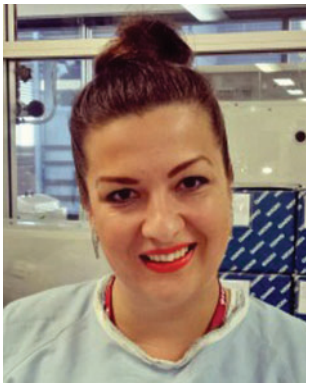
Chlamydia pecorum: successful pathogen of koalas or Australian livestock?



Martina Jelocnik

Centre for Animal Health Innovation (CAHI), Faculty of Science, Health, Education and Engineering
University of the Sunshine Coast
Locked Bag 4, Maroochydore
Qld 4558, Australia
Tel: +41 7 5456 3585,
Email: martina.jelocnik@research.usc.edu.au

Adam Polkinghorne

Centre for Animal Health Innovation (CAHI), Faculty of Science, Health, Education and Engineering
University of the Sunshine Coast
Locked Bag 4, Maroochydore
Qld 4558, Australia

In Australia, the obligate intracellular bacterium *Chlamydia pecorum* is best known as the notorious koala pathogen that causes debilitating ocular and urogenital tract disease. While globally published data suggests that this species is essentially ubiquitous in livestock, little is known about the epidemiology of livestock *C. pecorum* infections here in Australia. My research is focused on investigating the genetic diversity and transmission patterns of *C. pecorum*, and why it causes disease. Using our newly developed *C. pecorum*-specific molecular epidemiology typing scheme we provided the first epidemiological data on infections in sheep and cattle in Australia, identifying strains associated with a range of diseases in livestock, and uncovering an unexpected level of diversity for this pathogen. Most importantly, we observed that the same strain can infect koala and sheep, indicating on ongoing cross-host transmission and 'spill-over' risks to wildlife. Further, by dissecting koala, sheep, cattle and pig *C. pecorum* strains genomes, we have also identified novel virulence-associated factors that could be explored as vaccine candidates for both livestock and koala infections.

Chlamydiae, known for their obligatory parasitic lifestyle and distinct biphasic life-cycle[1], are diverse and enigmatic bacteria that infect an astonishing range of hosts[2]. This phylum includes *Chlamydia pecorum*, a species that constantly remains in the spotlight due to its role as a major threat to the survival of the iconic koalas. In koalas, highly prevalent *C. pecorum* infections cause ocular disease that can eventually lead to blindness, and urogenital disease that can lead to infertility[3,4]. The pathogenic potential of *C. pecorum* in economically significant livestock species (cattle, sheep, goats and pigs) has been well documented in Europe and USA, and to a lesser degree elsewhere including Australia. The spectrum of *C. pecorum* infections in livestock hosts ranges from subclinical to acute disease manifestations such as polyarthritis, sporadic bovine encephalomyelitis, pneumonia and conjunctivitis, with (asymptomatic) gastrointestinal shedding as the common feature[5]. *C. pecorum* infections in free-range ruminants such as Alpine ibex and chamois from Europe have also been reported[6,7].

The curious case of *Chlamydia pecorum* infections in Australia

While the koala chlamydial infections are easily the most intensively studied of any wildlife species, almost nothing is known about the prevalence and epidemiology of *C. pecorum* infections in Australian livestock. Veterinarians from agriculturally productive areas throughout Australia regularly report cases of chlamydiosis in sheep and cattle[8,9], but the information about the genetic diversity of strains infecting livestock is lacking. A range of previous molecular studies suggested that *C. pecorum* strains infecting koala are genetically diverse[10,11]; however, none of the studies investigated how and whether these strains are related to livestock strains. It has been hypothesised that the origin of koala *C. pecorum* infections is associated with the import of 'presumably *C. pecorum* infected' livestock in the late 1780s[3]. The encroachment of koala habitats by sheep and cattle farming along the east coast of Australia is common and raises serious questions over the relationships and potential role that cross-host transmission may have in the epidemiology and origin of chlamydial disease in koalas[3]. Despite the ongoing field reports, the epidemiology,

10.1071/MA17042

transmission, and reservoirs of *C. pecorum* in Australian sheep and cattle remain poorly understood[5,12].

