final report

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Besnoitiosis in Australian wildlife and significance to cattle

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Abstract

An investigation of rare cases of nose-bleeds in Western Grey kangaroos revealed *Besnoitia*-like organisms in their nasal passages. Serological and molecular investigations demonstrated them not to be *Besnoitia besnoiti*, the pathogen of cattle that is re-emerging as a major disease in Europe in recent years.

Executive summary

A previous study of South Australian cattle published in 2012 demonstrated low level antibodies to *Besnoitia besnoiti* in a large proportion of the surveyed cattle. At the same time, Western Grey kangaroos in South and Western Australia had been reported to be suffering, and occasionally dying from nose-bleeds (epistaxis), and the presence of *Besnoitia*-like organisms demonstrated in the nasal flushes of these clinical cases.

Investigations of one property in the Tailem Bend area of South Australia and of a serum bank of a further 299 sera by serological (ELISA and Western Blot) means and with molecular tools (PCR) demonstrated that these *Besnoitia*-like organisms were *not Besnoitia besnoiti.*

Attempts were made to isolate the organisms and a cell culture was established from a macropod animal (pouch-young of a Yellow-footed rock wallaby, grown at 35^{o}C). Unfortunately no more fresh clinical cases have since become available, and isolation efforts have as yet been unsuccessful.

In the meantime, there have been reports from Europe that the initial ELISA used in the first South Australian cattle study has also caused non-specific reactions in European surveys and has since been improved. *B besnoiti*, the cause of Elephant Skin Disease in cattle, has been reported as re-emerging as a major disease in recent years.

Thus, in conclusion: while the exact nature and identity of the *Besnoitia*-like organisms involved in the nose-bleeds of Western Grey kangaroos could not (yet) be accomplished, the evidence suggests it not to be the *Besnoitia besnoiti* of cattle and should therefore not be considered to be a known threat to the Australian cattle industry.

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1. Background

Previous work by the University of Adelaide had reported some evidence of anti-*Besnoitia besnoiti* antibodies in South Australian cattle (Nasir et al. 2012). Although there were no clinical signs of infection at that time reported in Australian cattle, the infection is emerging as a serious threat to European cattle in recent years (EFSA, 2010).

At about the same time, owners of a property near the Tailem Bend area of South Australia had been reporting clinical signs in Western Grey kangaroos (epistaxis) suspicious of *Besnoitia* infection, and provided animals to be examined at the School of Animal and Veterinary Sciences, University of Adelaide at the Roseworthy campus. Nasal tissue samples and serum samples were collected under general anaesthesia. A *Besnoitia*-like organism (BLO) was visualised in swabs from three individuals.

In addition, we were able to sample a further 20 kangaroos located at the property that had previously shown signs of epistaxis (nose-bleeding), obtained nasal swabs for examination (Figures 1-3), were able to visualise some *Besnoitia*-like organisms (BLOs) in those flushes (Figure 4 and 5) and kept blood samples for future serology.

