A spiroplasma associated with tremor disease in the Chinese mitten crab (*Eriocheir sinensis*)

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An epidemic of tremor disease has been a serious problem in Chinese mitten crabs, *Eriocheir sinensis*, in China in recent years. The disease-causing agent was previously considered to be a rickettsia-like organism. Here, analysis of the 16S rRNA gene sequence, light and electron microscopy and cultivation *in vitro* were used to identify the agent. Sequence analysis of the 16S rRNA gene found it to have 98% identity with that of *Spiroplasma mirum*. The agent was able to be passed through membrane filters with pores 220 nm in diameter and could be cultivated by inoculating the yolk sac of embryonated chicken eggs and M1D medium. Rotary motion and flexional movement were seen by light microscopy, and electron microscopy showed that the organism had a helical morphology and lacked a cell wall. The organism produced small colonies with a diameter of 40–50 μm after 17–25 days of incubation on solid M1D medium. The agent was found in blood cells, muscles, nerves and connective tissues of crabs inoculated with a filtrate of yolk sacs or with cultures grown in M1D medium, and it was similar in structure to those grown in eggs and cultivation broth. Disease was reproduced by experimental infection with the cultivated organisms. This study has demonstrated that the causative agent of tremor disease in the Chinese mitten crab is a member of the genus *Spiroplasma*. This is believed to be the first time a spiroplasma has been found in a crustacean. These findings are not only significant for studies on pathogenic spiroplasmas, but also have implications for studies of freshwater ecology.

INTRODUCTION

The Chinese mitten crab, *Eriocheir sinensis*, is an important species in freshwater aquaculture in China. An epidemic of tremor disease has been a serious problem in this species in recent years. The disease first appeared in 1994 and spread quickly in the Jiangsu, Anhui and Zhejiang provinces in southeast China, causing 30–90% mortality. By 1998, the disease had spread to most aquaculture facilities for mitten crabs in China (Huang, 2000). Random samples collected in Anhui province in 1998 suggested the prevalence was 34–3% (Wei, 1999). The disease occurs seasonally, with most disease associated with high water temperatures (19–28 °C) during July and August. Infected crabs exhibit signs of weakness, anorexia, intense paroxysmal tremors and death. During outbreaks the disease spreads quickly, is difficult to control and causes serious losses (Wang & Gu, 2002).

The causal agent of tremor disease was previously thought to be a rickettsia-like organism based on morphological and pathological studies (Wang & Gu, 2002; Wang *et al.*, 2002). The agent lacked a nucleus but contained a nucleoid, and was 0.2 x 0.9 μm when bacilliform and between 0.22 and 0.35 μm in diameter when spherical. It had a preference for infecting nerve, muscle and connective tissues (Wang *et al.*, 2001). Haemocytes played a major role in propagation and transmission (Wang & Gu, 2002). Microcolonies of the agent within membrane-bound vacuoles were seen in tissue smears stained with Giemsa. Characteristics such as transverse binary division in cytophasic vacuoles, formation
of inclusion bodies or appearance as single cells in the cytoplasm, and possible inhibition of phagosome-lysosome fusion (no lysosome function was observed in infected cells), suggested that this agent might be a member of the genus *Ehrlichia* (Wen, 1999).

The 16S rRNA gene sequence (Gasparich, 2002) was analyzed in order to confirm the taxonomic position of the agent. The results showed that the agent was not a rickettsia, but rather a spiroplasma, with its 16S rRNA gene having 98% sequence identity with that of *Spiroplasma mirum*. This result prompted a review of our previous studies and re-examination of the agent's status, including investigation of its cultivability and the capacity of the isolated agent to cause disease in experimentally inoculated crabs.

**METHODS**

**Determination and analysis of the 16S rRNA gene sequence of the tremor-disease-causing agent.** DNA was extracted from the haemolymph (5 samples), pectoral ganglion (15 samples) and cardiac muscles (4 samples) dissected from infected and healthy crabs (15 control samples), as well as colonies cultured on M1D agar (3 samples). These DNA samples were used for 16S rRNA gene sequence analysis. The DNA was extracted using a spin column kit (Qiagen) or glass powder (BioDev) according to the manufacturer's instructions. The 16S rRNA gene was amplified using a PE2400 thermocycler (95°C for 5 min, then 30 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 20 s, with a 7 min extension for the last cycle) using two universal primers for bacterial 16S rRNA gene sequences (P16S-907B, 5'-CCG TCA ATT CGA C/T TTG AGT TT-3' and P16S-894, 5'-AGA GTT TGA TC(A) TGG CTC AG-3'). The PCR products were purified with glass powder (BioDev) according to the manufacturer's instructions and cloned into the pGEM-T vector (Promega). Plasmid DNA was isolated from the clones and analyzed by restriction endonuclease digestion.