C. pecorum molecular epidemiology

Multi-locus sequence typing (MLST) is a widely used epidemiological tool for strain identification and estimation of intra-species relationships for many pathogens. Even today, with whole genome sequencing readily available, MLST is still often used as the first point to barcode a strain[13]. In an effort to evaluate the overall genetic diversity and relationships between Australian koala, sheep and cattle *C. pecorum* strains, we developed and applied a *C. pecorum*-specific MLST to a range of isolates and *C. pecorum*-positive clinical samples from koalas, Australian sheep and cattle[14–16].

The newly developed MLST proved to be an effective fine-detailed molecular epidemiology tool in characterising novel *C. pecorum* genotypes infecting livestock and koalas, highlighting an unexpected level of diversity for this pathogen. The genetic diversity of *C. pecorum* extends from the individual to the population level with repeated molecular evidence that: a) a single host can harbour two distinct *C. pecorum* strains at different anatomical sites; b) discrete populations can be infected with a mix of genetically diverse strains and c) the same strain can infect two different hosts (e.g. koala and sheep; sheep and cattle)[14] (Figure 1*a*). This observation was also repeated in the recent MLST of *C. pecorum* infecting koalas from French Island in Victoria. In this study, it was also observed that the certain koala strains were more similar to livestock strains than they were to other koala strains[17].

C. pecorum-specific MLST and Bayesian phylogenetic analyses of strains infecting Australian sheep with conjunctivitis, polyarthritis, or with no clinical signs of disease and cattle with sporadic encephalomyelitis revealed important observations of the genetic identity and relationships between *C. pecorum* strains found at different anatomical sites and the range of associated diseases[15,16]. We observed two distinct *C. pecorum* genotypes associated with disease, one denoted sequence type (ST) 23 found in association to sheep with polyarthritis and conjunctivitis and cattle with encephalomyelitis, and the other denoted ST 69 found in association with ovine conjunctivitis as well as previously detected in koala urogenital tract and ocular infections (Figure 1*a*). The majority of *C. pecorum* strains detected at vaginal and rectal sites of clinically healthy as well as diseased animals clustered together in a distinct and separate clade, away from strains associated with disease. *C. pecorum* ST 23 strains are also readily found in association with disease in European and USA livestock hosts, indicating a potential global spread of such clonal 'pathogenic' *C. pecorum*[14,18].