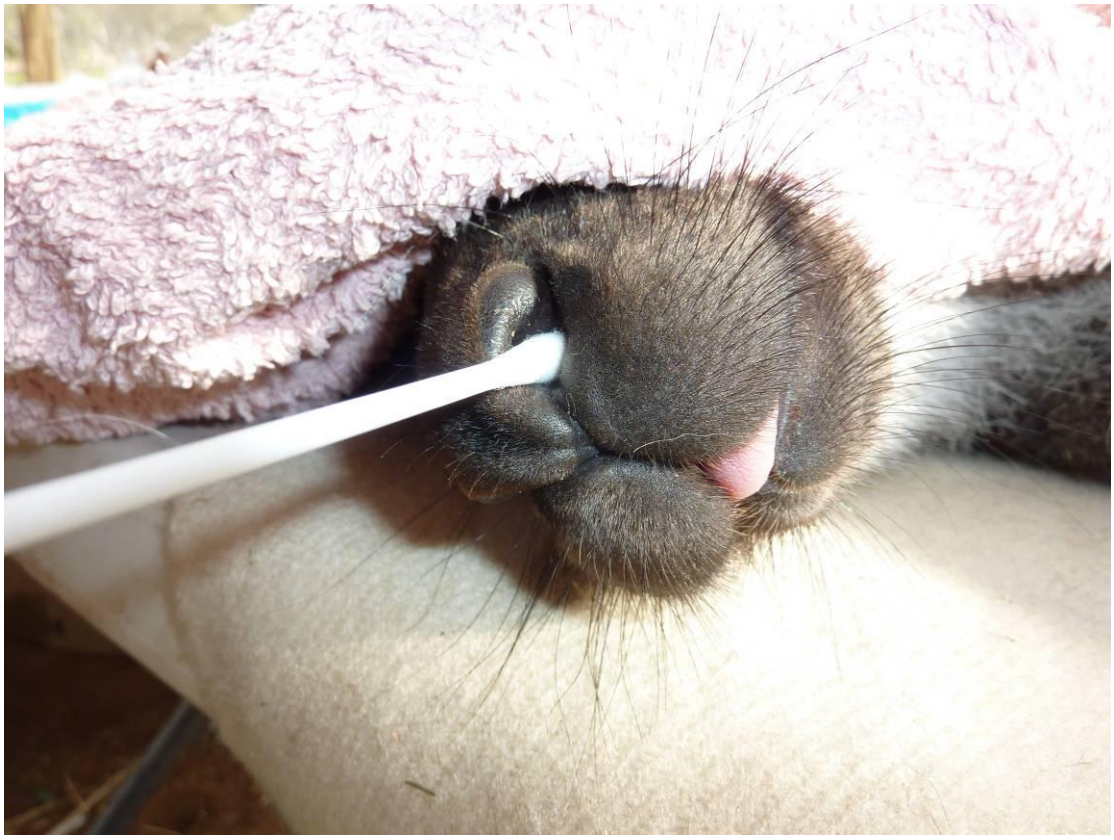

Figure 1: Nasal swabbing of an immobilised kangaroo

Figure 2: Nasal flushing of an immobilised kangaroo

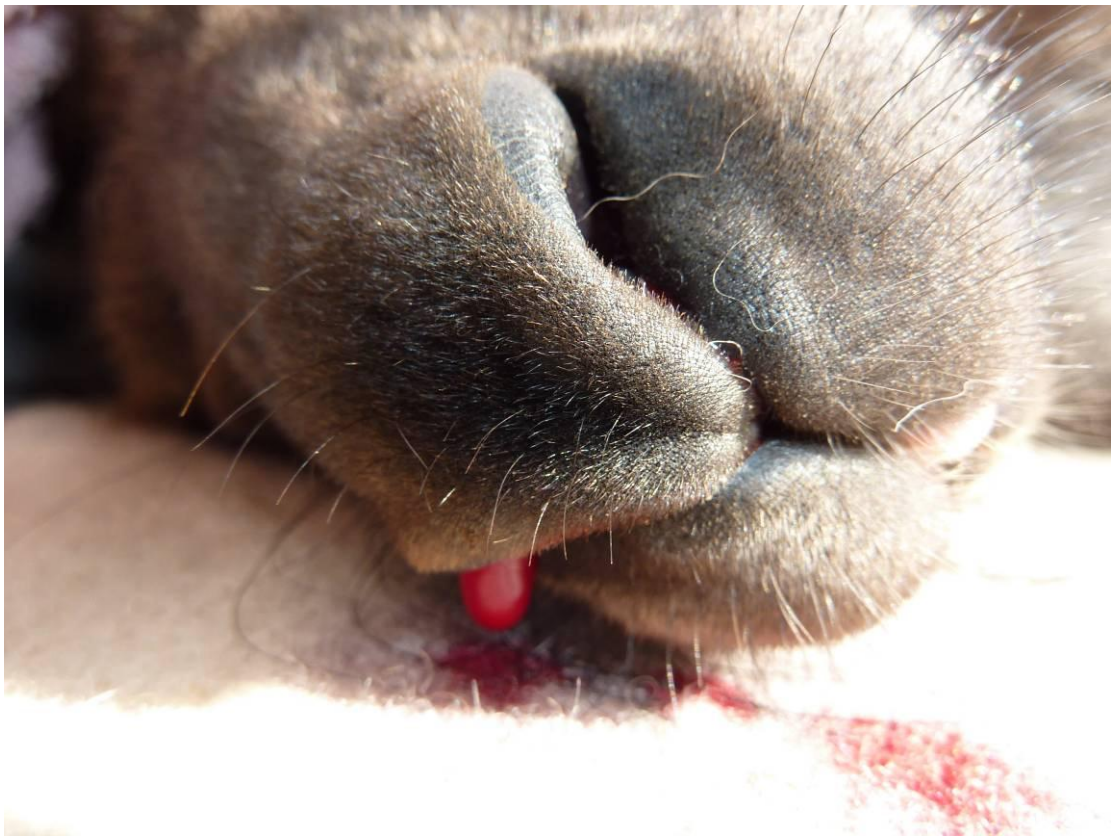

