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Evaluation of the relationship between *Chlamydia pecorum* sequence types and disease using a species-specific multi-locus sequence typing scheme (MLST)

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Abstract

Chlamydia pecorum is globally associated with several ovine diseases including keratoconjunctivitis and polyarthritis. The exact relationship between the variety of *C. pecorum* strains reported and the diseases described in sheep remains unclear, challenging efforts to accurately diagnose and manage infected flocks.

In the present study, we applied *C. pecorum* Multi Locus Sequence Typing (MLST) to *C. pecorum* positive samples collected from sympatric flocks of Australian sheep presenting with conjunctivitis, conjunctivitis with polyarthritis, or polyarthritis only and with no clinical disease (NCD) in order to elucidate the exact relationships between the infecting strains and the range of diseases. Using Bayesian phylogenetic and cluster analyses on 62 *C. pecorum* positive ocular, vaginal and rectal swab samples from sheep presenting with a range of diseases and in a comparison to *C. pecorum* sequence types (STs) from other hosts, one ST (ST 23) was recognised as a globally distributed strain associated with ovine and bovine diseases such as polyarthritis and encephalomyelitis. A second ST (ST 69) presently only described in Australian animals, was detected in association with ovine as well as koala chlamydial infections. The majority of vaginal and rectal *C. pecorum* STs from animals with NCD and/or anatomical sites with no clinical signs of disease in diseased animals, clustered together in a separate group, by both analyses. Furthermore, eight/13 detected STs were novel.

This study provides a platform for strain selection for further research into the pathogenic potential of *C. pecorum* in animals and highlights targets for potential strain-specific diagnostic test development.

Keywords: *Chlamydia pecorum*, sheep, conjunctivitis, polyarthritis, MLST

1. Introduction

The obligate intracellular bacterial pathogen *Chlamydia pecorum*, is globally associated with several diseases in sheep including keratoconjunctivitis and polyarthritis (Andrews et al., 1987; Page and Cuttlip, 1968; Polkinghorne et al., 2009; Yousef Mohamad and Rodolakis, 2010). Although rare, *C. pecorum* has also been associated with abortions in sheep as well (Rekiki et al., 2004). Regardless of the health status of studied animals, *C. pecorum* is also readily found in the gastrointestinal tract and subsequently shed in faeces (Clarkson and Philips, 1997; St George, 1971).

Chlamydial polyarthritis can appear rapidly and typically affects growing lambs from four to eight months of age. Affected animals present with variable degrees of lameness, stiffness, and occasionally swollen joints. Consequently, some animals may develop debilitating arthritis that interferes with grazing and leads to weight loss and growth retardation (Page and Cuttlip, 1968; Watt, 2011). Chlamydial conjunctivitis (often concurrent with polyarthritis) is also commonly observed and the severity of this disease can range from mild, with clear ocular discharge to severe, with purulent discharge, oedema, hyperaemia, corneal opacity and blindness (Hopkins et al., 1973; Walker, 2013; Watt, 2011). Complicating our understanding of the relationship between *C. pecorum* infection and disease, asymptomatically infected animals can also shed high levels of *C. pecorum* faecally (Lenzko et al., 2011), an observation further confused by the recent suggestions that asymptomatically infected cattle have significant levels of subclinical pathology (Poudel et al., 2012; Reinhold et al., 2011).

Molecular studies of *C. pecorum* strains from livestock (Jelocnik et al., 2013; Kaltenboeck et al., 2009; Yousef Mohamad et al., 2008) and strains associated with devastating infections in the koala (Jackson et al., 1997; Kollipara et al., 2013; Marsh et al., 2011), a native Australian

marsupial whose survival is threatened by chlamydial disease (Polkinghorne et al., 2013), have revealed that this pathogen appears to be genetically diverse. Interestingly, an outcome of these molecular studies was a potential revelation that certain *C. pecorum* strains may be associated with disease outcome (Sait et al., 2014; Yousef Mohamad et al., 2008), an observation that would appear to be reinforced by (a) a limited subset of experimental infection studies that showed the presence of potentially "pathogenic" and "non-pathogenic" strains of this species (Rekiki et al., 2004; Rodolakis et al., 1989) ; and (b) evidence that certain *C. pecorum*-infected populations of koalas and livestock appear unaffected by their infection while other populations develop severe disease (Kaltenboeck et al., 2005; Wan et al., 2011).