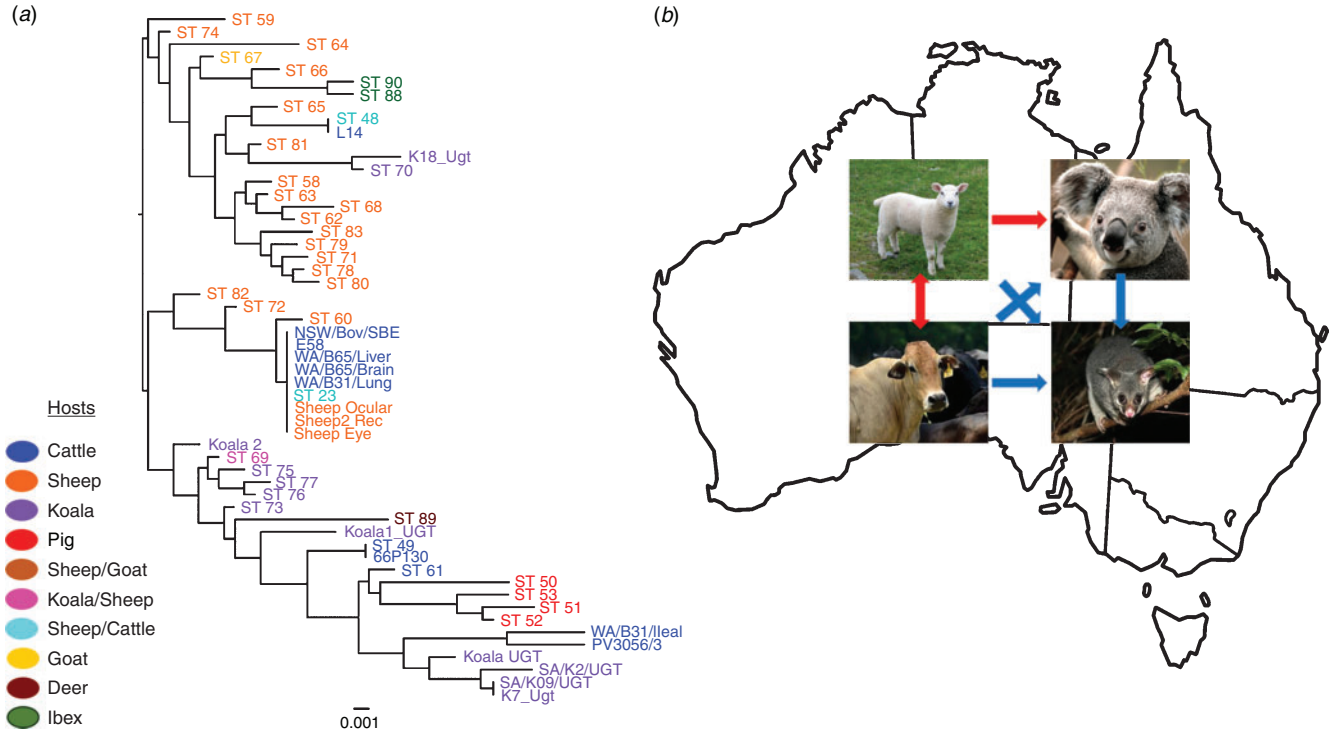


Figure 1. Genetic relationships of *C. pecorum* strains found in various hosts. (*a*) Phylogenetic analyses of the concatenated seven MLST gene fragments sequences from the previously described *C. pecorum* STs available from *Chlamydiales* PubMLST (http://pubmlst.org/chlamydiales/) and newly generated *C. pecorum* STs from our database. The mid-point rooted phylogenetic tree was generated using FastTree, as implemented in Geneious 9.1 software. Hosts are denoted by different colours in the legend. (*b*) Schematic representation of possible and present cross-host transmission of *C. pecorum* infections in livestock, koalas and other wildlife hosts (depicted by a possum). Red arrows indicate the most likely (molecularly evidenced) routes of cross-host transmission (e.g. sheep and cattle, and sheep and koala), while blue arrows indicate likely (hypothesised) routes of cross-host transmission (e.g. cattle and koalas, cattle or sheep and other native wildlife, and between wildlife animals).

Similarly, genetically identical the 'non-pathogenic' vagino-rectal strains characterised in our studies were also detected in the faeces of clinically healthy sheep from Europe (Figure 1*a*).

Chlamydial infections can spread rapidly in a flock/herd, potentially developing into diseases such as polyarthritis, conjunctivitis and encephalomyelitis, leading to increased morbidity and mortality on farms[19]. Detection of the genetically different *C. pecorum* strains at various anatomical sites in a single host and a plausible association of certain genotypes with disease/virulence in our and other studies[18,20] raises questions over the existence of 'pathogenic and non-pathogenic' *C. pecorum* and their role in pathogenesis of disease and impact on an animal's health[12].

The role of cross-host transmission in the epidemiology and origin of *C. pecorum* infections

The repeated evidence that identical or closely related *C. pecorum* strains infect koalas and livestock raises serious questions over the role of contemporary and historic spill over of this pathogen from domesticated animals to koalas[3,21] (Figure 1*b*). Ongoing work in our laboratory also provided evidence of *C. pecorum* infections in other Australian native marsupials *(unpublished observations)*, raising the possibility of cross-host transmission to the native wildlife as well (Figure 1*b*). Although ecologically distinct, livestock and wildlife host species could have an indirect contact allowing an opportunity for infection spill-over, most likely via the faecal-oral route[12,22]. For livestock *C. pecorum* infections, sheep and cattle are strong reservoir candidates as evidenced by ubiquitous faecal shedding of presumably 'non-pathogenic' strains[16,22] (Figure 1*b*). Evidence for cross-host transmission was also observed in our typing study of *C. pecorum* strains detected in European free-range ruminants[7] where, in limited examples, we observed a phylogenetic relatedness of the Alpine ibex *C. pecorum* with sheep faecal strains.

