First human case of infection with *Arsenophonus nasoniae*, the male-killer insect pathogen

Sir,

Many arthropod-borne human infections have been recognised in recent years, but this is the first reported case of honeybee-associated *Arsenophonus nasoniae* bacteraemia.

A 37-year-old male who presented to the hospital emergency department with sudden onset of fever, rigors, and myalgia five hours after sustaining a bee sting to his left thumb that occurred in the evening hours during April 2018 (fall) behind his home garage. This was unusual for him as he had sustained several bee stings in the past without any severe reaction. His other medical problems included eczema well-controlled with topical corticosteroid cream and chronic neck pain.

On presentation, he was well-perfused with blood pressure of 137/81 mmHg but was tachypnoeic with a respiratory rate of 30 breaths per minute and tachycardic with a heart rate of 145 beats per minute. He was febrile with a temperature of 39.0°C. Oxygen saturation was 98% on room air. His left thumb was inflamed but there were no signs of any embedded bee body parts after removal by his wife. The remainder of his physical examination was unremarkable.

Investigations revealed a normal white cell count 10.5 x 10^9/L (reference range: 3.50-11.00) with a mild neutrophilia of 8.29 x 10^9/L (reference range: 1.7-7.0). He had normal renal and liver function test results and his urinalysis, electrocardiogram and chest-X-ray were all unremarkable. One set of blood cultures was collected into BD BACTEC™ Plus Aerobic/F and Plus Anaerobic/F culture vials. They were incubated in the BD BACTEC™ 9000 automated blood culture system. After four hours observation and conservative management in the emergency department (ED), he was discharged home. He was recalled to the ED 48 hours later for further assessment when his aerobic blood culture vial signalled

positive with Gram-negative bacilli (Figure 1). Clinically, he was doing well with no fever, thus he was not started on any antibiotic and was discharged home again.

The contents of the aerobic vial were sub-cultured onto horse blood, chocolate and MacConkey agars and these media were incubated at 35°C. Growth was evident only on horse blood agar and chocolate agar incubated in 5% CO_2 after 48 hours incubation. There was no growth on horse blood agar incubated anaerobically and no growth on MacConkey agar incubated in ambient air. Colonies were mucoid, grey-white, round, and convex with entire edges (Figure 2). Cells were non-motile, non-spore forming, long rods, occasionally filamentous in young cultures.

The blood culture isolate was further identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry using a Bruker MALDI Biotyper with Bruker Biotyper 3.4 software and library version V4.0.0.1 (Bruker Daltonik, Bremen, Germany), according to the manufacturer's instructions with a spectral score of 1.78 as *Arsenophonus nasoniae* with good confidence to the genus level.

Confirmation of the species identification was achieved by 16S rRNA PCR and sequencing. DNA extraction was performed from cultures by crude lysate preparation (100ºC for 10 minutes in molecular grade water).

Antimicrobial susceptibility testing of the *A. nasoniae* blood culture isolate was performed using Vitek 2® (bioMerieux, USA) semi-automated System Version: 08.01 with the commercially available Vitek 2 AST-N246 card. Antimicrobial susceptibility results are shown (Table 1).

The patient decided to keep the dead bee in a resealable plastic bag and its remnants were sent to Hawkesbury Institute for the Environment, Western Sydney University for further identification and possible detection of the causative bacterial DNA, six weeks after he was contacted.

The bee was morphologically confirmed as *Apis mellifera* before being homogenized in a TissueLyser (Qiagen) at 30 Hz for 4 minutes with a 2mm sterile steel ball. Total DNA was then extracted using TRIsure (Bioline) following manufacturer's recommendations. To detect the presence of *Arsenophonus sp*. in the sample, PCR was performed using the Bioline MyTaq Red PCR kit and primer set Ars23S1 (CGTTTGATGAATTCATAGTCAAA) / Ars23S2 (GGTCCTCCAGTTAGTGTTACCCAAC). 200ng DNA was used in a 10ul reaction (2ul DNA, 2ul MyTaq red mix, 0.2ul Taq polymerase, 0.4ul 5pmol F primer, 0.4ul 5pmol R primer, 10ul H_2O). Reaction conditions were as follows: Initial denaturation 95ºC for 5 minutes, then 30 cycles of denaturation for 30 seconds, annealing at 50ºC for 30 seconds and extension at 72 ºC for 60 seconds, followed by a final extension 72 ºC for 10 minutes. Resulting fragments were subjected to Sanger sequencing (in each direction) using the PCR primers. Sequences were then inspected and trimmed in Geneious v10.2.6 (Biomatters) before using a BLASTn search against the NCBI nucleotide database to confirm identity of the fragments. The PCR reaction amplified an approximately 800bp fragment, which was confirmed using a BLASTn search as showing 97.68% similarity to *A. nasoniae* genome (accession number CP038613.1).