Figure 3: Nose bleed observed after nasal flushing

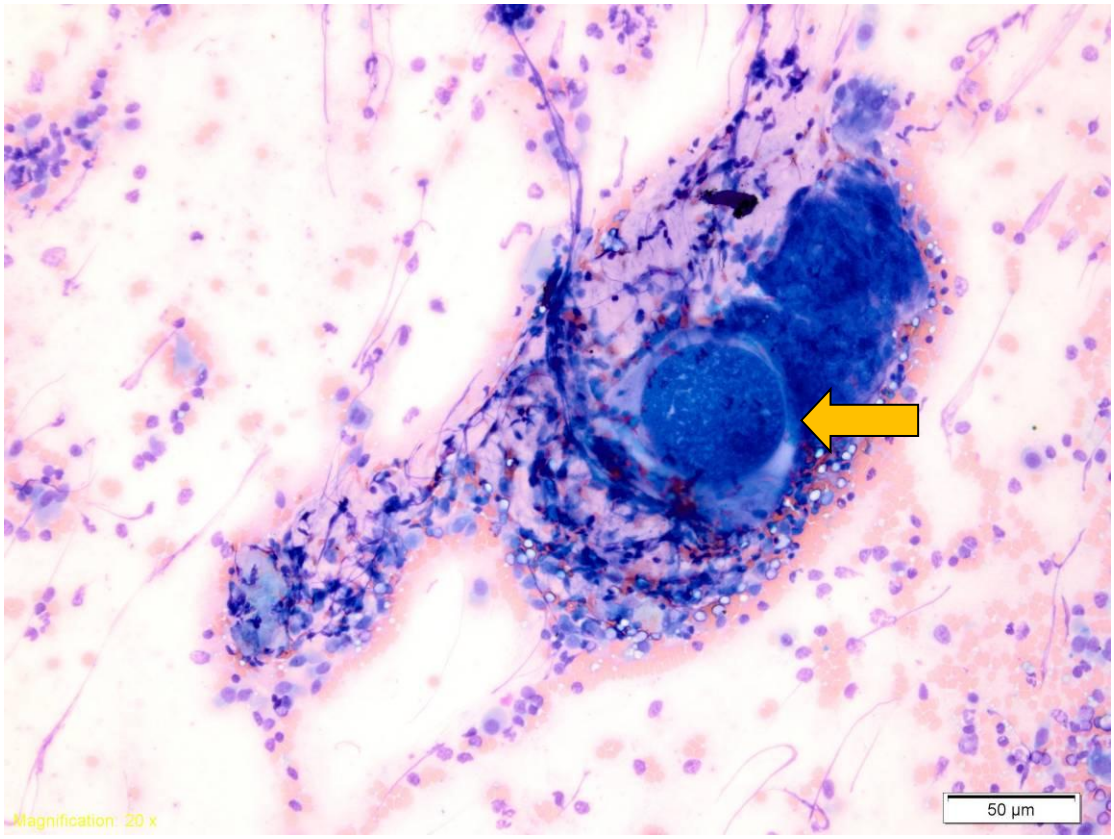

Figure 4: Parasite-containing cell visualised in the nasal swab of a "roo"