The cloned 16S rRNA gene fragments were sent to Shanghai Biological and Engineering Inc. for DNA sequence determination (ABI PRISM 377-96; BigDye Terminator v2.0). Sequences were compared to those in the GenBank database using the program BLASTN. The 16S rRNA gene sequences obtained from DNA extracted from the haemolymph (Crab22, CrabSP6), pectoral ganglion (Crab10, CrabT7), and cardiac muscle (Crab25), and from cultivated organisms (CrabSP6Cult) were used for phylogenetic analysis. The 16S rRNA gene sequences from the samples above were aligned with 16S rRNA gene sequences from representatives of the major spiroplasma clades and appropriate outgroups using CLUSTAL_W (Thompson et al., 1994) and then aligned manually in MacClade (Maddison & Maddison, 1992). Phylogenetic reconstruction was performed using maximum-likelihood. *Acholeplasma laidlawii* was used as an outgroup. A total of 1428 characters were used for the majority of sequences. The analysis used a heuristic search and the tree bisection-reconnection maximum-likelihood algorithm for branch swapping. The dataset was resampled 500 times to obtain bootstrap percentage values using PAUP (version 4.0b10; Swofford, 1998).

**Primary isolation and cultivation of the tremor-disease-causing agent.** The tremor-disease-causing agent was first cultivated in the yolk sacs of embryonated chicken eggs. Blood from a crab with tremor disease was passed through a membrane filter with mean pore diameter of 220 nm and the filtrate was inoculated into the yolk sac of ten 7-day-old chicken embryos. Four further embryos were inoculated with a filtrate of blood from healthy crabs. Eggs were also inoculated with 100 U penicillin to inhibit bacterial growth. Eggs were incubated at 35±1°C and candled daily to check embryo viability.

M1D broth (Whitcomb, 1983) was used for further cultivation of the agent. Tubes containing 1-5 ml medium were inoculated with 0-05 ml fluid from embryonated chicken eggs, incubated at 30°C and observed daily. After acidification of the cultures, dark-field microscopy was used to examine the medium for the presence of organisms and to determine their morphology. Subcultures were made to ensure that growth was sustained by the medium and at least 10 additional passages were carried out. M1D agar was prepared by inclusion of 1.5% Noble agar in the broth medium and the plates were inoculated with the tenth passage culture in M1D broth.

**Crab inoculation experiments.** A total of 28 healthy crabs were used for inoculation experiments. Eighteen healthy crabs were each inoculated with 0-1 ml of a filtrate of yolk sac fluid diluted in PBS (pH 6-8) and 10 crabs were each inoculated with 0-1 ml of saline as a control. A group of 25 healthy crabs were used for another inoculation experiment. Ten crabs were each inoculated with 0-1 ml of the tenth passage of the agent in M1D and another 10 crabs were inoculated with 0-1 ml of a suspension of colonies from M1D agar in PBS (pH 6-8). Five crabs were each inoculated with 0-1 ml medium as a control. All the crabs were observed daily and tested by a blood smear method every 5 days to determine whether they were infected. The body of the crab was cleaned with water and disinfected using 75% alcohol, and a needle was introduced into the crab through the joint between the pereiopod and the thorax. A small aliquot of blood (about 0-05 ml) was obtained with a 1 ml syringe and smeared on a glass slide. The smear was fixed in 4% formaldehyde and the slide was air-dried and stained with Giemsa solution diluted 1:10 with phosphate buffer (pH 6-8) for 15–20 min. The film was washed with water and examined by light microscopy. Pink to bluish-purple inclusion bodies were identified in cells in smears from diseased crabs. Impression smears of the heart, gills, hepatopancreas, pereiopod muscles, thoracic ganglion and gonads from diseased and healthy crabs were also prepared and stained using the same method.

**Electron microscopy.** For negative staining, samples of the organism cultured in M1D were placed on Formvar-coated copper grids. The excess fluid was removed by touching the grids with filter paper and the preparation was fixed with 2.5% glutaraldehyde in PBS for 1 h. After removal of excess fluid by touching with filter paper, the grids were stained with 2% sodium phosphotungstate for 30–40 s and were air-dried before examination. For ultrathin sectioning, samples of blood cells, cardiac and pereiopod muscle, and pectoral ganglia of both control crabs and crabs inoculated with M1D cultivated organisms were fixed with 2.5% glutaraldehyde in phosphate buffer. The colonies on M1D agar were embedded first in 1.5% Noble agar and then fixed with 2.5% glutaraldehyde in phosphate buffer. All samples were secondarily fixed with 1% osmium tetroxide in the phosphate buffer, then dehydrated with a series of acetone washes (10%, 20%, 30%, 50%, 70%, 90%, 100%) and embedded in Epon 812. Ultrathin sections of 50–80 nm were made using a Reichert–Jung ultramicrotome and stained with uranyl acetate and lead citrate. The sections were examined using a Hitachi 600-2A transmission electron microscope.