To establish the relatedness of *A. nasoniae* from human blood culture isolate to the honeybee DNA extract, DNA extraction of the blood culture isolate was performed using the HiYieldTM Genomic DNA Mini Kit and Nextera library preps, made for each sample according to the standard protocol. The libraries were subjected to whole-genome sequencing using the NovaSeq 6000 Sequencer (Illumina) according to the manufacturer's instructions. Approximately 20 Gb of 2 x 150nt paired-end reads were generated. Reads were cleaned and trimmed using fastp with default parameters. Megan 6.14.2 in 1000 reads for each sample was run. Diamond blastx against NR (2019) was used to map the read to known peptides. Snippy (https://github.com/tseemann/snippy v4.6.0) was used to call SNPs based on the

public reference genome. SNP frequencies were extracted from the Snippy output with custom per scripts. Metagenomic analysis detected *A. nasoniae* from the human blood culture isolate DNA whereas in the honeybee DNA extract *Arsenophonus sp, Photorhabdus luminescens*, *Proteus mirabilis* and *Haemophilus influenzae* were detected. Analysis showed 95% homology with only 1928 single nucleotide polymorphisms (SNPs) between the 20 Gb of data from the honeybee *Arsenophonus sp* DNA and the *A. nasoniae* DNA from the blood culture isolate.

Bee stings causing infection are rare and have not been extensively studied as a medical phenomenon. A range of skin, soft tissue and disseminated infections have been described, including death[1]. Previously isolated bacteria included *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Enterococcus faecalis, Stenotrophomonas maltophilia,* and coagulase-negative *Staphylococcus* species[1]. There is a case report of co-infection with *A. nasoniae* and *Orientia tsutsugamushi* in a traveller to Cambodia, both detected in a skin eschar by qPCR, and with seroconversion to *O. tsutsugamushi* by immunofluorescence and Western Blot[2]. The role played by *A. nasoniae* in the disease pathogenesis was not clear[2]. The patient was clinically diagnosed with rickettsiosis and recovered following treatment with 200 mg / day of doxycycline.

Arsenophonus nasoniae is a ϒ-proteobacterium from the family *Enterobacterales*. The genome of *A. nasoniae* is 3.5Mbp in size, a relatively large genome for an insect symbiont[3]. *Arsenophonus nasoniae* induces a male-killing phenomenon in the wasp *Nasonia vitripennis*[4]. In the honeybee, the presence of *A. nasoniae* is one of several microbial factors that has been suspected in Colony Collapse Disorder, a mysterious condition involving the sudden disappearance of adult worker bees, which may be related to the poor health of the bee[5]. Thus far *A. nasoniae* has not been detected in Australian honeybees, but that this may be due to limited screening.

The mechanism of *A. nasoniae* transmission to our patient during the bee sting was unclear. We postulate that the honeybee was in poor health and carried high count of *A. nasoniae* in its hemolymph, or on its body, and the sting organ might have been covered by its faeces contaminated with *A. nasoniae* and transmitted it to our patient during the stinging process. The sting organ of the honeybee is composed of a stylet, 2 barbed lancets, and a venom sac. When the bee utilizes its sting, the sting becomes deeply embedded in the skin and quickly advances by alternating the thrusts of both lancets, which, because of the direction of their barbs, can only move forward. Meanwhile, the venom from the sac is injected into the victim. Eventually, the whole stinging mechanism separates from the body of the bee (which kills the bee); however, because of the automatism of the intrinsic muscles, the lancets continue to advance, and the venom is continuously pumped[6]. In this manner, any bacteria on either the insect's body or its sting, or on the surface of the victim's skin, can be inoculated under the epidermis. Unless meticulously removed, the sting remains in the wound and may facilitate introduction of the infection. Further research is needed to determine the relationship between honeybee stings and infection.