The lessons from ongoing *C. pecorum* genomics

While the MLST provided us with preliminary characterisation of *C. pecorum* strains from various hosts, comparative genomics provides valuable insights into the lifestyle, within-host adaptation, virulence factors and population structure[12,23]. The ongoing

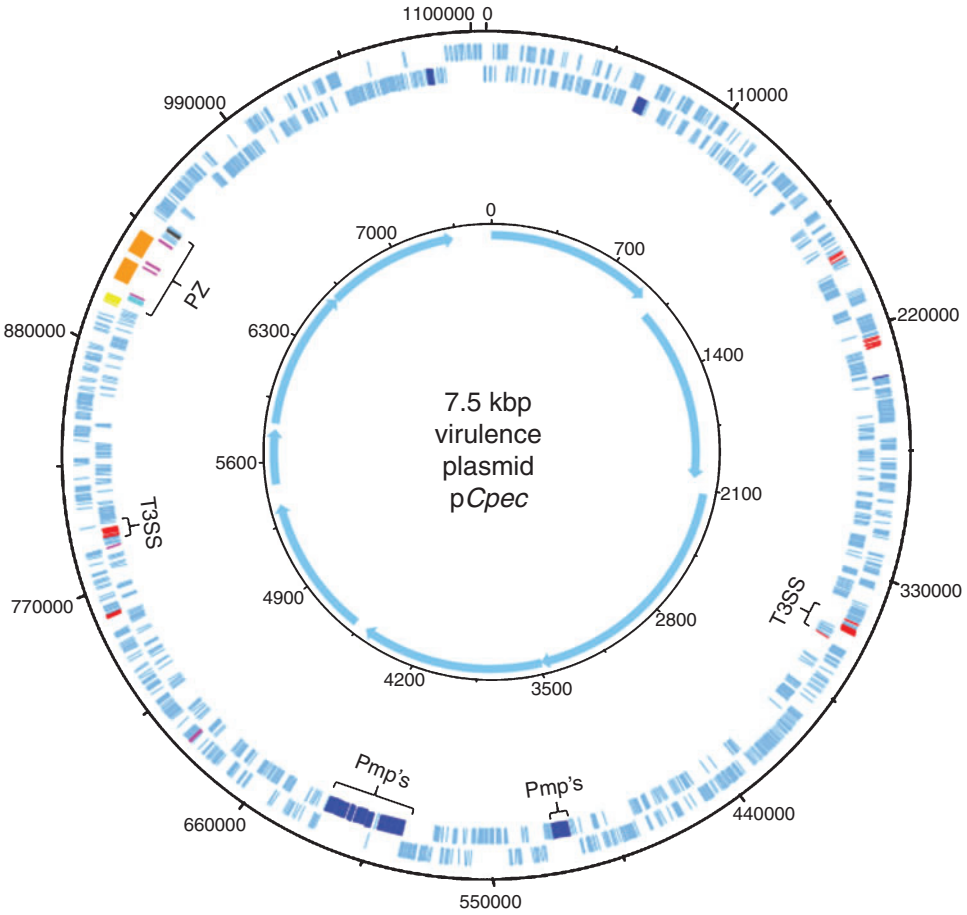


Figure 2. *C. pecorum* genome and plasmid composition. Circular representation of the *C. pecorum* chromosome: First ring denotes CDSs (in light blue) in forward direction, while the second ring denotes CDSs in reverse direction. Genomic location of important genomic elements such as Type III secretion system (T3SS) in red, polymorphic membrane proteins (*pmp*'s) in blue as well as the PZ (with cytotoxin genes in orange, and Phospholipase D genes in pink) are also outlined on the *C. pecorum* genome plot. Inset: Circular representation of the chlamydial plasmid (pCpec), where present in the genome. Image was generated with the DNAplotter.