**RESULTS**

**16S rRNA gene sequence analysis**

PCR amplification of the 16S rRNA gene from infected crabs and an isolate of the organism cultured on M1D agar yielded products of approximately 900 bp. Products of the
same size were obtained from 15 of 15 pectoral ganglia, five of five haemolymph samples and four of four cardiac muscle samples from crabs from geographically distinct areas and from three of three colonies on M1D agar, but not from any of 15 control samples. Direct sequencing of the amplified PCR products confirmed the presence of similar rRNA genes in all 27 of the samples tested. Comparison of these sequences with those in the GenBank database showed that they were 98% identical to those of *S. mirum*. The sequence was submitted to GenBank and has the accession number M24662. A phylogenetic tree was inferred using the 16S rRNA gene sequences. Of the 16S rRNA gene sequences, two from the haemolymph (Crab22, CrabSP6), two from the pectoral ganglion (Crab10, CrabT7), one from the cardiac muscle (Crab25) and one from an isolate cultured on M1D agar (CrabSP6Cult) were analysed, and all clustered together with *S. mirum* (Fig. 1). The dataset was resampled 500 times and the bootstrap percentage values are given for each branch of the tree.

**Primary isolation and cultivation of the tremor-disease-causing agent**

All chick embryos inoculated with material from infected crabs died within 5–8 days, while those inoculated with material from healthy crabs remained viable. Motile, round microorganisms were seen by light microscopy in yolk fluids of all embryos inoculated with material from infected crabs, but none were seen in fluids from eggs inoculated with material from healthy crabs.

The tremor-disease-causing agent grew well in M1D broth at 25–30°C, with a doubling time of 69 h within 10 passages. Doubling times were estimated using the method of Konai et al. (1996). On M1D agar the agent produced colonies with a diameter of 0.4–0.5 mm after 17–25 days of aerobic incubation at 30°C (not shown). Neither ‘fried egg’ colonies nor satellite growth around central zones were seen.

**Crab inoculation experiments**

All 18 crabs inoculated with the filtrate of the yolk sac fluid developed typical signs of tremor disease (tremor of pereiopods) within 8–15 days and died within 25 days, whereas 10 crabs inoculated with control yolk sac fluid remained healthy. The agent could be detected in Giemsa-stained smears of tissue from all 18 crabs and in ultrathin sections of tissue from all crabs by electron microscopy (Wang & Gu, 2002). Ten crabs inoculated with the tenth passage of the agent in broth and another 10 crabs...
inoculated with organisms from colonies on M1D agar showed typical signs of tremor disease (tremor of pereiopods) within 7–14 days and died within 23 days, whereas five crabs inoculated with 0·1 ml of M1D medium as a control were healthy. The agent was detected by light and electron microscopy in haemolymph, muscles, nerves and connective tissues of the crabs inoculated with the filtrate of yolk sacs or with cultivated organisms. The agents were similar in structure to those grown in eggs and broth (Fig. 2), and experimental infection produced disease similar to that seen in natural infection.

Light and electron microscopy

The agents cultured in either embryonated chicken eggs or M1D broth were seen to be motile using phase-contrast and dark-field microscopy. The agent showed typical rapid rotary and flexing movements. It was detected in haemolymph, muscles, nerves and connective tissues of the crabs inoculated with the isolate from eggs or organisms cultivated in broth or on agar. The helical morphology of the agent could be seen by transmission electron microscopy (Fig. 3). Ultrastructural examination of the agent showed that it lacked a cell wall (Fig. 4) and was 50–200 nm in diameter and 3–12 μm in length. The cultivated agent had the same structure as those seen in the crabs with tremor disease (Fig. 4).

As in naturally occurring cases of tremor disease, experimentally infected crabs had inclusions that stained purple with Giemsa stain in cells in infected tissues. Ultrastructural studies showed the presence of the agents in the connective tissue of cardiac and pereiopod muscles and the pectoral ganglia of the crabs. They were not seen in the tissues of uninfected crabs. In the early stages of infection, the agents were mainly seen in the haemolymph cells and aggregated near the nuclei to form inclusion bodies. Later, they were seen in most tissues. Muscles and nerves were the main tissues affected. The highest concentrations of the agent were found in the sarcolemma of muscles, in nerve cells and sometimes at the motor end-plate between the nerve and muscle fibres. The pectoral ganglion is a nerve plexus controlling the movement of pereiopods. The fact that the agent infected this tissue and the motor end-plate correlates with the clinical signs of paroxysmal tremor of the pereiopods. The pathology seen in experimentally infected crabs

**Fig. 2.** Electron micrograph of the cardiac muscle of a crab inoculated with the organisms cultivated on M1D agar. The micrograph shows the trilaminar membrane, the lack of a cell wall and the width of the membrane (solid arrow) compared to that of the muscle cell membrane (open arrow). Scale bar, 0·1 μm.