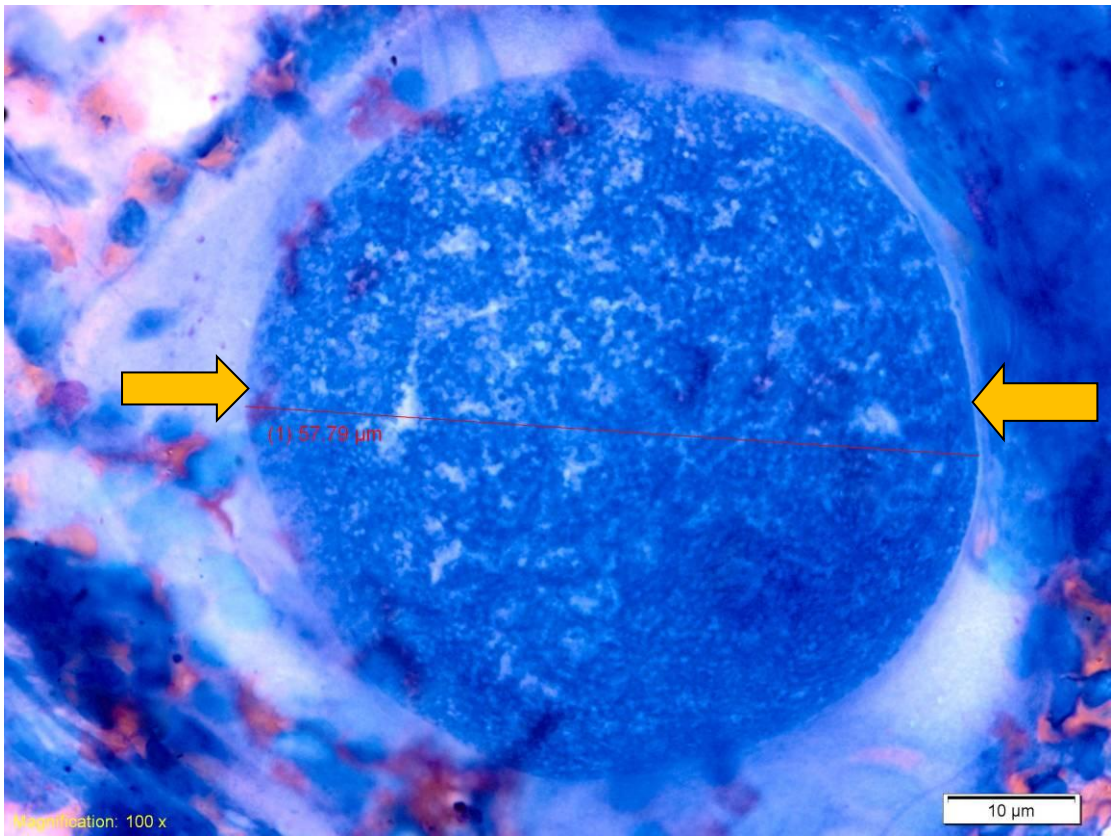

Figure 5: Higher magnification showing hundreds of organisms within a round cyst (between arrows) within cell visualised in the nasal swab of a clinically affected "roo"

2. Serology

Toxoplasma gondii (ToxoReagent, Eiken, Tokyo, latex agglutination assay), *Neospora caninum* (utilising the Neospora Idexx ELISA with an anti-kangaroo conjugate; Bethyl Laboratories, Rabbit anti-Kangaroo Whole Serum Antibody, Catalog No. A140-105) and *Besnoitia besnoiti* ELISA (PrioCHECK® Besnoitia Ab 2.0, Prionics, again with before-mentioned anti-kangaroo conjugate) were assayed by enzyme-linked immunosorbent (ELISA) assay in the 23 sera originating from Tailem Bend.

In addition, *Besnoitia besnoiti* antibodies were also assayed by Western blot (Schares *et al*. 2013) (through collaboration with the Friedrich Loeffler Institute (FLI) for Animal Health, Greifswald, Riems Island, Germany (Dr Gereon Schares), a leading researcher into *Besnoitia besnoiti*), all with negative results (Figure 6) (although three showed weak reactions to *T. gondii* tachyzoites).