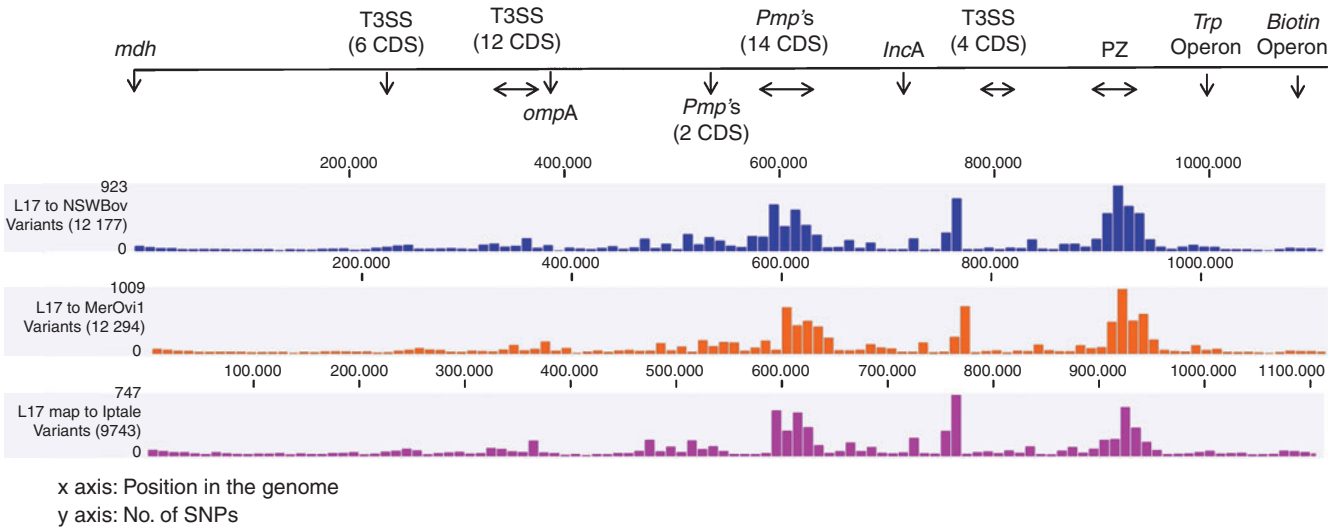


Figure 3. SNPs distribution in *C. pecorum* genomes. Genomic position of T3SS, *pmp*'s, inclusion proteins (*Inc*), major outer membrane protein (*omp*A), PZ, tryptophan (*Trp*) and biotin (*bio*) operons are indicated on a line above genomes, while the number and distribution of SNPs across the pig *C. pecorum* genome in comparisons to cattle (in blue), sheep (in orange) and koala (in pink) genomes are depicted as histograms. SNPs, represented as histograms of a 10 kb size, were determined in CLC Genomics (CLCBio, Qiagen) platform. All genomes start from malate dehydrogenase (*mdh*) gene.

comparative genomics of koala, sheep, cattle and pig *C. pecorum* genomes are building the global population framework of this enigmatic species[20,23–25]. Highly conserved and syntenic, the main genetic differences between *C. pecorum* strains are limited to single nucleotide polymorphisms (SNPs), mainly accumulating in the highly polymorphic membrane protein (*pmp*'s) genes and the plasticity zone that harbours virulence factors, such as cytotoxin, phospholipase D and Mac-Perforin genes[25] (Figures 2 and 3). The unexpected genomic finding in our studies was the identification of a chlamydial virulence plasmid (p*Cpec*) (Figure 2). p*Cpec* seems to be highly distributed in strains, however plasmidless strains are also common, raising questions over its function in disease pathogenesis[26]. Culture-independent genome sequencing of sheep, cattle and koala *C. pecorum* positive clinical swabs demonstrated that not only could multiple strains infect a single host but that a single anatomical site can harbour multiple *C. pecorum* strains[27], somewhat confirming observations from molecular epidemiology but also reiterating complex epidemiology of these infections. *C. pecorum* genome-derived phylogenies highlighted the polyphyletic history of the pathogen itself, and provided important clues about the evolutionary origins of koala strains, giving more confidence to our hypothesis that the origin of *C. pecorum* infections in Australia is associated with importation of domesticated animals into Australia with European colonisation[3,25,27].

Future directions

Drawing on the knowledge acquired in our studies, we have the opportunity to minimise cross-host transmission and improve on the diagnosis and management of these infections. We have recently developed a prototype anti-*C. pecorum* sheep[28] and improved koala vaccine formulation[29] using novel antigens identified in our genomic studies that could be used as a valuable tools to manage and control *C. pecorum* infections in the future. Improvements to diagnostic testing are now possible and laboratory and Point-of-Care assays for use in the lab and field are now also in development.

