of 'brown spot' lesions and the emergence of new cases in non-infected crocodiles under treatment, the protocol for administering the copper sulphate through the water strongly suggests that the animals did not receive sufficient exposure to medication. As such, animals recovering from a sub-therapeutic dose could be the source of recurrent and prolonged infections. Furthermore, a water temperature below 32°C overnight probably contributed to the recurrence of infection (Buenviaje *et al*., 1994) after the immersion heaters failed.

It was concluded that the concentration of 1 ppm copper sulphate can only be maintained if 'dipping bath' pens are provided. In a management system employing flowing water, treatment was given twice to help maintain therapeutic levels of copper sulphate. There was a response to the medication, only when infected animals were immersed in copper sulphate-treated water for at least 15 minutes twice a day. Based on the four copper sulphate treatment protocols evaluated, the forced immersion of crocodiles in 1 ppm of copper sulphate was the most effective method for the treatment of skin lesions. However, the handling of animals should be minimised to avoid unnecessary stress.

Although it appears that flowing water did restore the normal appetite of infected animals earlier than those kept in static water, there was no effect on the control of 'brown spot' lesions. The practice of providing flowing water in most crocodile farms in hatchling and grower pens probably should be re-evaluated to consider the cost and the effectiveness in preventing 'brown spot' disease. As flowing water did not prevent or reduce the occurrence of 'brown spot' disease it is probably more practical and cost effective to clean and replenish with clean water daily.

Poxvirus infection is another major concern in crocodile farms in Australia. In the epidemiological study on skin diseases of farmed crocodiles, the incidence of poxvirus infection is lower and presently not as important as in South Africa (Pandey *et al*., 1990; Gitao and Mwendia, 1999). However, the diagnosis of poxvirus infection in 11 of 16 (68.8%) hatchlings and the severity of lesions in a separate transmission study on dermatophilosis is a cause for concern. The possibility of future outbreaks in farmed crocodiles must be considered. The source of infection could not be established because the infected hatchlings were hatched from eggs collected from the wild, and were kept in a separate
enclosed pen not in contact with other crocodiles. The role of mosquitoes or other insect vectors implicated in outbreaks of poxvirus infection in poultry (Tripathy and Cunningham, 1984) needs to be investigated.

In conclusion, the work undertaken in this research project has contributed substantially to our understanding of a wide range of skin diseases in farmed crocodiles in Australia. The objectives of this project as defined at the commencement of the study were achieved. The pathology of the skin diseases particularly 'brown spot' disease was described both macroscopically and microscopically. 'Brown spot' disease was identified as the most prevalent and probably the most important disease in farmed crocodiles in Australia. The aetiological agent of 'brown spot' disease was identified, and a new species of *Dermatophilus* was proposed. Copper sulphate at 1 ppm concentration was proven to be effective against 'brown spot' disease, however it is highly recommended that the crocodile farms should provide 'dipping bath' pens for treatment of sick animals.

8.1 Recommendations

There are several research topics that require further investigation in order to fully understand skin diseases of crocodiles and the most appropriate method for treatment.

- Further evaluation on the application of copper sulphate in water in different management systems as prophylaxis against diseases affecting the skin, with particular emphasis on 'brown spot' disease.

- Further DNA studies utilising DNA-DNA hybridization techniques and infectivity studies of crocodile isolate TVS 96-490-9B, which is phenotypically similar to crocodile isolates TVS 96-490-7B and TVS 367-15.

- Poxvirus infection in crocodiles needs to be re-investigated. This includes the isolation and propagation of the virus, and detailed characterisation of the virus, conduct experimental transmission studies and determine the pathogenesis of the disease.
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