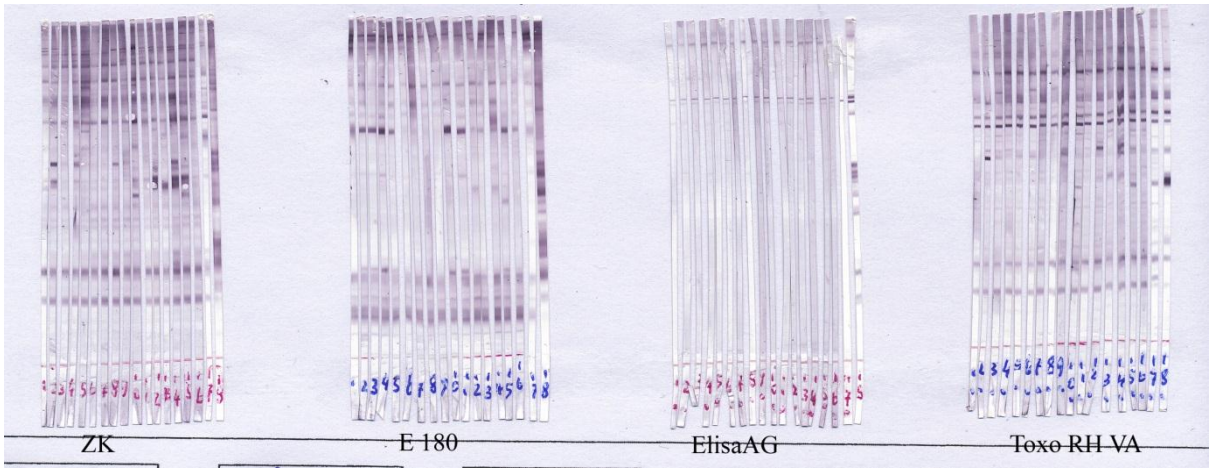

Figure 6: Western Blot of selected "roo" sera against Besnoitia tachyzoites (ZK), *Besnoitia* bradyzoites (E180); *Besnoitia* ELISA-AG; *Toxoplasma* tachyzoites (as supplied by Dr Gereon Schares)

Additional serum samples were assayed from a captive population of 45 individual "malas" (rufous hare-wallaby (*Lagostrophus hirsutus*) at Scotia Sanctuary in the Southwestern plains of New South Wales, adjacent to the South Australian border and 150km south of Broken Hill, all with negative results.

Blood samples from 254 bridled nailtail wallabies *(Onychogalea fraenata)* in Scotia Sanctuary, NSW had been collected in 2008 and since been stored at -80°C and were also available for testing.

Serology results

All Tailem Bend and all sera available from the Scotia sanctuary were negative for *Besnoitia besnoiti* antibodies by PrioCHECK® *Besnoitia* Ab 2.0 ELISA.

PCR Methodology

DNA extraction and Conventional PCR
DNA from the nasal washes of Western Grey kangaroos were extracted using the QIAamp DNA Blood Mini Kit (Qiagen) and stored at -20 C in 60 µL aliquots. Purified

B. besnoiti DNA provided by Dr Gereon Schares, FLI, Riems Island, Germany, was diluted to a working concentration of 9.1 ng/µL and used as a positive control for the conventional PCR. Conventional PCR was performed on extracted samples using the BbRT2 primers from Schares *et. al.* (2011) (F: CAACAAGAGCATCGCCTTC, R: ATTAACCAATCCGTGATAGCAG), which target the *B. besnoiti* internal transcribed spacer 1 (ITS1) partial sequence (GenBank AF076859.1). Primers were synthesised by Geneworks and optimised to 0.5 µM concentration in a final reaction mix volume of 25 µL including 200 µM dNTP (Fisher Biotech) and 0.25 U/25 µL of MyTaq DNA polymerase in 5 x MyTaq reaction buffer (Bioline). Reaction mix containing no sample template was served as a negative control.

Conventional PCRs were run in a BioRad Thermal cycler (T100) with the following reaction conditions: Initial denaturation, 95 C for 5 min followed by 35 cycles of denaturation at 95 C for 0.5 min, annealing at 65 C for 0.5 min and extension 72 C at 0.5 min. A final extension at 72 C for 0.5 min completed the PCR reaction. Gel electrophoresis of GelRed (BioTium) stained amplified PCR products in a 2.5% agarose gel at 100V for 54mins, allowed for the visualisation of positive samples under ultra violet light using the E-Box (Vilber). The correct band length (112 bp) was identified by comparing to an Axygen 100bp marker.