References

1. Hammerschlag, M.R. (2002) The intracellular life of chlamydiae. *Semin. Pediatr. Infect. Dis.* **13**, 239–248. doi:10.1053/spid.2002.127201
2. Taylor-Brown, A. *et al*. (2015) Twenty years of research into *Chlamydia*-like organisms: a revolution in our understanding of the biology and pathogenicity of members of the phylum *Chlamydiae*. *Pathog. Dis.* **73**, 1–15. doi:10.1093/femspd/ftu009
3. Polkinghorne, A. *et al*. (2013) Recent advances in understanding the biology, epidemiology and control of chlamydial infections in koalas. *Vet. Microbiol.* **165**, 214–223. doi:10.1016/j.vetmic.2013.02.026
4. Griffith, J.E. and Higgins, D.P. (2012) Diagnosis, treatment and outcomes for koala chlamydiosis at a rehabilitation facility (1995–2005). *Aust. Vet. J.* **90**, 457–463. doi:10.1111/j.1751-0813.2012.00963.x
5. Walker, E. *et al*. (2015) *Chlamydia pecorum* infections in sheep and cattle: a common and under-recognised infectious disease with significant impact on animal health. *Vet. J.* **206**, 252–260. doi:10.1016/j.tvjl.2015.09.022
6. Holzwarth, N. *et al*. (2011) Occurrence of *Chlamydiaceae, Mycoplasma Conjunctivae*, and pestiviruses in Alpine chamois (*Rupicapra r. Rupicapra*) of Grisons, Switzerland. *J. Vet. Diagn. Invest.* **23**, 333–337. doi:10.1177/104063871102300223
7. Jelocnik, M. *et al*. (2015) Novel sequence types of *Chlamydia pecorum* infect free-ranging Alpine ibex (*Capra ibex*) and red deer (*Cervus elaphus*) in Switzerland. *J. Wildl. Dis.* **51**, 479–483. doi:10.7589/2014-08-220
8. Watt, B. Chlamydial infection in sheep, Proceedings of the 92nd District Veterinarian's Conference, District Veterinarian's Association, Dubbo, New South Wales, Australia.
9. Pitman, B. (2010) A case of Sporadic Bovine Encephalomyelitis (SBE) in Hume LHPA, Proceedings of the 91st District Veterinarian Conference 2010, Dubbo, New South Wales, Australia.