**Fig. 3.** Negatively stained electron micrograph of agent from the cardiac muscle of a crab with tremor disease showing its helical structure. Scale bar, 0·5 μm.

**Fig. 4.** Electron micrograph of a cloned isolate of the organism on M1D agar. The higher-magnification micrograph (inset) shows the same structure as that in crabs with tremor disease. The solid arrow indicates the membrane and the open arrow indicates the branching point. Scale bars, 3 μm and (inset) 0·2 μm.
was similar to that seen in naturally occurring tremor disease (Wang et al., 2001).

Ultrathin sections of the organisms showed a typical trilaminar membrane with no cell wall, but with an outer layer (Fig. 2, Fig. 4, solid arrows). This structure was previously mistaken for a cell wall because the outer layer was electron-dense. At higher magnification, it can be seen that the width of its membrane is similar to that of the host cell membrane (Fig. 2, solid arrow).

**DISCUSSION**

The results of 16S rRNA gene analysis and the microbiological and morphological studies indicate that the tremor-disease-causing agent is a member of the genus *Spiroplasma*. The usual hosts of spiroplasmas are insects and plants (Whitcomb et al., 1997, 1999). Taxonomically, spiroplasmas are in the domain *Bacteria*, phylum *Firmicutes*, class *Mollicutes*, order *Entomoplasmales*, family *Spiroplasmataceae* and genus *Spiroplasma*. They are unique among the *Mollicutes* in having helical morphology (Whitcomb, 1980; Whitcomb et al., 1999). Several revisions have been made to the classification of the genus *Spiroplasma* since the original description of *Spiroplasma* species (Junca et al., 1980; Whitcomb et al., 1987). The most recent revision describes 34 spiroplasma groups and 14 subgroups (Williamson et al., 1998). Spiroplasmas have been found in a wide range of hosts and cause several plant and insect diseases. They have been isolated from both the surfaces of flowers and the sap in the sieve tube elements of phloem. As many insects feed primarily on nectar it has been suggested that the spiroplasmas found on flower surfaces are deposited there by feeding insects and thus the flowers serve as a site for transmission of the bacteria from one host to another (Hackett & Clark, 1989).

The *Spiroplasma mirum* cluster contains a single species (group V) from rabbit ticks (Tully et al., 1983). Although spiroplasmas have been well documented as causes of disease in insects, they have not been detected in aquatic animals or crustaceans. Our detection of spiroplasmas in the mitten crab may increase understanding of the host range of these organisms and provoke investigation of other possible hosts in water, as well as possible relationships between terrestrial hosts and aquatic hosts. These findings have implications not only for studies on pathogenic spiroplasmas, but also for studies of freshwater ecology and epidemiology.

Further work is needed to explore the origin of this pathogen. Studies on the biological properties of this agent and its infection cycle in crabs will be valuable in the development of methods to control tremor disease. Since the agent responsible has now been confirmed to be a spiroplasma, the disease might be more appropriately described as spiroplasmosis.

There are some differences between *S. mirum* and the tremor-disease-causing agent, although they have 98% identity in their 16S rRNA genes. The colonies on M1D agar appeared only after 17–25 days of incubation at 30 °C, which is 7–11 days longer than that taken for *S. mirum*. The ‘fried egg’ colonies and satellite growth around central zones that are typical of *S. mirum* were not seen. The three criteria most useful in spiroplasma taxonomy are the 16S rRNA gene sequence, DNA–DNA hybridization analyses and serology. The highest resolution is provided by 16S rRNA gene sequence analysis, which is useful for discrimination of most species (Whitcomb et al., 1999). However, the evolutionary distances between members of groups VIII and XVI are much smaller and rRNA gene sequences are unable to distinguish them at the subgroup level (Gasparich, 2002). The inability of 16S rRNA gene sequences to discriminate closely related microbial species has been reported previously (Fox et al., 1992). Our study has shown that the organism isolated from Chinese mitten crabs is indeed a member of the *Spiroplasma* genus. However, further study is needed to identify the actual species, which is closely related to *S. mirum*, but which may be distinct.

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