3. PCR Results

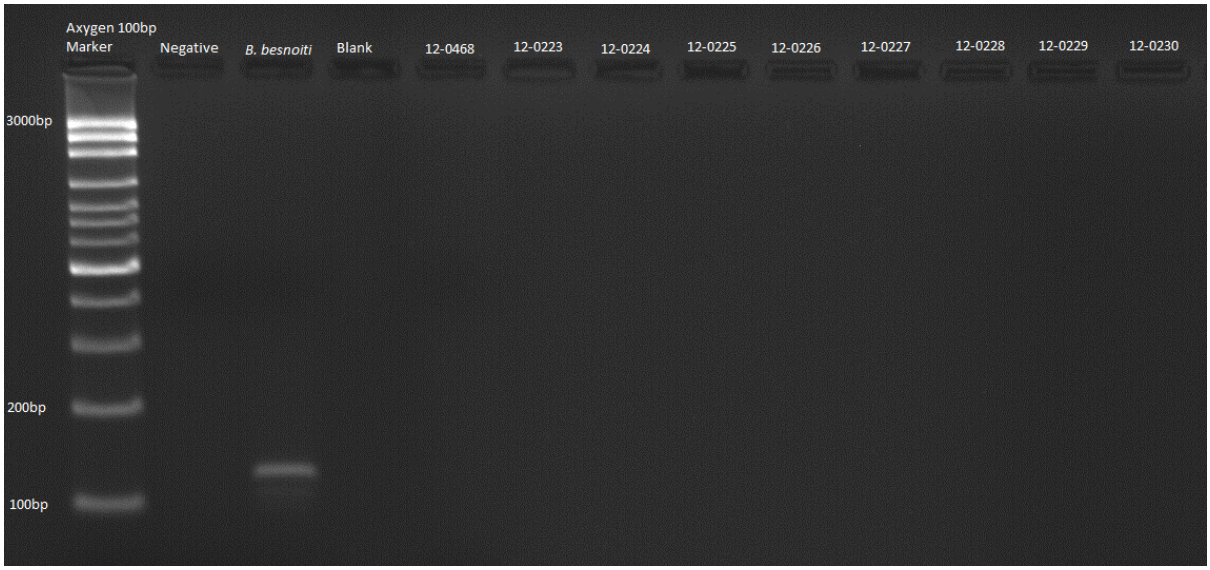

Figure 7: Selected tissue nasal flush extracts tested for *Besnoitia besnoiti*

Cell culture

As the organisms we could visualise did not appear to be *Besnoitia besnoiti* and the tissues and sera tested negative in specific assays, we tried to isolate the organism and to this end established a cell-line (fibroblasts) from the nasal passages of a pouch-young Yellow-footed rock wallaby at 35^{o}C. This may be useful in the future, should further clinical cases be submitted, for the isolation, and subsequent identification of the BLOs.

Unfortunately, no further suspect clinical case material with BLOs has been submitted to the diagnostic laboratory since December 2012 and therefore no further isolation attempt could be made.

4. Discussion and conclusions

B. besnoiti has re-emerged as an important disease of cattle in Europe (EFSA, 2010). A recent study conducted In South Australia (Nasir *et al.* 2012) also suggested the presence of anti-*B. besnoiti* antibodies in South Australian cattle, although the clinical symptoms of benoitiosis have not been observed. Subsequent work by Schares *et al.* (2013), however, indicated concern about the specificity of the earlier version of that enzyme-linked immunosorbent assay (as had also been used in the Nasir study). Also around the same time, there were reports of epistaxis (nose-bleeds) in Western grey kangaroos around Australia, including South Australia, that suggested the presence, in the nasal passage of these marsupials, of *Besnoitia*-like organisms (BLOs). The present study aimed to characterise those organisms further. Three clinical cases (with epistaxis) were closely examined, as well as serological and molecular assays performed.

The histopathological, serological (ELISA and immunoblot) and molecular investigations demonstrated these BLOs not to be *Besnoitia besnoiti*. The study also established a marsupial cell-line that can be used for further isolation attempts from future cases of epistaxis in marsupials. The investigation was unable to isolate the BLOs that were visualised from clinical cases, or to determine the identity of these organisms due to the very small number of clinical cases being available.

It is important to note that although this study demonstrated that the organism is not *B. besnoiti*, the host-parasite relationship with cattle (first discovered through serum antibodies in Australian cattle) still needs clarification.

5. Acknowledgements

The collaboration and participation of Drs Wayne Boardman, Lucy Woolford, Milton McAllister, Charles Caraguel and advice from Prof John T Ellis are gratefully acknowledged, as is the technical assistance of Sarah Manning, Damien Rixon, Alisha Richardson, Julia Boehner and Widi Nugroho and technical expertise provided by Dr Gereon Schares, Greifswald, Germany.

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