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Short Communication

First confirmed report of a bacterial brood disease in stingless bees

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ABSTRACT

Susceptibility to brood pathogens in eusocial stingless bees (Meliponini), alternative pollinators to honey bees, is unknown. Brood losses in managed colonies of the Australian stingless bee, *Tetragonula carbonaria*, were studied over 20 months. We isolated a disease-causing bacterium, *Lysinibacillus sphaericus* (Firmicutes, Bacillaceae), from worker and queen larvae, brood cell provisions and honey stores. Pathogenicity experiments confirmed this bacterium as the causal organism. It took 22 days from infection to first appearance of brood disease symptoms. This is the first confirmed record of a brood pathogen in stingless bees.

1. Introduction

Brood diseases are of concern for beekeeping because of the effects they have on hive population numbers. Control measures can include antibiotics; however, bacterial resistance is common. *Apis mellifera* can be infected with a number of diseases caused by pathogenic bacteria and fungi (Aronstein and Murray, 2010; Genersch, 2010; Genersch et al., 2010) whereas stingless bees appear to have few brood diseases. The only report is by Kerr (1948), who noted diseased pupae in the Brazilian stingless bees *Melipona quadrifasciata* and *Melipona bicolor bicolor. Bacillus paraalvei* was identified in 1957 as the possible causal organism (Nogueira-Neto, 1997). However, at the time only microscopic investigations were performed without any description of pathogenicity, symptoms of the infection or molecular diagnostics. Apart from this, no other reports have been published. Prior to the current study, there were no recorded cases of brood disease in Australian stingless bees.

The Australian stingless bee, *Tetragonula carbonaria*, has been reported to be an effective crop pollinator (Dollin et al., 1997), due to its generalist foraging behaviour (Slaa et al., 2006). *Tetragonula carbonaria* is the most commonly kept (Halcroft et al., 2013) and widespread species of stingless bee, distributed from the warm tropical areas of coastal Queensland (QLD) (Cape York, 16°S, 145°E) to the temperate areas of southern New South Wales (NSW) (Bega, 36° 40.27′S, 149° 50.34′E), and has a flight temperature range between 18 and 35 °C (Rayment, 1935). We report the first detection and identification of a bacterial brood disease, discovered in a *T. carbonaria* hive showing brood losses from December 2012. Over a 20-month observational period starting from March 2013, a further seven colonies showed symptoms of infection.

2. Materials and methods

Tetragonula carbonaria colonies were kept at the Hawkesbury campus apiary, Western Sydney University in Richmond (33° 36.42′S, 150° 44.44′E), NSW, Australia, within their natural range. They were originally sourced from south-east QLD (Australian Stingless Native Bees, Hatton Vale). Four colonies arrived at the apiary in March 2011, and appeared healthy. From then to the first symptoms of disease they experienced normal environmental conditions, with ambient temperatures ranging between 1.5 and 37 °C. However, in December 2012 (after 18 months), upon opening, one hive showed atypical spiral brood structures containing discoloured larvae. The hive was subsequently assessed for its internal colour, structural appearance of the brood, texture of storage pots, involucrum appearance and coverage, formation of brood nest, worker population, in-hive worker behaviour, and colony odour. All brood cells were removed from the colony, and any larvae showing discolouration or fluid appearance in opened cells were separately placed into 1.5 mL sterile Eppendorf tubes. Samples were processed under aseptic conditions, using procedures reported for diagnosis of *Paenibacillus larvae*-infected honey bee larvae (World Organisation for Animal Health, 2013). Individual

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samples were plated onto Sheep Blood Agar (SBA) containing the antibiotic nalidixic acid (3 mg/mL), and incubated for 24–48 h at 37 °C in 5% CO_2 for further characterisation and pathogenicity testing. After incubation, discrete colonies were observed. Individual colonies were plated onto fresh SBA plates. The SBA cultures were subsequently subcultured and maintained on nutrient agar at 37 °C in 5% CO_2. Samples were prepared for Gram staining and biochemical profiling via catalase testing (Reiner, 2010; World Organisation for Animal Health, 2013).

The pathogenicity of the isolated bacterium was tested against healthy *T. carbonaria* brood. In October 2013, two strong hives of equal strength with regard to honey and pollen provisions, workers and brood, determined by estimating the brood populations (Michener, 1961; Roubik, 1979), were selected. Before sunrise (i.e. prior to bee flight activity), one hive was split into two halves; these two halves were used as the uninoculated control. Each of the two control halves were treated with a total of 105 mL of sterile water. Water was applied first using a 10 mL sterile plastic syringe to dispense the water directly into all opened brood cells and storage pots, after which any remaining water was uniformly applied using a 500 mL sterile plastic hand sprayer with fine spray nozzle over the entire brood chamber, storage pots and nest structures. A 3 mm thick acrylic lid was placed onto the open side and sealed with 48 mm wide masking tape. Both control box halves were placed in a temperature controlled room at 26 °C. The second hive was split and processed in the same manner as the control, but treated with a highly concentrated spore suspension (total 105 mL) containing approximately 400 million CFUs of the isolated bacterium. To reduce possible environmental contamination, the colonies were not allowed to forage; however, in-hive stores were plentiful.