10. Marsh, J. *et al*. (2011) Novel molecular markers of *Chlamydia pecorum* genetic diversity in the koala (*Phascolarctos cinereus*). *BMC Microbiol.* **11**, 77. doi:10.1186/1471-2180-11-77
11. Kollipara, A. *et al*. (2013) Genetic diversity of *Chlamydia pecorum* strains in wild koala locations across Australia and the implications for a recombinant *C. pecorum* major outer membrane protein based vaccine. *Vet. Microbiol.* **167**, 513–522. doi:10.1016/j.vetmic.2013.08.009
12. Jelocnik, M. (2016) Molecular epidemiology and comparative genomics of *Chlamydia pecorum* infecting livestock and koalas. PhD Thesis, University of the Sunshine Coast, Australia.
13. Maiden, M.C.J. *et al*. (2013) MLST revisited: the gene-by-gene approach to bacterial genomics. *Nat. Rev. Microbiol.* **11**, 728–736. doi:10.1038/nrmicro3093
14. Jelocnik, M. *et al*. (2013) Multi-locus sequence analysis provides insights into the molecular epidemiology of *Chlamydia pecorum* infections in Australian sheep, cattle and koalas. *J. Clin. Microbiol.* **51**, 2625–2632. doi:10.1128/JCM.00992-13
15. Jelocnik, M. *et al*. (2014) Molecular and pathological insights into *Chlamydia pecorum*-associated sporadic bovine encephalomyelitis (SBE) in Western Australia. *BMC Vet. Res.* **10**, 121. doi:10.1186/1746-6148-10-121
16. Jelocnik, M. *et al*. (2014) Evaluation of the relationship between *Chlamydia pecorum* sequence types and disease using a species-specific multi-locus sequence typing scheme (MLST). *Vet. Microbiol.* **174**, 214–222. doi:10.1016/j.vetmic.2014.08.018
17. Legione, A.R. *et al*. (2016) *Chlamydia pecorum* infection in free-ranging koalas (*Phascolarctos cinereus*) on French Island, Victoria, Australia. *J. Wildl. Dis.* **52**, 426–429. doi:10.7589/2015-10-276
18. Mohamad, K.Y. *et al*. (2014) Host adaptation of *Chlamydia pecorum* towards low virulence evident in co-evolution of the *omp*A, *inc*A, and ORF663 Loci. *PLoS One* **9**, e103615. doi:10.1371/journal.pone.0103615
19. Walker, E. *et al*. (2016) Clinical, diagnostic and pathologic features of presumptive cases of *Chlamydia pecorum*-associated arthritis in Australian sheep flocks. *BMC Vet. Res.* **12**, 193. doi:10.1186/s12917-016-0832-3
20. Sait, M. *et al*. (2014) Genome sequencing and comparative analysis of three *Chlamydia pecorum* strains associated with different pathogenic outcomes. *BMC Genomics* **15**, 23. doi:10.1186/1471-2164-15-23
21. Burnard, D. and Polkinghorne, A. (2016) Chlamydial infections in wildlife–conservation threats and/or reservoirs of 'spill-over' infections? *Vet. Microbiol.* **196**, 78–84. doi:10.1016/j.vetmic.2016.10.018
22. Reinhold, P. *et al*. (2011) *Chlamydiaceae* in cattle: commensals, trigger organisms, or pathogens? *Vet. J.* **189**, 257–267. doi:10.1016/j.tvjl.2010.09.003
23. Bachmann, N.L. *et al*. (2014) *Chlamydia* genomics: providing novel insights into chlamydial biology. *Trends Microbiol.* **22**, 464–472. doi:10.1016/j.tim.2014.04.013
24. Bachmann, N.L. *et al*. (2014) Comparative genomics of koala, cattle and sheep strains of *Chlamydia pecorum*. *BMC Genomics* **15**, 667. doi:10.1186/1471-2164-15-667
25. Jelocnik, M. *et al*. (2015) Genetic diversity in the plasticity zone and the presence of the chlamydial plasmid differentiates *Chlamydia pecorum* strains from pigs, sheep, cattle, and koalas. *BMC Genomics* **16**, 893. doi:10.1186/s12864-015-2053-8
26. Jelocnik, M. *et al*. (2016) Molecular characterisation of the *Chlamydia pecorum* plasmid from porcine, ovine, bovine, and koala strains indicates plasmid-strain co-evolution. *PeerJ* **4**, e1661. doi:10.7717/peerj.1661
27. Bachmann, N.L. *et al*. (2015) Culture-independent genome sequencing of clinical samples reveals an unexpected heterogeneity of infections by *Chlamydia pecorum*. *J. Clin. Microbiol.* **53**, 1573–1581. doi:10.1128/JCM.03534-14
28. Desclozeaux, M. *et al*. (2017) Safety and immunogenicity of a prototype anti-*Chlamydia pecorum* recombinant protein vaccine in lambs and pregnant ewes. *Vaccine* **35**, 3461–3465. doi:10.1016/j.vaccine.2017.03.091
29. Desclozeaux, M. *et al*. (2017) Immunization of a wild koala population with a recombinant *Chlamydia pecorum* major outer membrane protein (MOMP) or polymorphic membrane protein (PMP) based vaccine: new insights into immune response, protection and clearance. *PLoS One* **12**, e0178786. doi:10.1371/journal.pone.0178786

Biographies

Dr Martina Jelocnik is an Early Career Research Fellow at the University of the Sunshine Coast's Centre for Animal Health Innovation. In June 2016, she has been awarded PhD in Microbiology from the University of the Sunshine Coast. Her research is focused on investigating the genetic diversity, molecular epidemiology and phylogenomics of chlamydial infections in livestock and wildlife, with particular interest in zoonotic events caused by chlamydial pathogens.

Associate Professor Adam Polkinghorne is a molecular microbiologist and the Director of the University of the Sunshine Coast's Centre for Animal Health Innovation. His work is primarily focussed on understanding the diagnosis, epidemiology and control of chlamydial infections in a variety of wild and domesticated animal species.