We demonstrated *A. nasoniae* transmission to our patient through the sting of the probable sick honeybee. Whether *A. nasoniae* releases endotoxin, like other Gram-negative bacteria, that triggers an intense inflammatory and immune response during infection, is yet to be established. Nevertheless, our patient recovered from this episode without major short-term complication given he was immunocompetent. If this were to occur in an immunosuppressed patient, the outcome may have been more detrimental. This case highlights the intricate interactions in nature, between microbes, vectors and people leading to zoonotic infection. Many other such relationships probably remain to be discovered as part of the One Health movement.

Conflicts of interest and sources of funding. No human genome was sequenced in this study. The authors state that there are no conflicts of interest to disclose. This study was investigators-initiated, supported by NSW Health Pathology and Hawkesbury Institute for the Environment, Western Sydney University, NSW, Australia.

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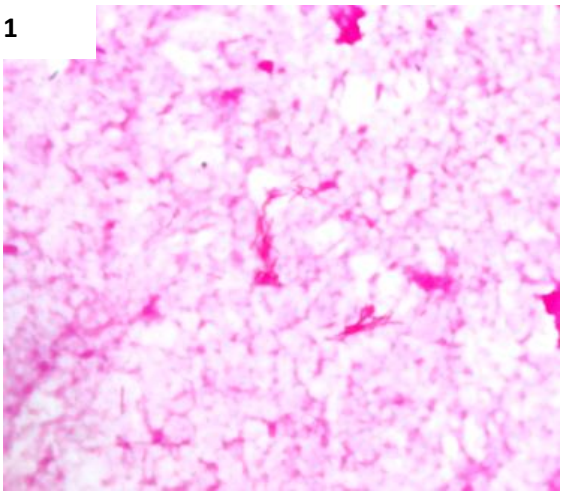
Figure 1. Gram stain of the blood culture showed filamentous Gram-negative bacilli in aerobic bottle (BACTEC™ Plus Aerobic/F). **Figure 2.** Chocolate agar showed mucoid, grey-white, round, and convex colonies with entire edges after 48 hours of incubation.

1



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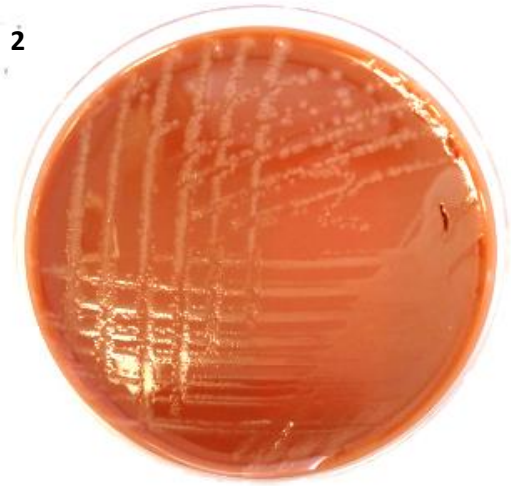

Table 1: Antimicrobial susceptibility of the *Arsenophonus nasoniae* blood culture isolate using VITEK 2 Systems Version: 08.01 and AST-N246 card.

MIC: minimal inhibitory concentration

Antimicrobial agent	**MIC (mg/L)**
Ampicillin	4
Amoxicillin/Clavulanic acid	<=2
Cefazolin	<= 4
Ceftriaxone	<=1
Ceftazidime	<=1
Cefepime	<=1
Meropenem	<=0.25
Amikacin	4
Gentamicin	<= 1
Tobramycin	<= 1
Ciprofloxacin	<= 0.25
Norfloxacin	<= 0.5
Nitrofurantoin	<= 16
Trimethoprim	<= 0.5
Trimethoprim/sulfamethoxazole	<= 20