Hives were observed 48 h after treatment, then weekly until first symptoms of disease. The inoculated lower hive half showed symptoms of disease after three weeks and was examined before sunrise. Samples of brood, callows (newly emerged workers), adult workers and honey were collected as described previously. The inoculated upper half was also removed and sampled similarly. Bacteria were isolated and cultured on SBA media as previously described. Furthermore, discrete, re-isolated colonies from these bacterial cultures were used for DNA extraction (Isolate II Genomic DNA Kit, Bioline, London, UK), following the manufacturer's protocol; and then amplified using 16S rDNA primers (530F and 1495R) (Madrid et al., 2001), with the following modifications: 95 °C, 5 min, 35 cycles of 94 °C, 1 min; 50 °C, 30 s; 72 °C, 1.5 min, followed by a final extension step at 72 °C for 10 min. This was followed by molecular cloning using the pGEM®–T Easy Vector System I (Promega, Madison, Wisconsin, USA). Cleaned colony PCR products were sent to Macrogen (Geumcheon, South Korea) for sequencing. In addition, the bacterial samples were subjected to multi locus sequence typing (MLST) analysis of four loci (*adk*, *glcK*, *glpF*, *glyA*) following the protocol outlined by Ge et al. (2011). The sequences of the MLST loci were aligned with sequences of eight previously characterised *L. sphaericus* isolates, four that are toxic (ST-2, ST-4, ST-5, ST-9) and four non-toxic (ST-3, ST-7, ST-8, ST-17) to mosquitos (Ge et al., 2011). Then, a neighbour-joining phylogenetic tree based on the Tamura 3-parameter using a discrete Gamma distribution (T92 + G) model was constructed for the 1622 bp concatenated sequence of the four MLST loci of *L. sphaericus*. Samples of brood collected from the initial infected hive were also sent to the State Veterinary Diagnostic Laboratory of the Elizabeth Macarthur Agricultural Institute, NSW Department of Primary Industries, for isolation and identification of the putative pathogen.

3. Results

The diseased hive identified in December 2012 had reduced forager activity on an ideal foraging summer day (warm and sunny). Most workers were observed to be moving very slowly or were motionless, not performing any tasks in the colony. The few workers that were performing normal nest behaviours were slower in performance and action than normal (Table 1). The brood structure was small, non-uniform, scattered, and with few brood cells (Fig. 1). The hive materials were dark in colour and thick in structure. Developing larvae had changed in appearance, colour and texture (Table 1, Fig. 1). Many infected larvae had been removed from their cells and were located singly or in small groups, mainly on the involucrum. A prominent rotting smell was detected in infected brood cells; cell provisions had a greenish-yellow colour (Fig. 1). It appeared that the queen was absent as no newly laid eggs were observed. SBA plates were dominated by one bacterial species, which was both Gram and catalase positive. In the pathogenicity tests, the bacterium induced disease symptoms (i.e. the presence of brown larvae) at 22 days after inoculation. The re-isolated bacterium (*ex* larvae, brood provisions and honey stores) was confirmed as *Lysinibacillus sphaericus* comb. nov. (Ahmed et al., 2007) formerly known as *Bacillus sphaericus* Meyer and Neide, 1904 (Firmicutes, Bacillaceae), by sequencing of the 16S rDNA (GenBank accession numbers: KR947300–KR947307).

Table 1
Comparison of healthy and unhealthy nest characteristics.

	Healthy	Unhealthy
Colony strength	Large adult population, with strong entrance activity of foragers and cleaners	Adult population reduced, little entrance activity
Odour	Strong smell of plant resins	Pungent, decaying, rotten smell
Storage pots	Oval, bright orange-brown, thin, with smooth appearance. Glossy honey and fresh, moist pollen	Oval, darker brown, thick and tough appearance. Honey and pollen may be present but do not appear to be newly collected
Involucrum	Orange, soft and malleable, smooth lines and finishes, strong network in nest and multiple (4+) thin layers covering brood chamber	Dark brown, tough and thick network, dry and brittle in advanced stages; may cover the brood chamber but limited to 1–2 layers
Brood chamber	Large, characteristic spiral formation, with leading edge of newly laid eggs, fresh healthy colour of newly made cerumen	Variety of sizes (depending on infection stage), may lack spiral formation, cells are scattered and may lack leading edge (absence of queen), lack of developing pupae, overall colour variable
Brood cell exterior	Oval, fully formed swollen caps, soft texture, bright yellow-orange colour	Oval or irregular oval with flattened caps, thick, dark orange
Cell provisions	Smooth, glossy, yellow to orange	Thick, dark yellow to green
Larvae	Glossy, white, soft, solid mass	Half to full brown to black in colour, fluid-like or dry, may form short rope with matchstick test, may be dumped singly or in small groups on nest structures
Worker behaviour	Actively moving (flying, walking, preening), performing hive tasks, will defend nest if opened	Motionless, lethargic, will walk/crawl out of nest, will not aggressively defend nest if opened