We recently developed a *C. pecorum* Multi Locus Sequence Typing (MLST) scheme and showed that this typing scheme appears to be congruent with other typing schemes for this species (Jelocnik et al., 2013). In a preliminary investigation into the genetic diversity and relationships between *C. pecorum* strains infecting Australian livestock and koalas, we made several important observations including that (a) a single host can harbour two distinct *C. pecorum* strains, one usually isolated from the clinically affected site (eyes, joints) and a second from faeces; (b) the same strain can infect two different hosts (eg. koala and sheep); and (c) an association between disease and *C. pecorum* sequence type (ST) is plausible (Jelocnik et al., 2013). The detection of mixed infections at different anatomical sites of the same animal was particularly important in light of the previous investigations of *C. pecorum* strain diversity and pathogenicity, the latter limited to analysis of cultured isolates sampled from a single infected site of the animal (Yousef Mohamad et al., 2008).

In the present study, we have expanded the application of the *C. pecorum*-specific MLST to a comprehensive collection of samples obtained from Australian sheep presenting with conjunctivitis, conjunctivitis with polyarthritis, polyarthritis only and with no clinical signs of

92 disease (NCD) from a sympatric region of Central New South Wales (NSW), Australia in
93 order to more fully elucidate the exact relationships between certain *C. pecorum* strains and
94 the range of diseases described in these animals.

95

96 **2. Materials and methods**

97

98 *2.1 Field examination of sheep flocks with chlamydiosis*

99 In an attempt to further understand the diversity of *C. pecorum* STs implicated in diseases of
100 Australian sheep, we collected a total of 198 swabs from clinically affected sites (e.g. eyes
101 and joints) and from unaffected sites (e.g. rectum, vagina and eyes). 74 lambs were examined
102 in total from five different sheep flocks across Central New South Wales (NSW).
103 Examinations and sample collection were carried out by Local Land Services (LLS) District
104 Veterinarians as a part of veterinary diagnostic investigations. Sheep flocks consisted of
105 animals with symptoms of conjunctivitis and/or arthritis. Some flocks had clinical disease
106 despite previous treatment with antibiotics. In addition, normal sheep were also present in
107 affected flocks (Table 1).

108

109 *2.2 C. pecorum qPCR screen*

110 Briefly, the 198 swab samples were processed by vortexing and centrifugation (Marsh et al.,
111 2011) and DNA was extracted using a QIAmp DNA kit (Qiagen, Doncaster, Australia). *C.*
112 *pecorum* positivity of the samples in duplicate was determined by the *C. pecorum* species-
113 specific qPCR screen, as previously described (Marsh et al., 2011). Samples with < 100

copies *C. pecorum* 16S rDNA were considered negative. Detected *C. pecorum* infectious loads are outlined in Table 1S. For this assay, animals were considered positive if *C. pecorum* DNA was detected at one or more anatomical sites. The sampling of these 198 swab samples has been considered by the Queensland University of Technology (QUT) Animal Ethics Committee and approved under Tissue Use Notification # 1100000362. *C. pecorum* was detected in a total of 151 swab samples from 62 sheep (as determined by the *C. pecorum* qPCR assay) (Table 1 and Table S1). 51 out of these samples were then further utilised for molecular analyses.

Additionally, we also included 14 previously screened *C. pecorum* positive swab samples collected from 17 sheep, also presenting with either signs consistent with chlamydiosis or with NCD from another five flocks from Central NSW (Table 1). For the purpose of this study, these swabs were grouped together as they were collected from individuals or only a limited number of animals from a single flock. Field examination of these sheep and *C. pecorum* screening of these samples was previously described (Jelocnik et al., 2013), with the exception of the For/Ovi4/Eye ocular sample collected from a sheep presenting with conjunctivitis, described here for the first time.

2.3 Statistical analyses

The *C. pecorum* 16S rDNA copy mean, range and standard error of the mean detected in the 151 *C. pecorum* positive ocular, joint, vaginal and rectal sheep swabs (Table S1) were determined using the one way ANOVA, as implemented in the http://epitools.ausvet.com.au/ (Sergeant, 2014). In order to compare whether differences in the means of the detected *C. pecorum* 16S rDNA copies on different anatomical sites of the animals are statistically significant, we used the two tailed 2-sample t-test for summary data, as implemented in

(http://epitools.ausvet.com.au/content.php?page=2-sample-t-test ; Sergeant, 2014), where a p value < 0.05 was considered statistically significant.

2.4 C. pecorum MLST

Out of the 151 *C. pecorum* positive samples, we typed 51 *C. pecorum* positive samples from 26 sheep, presenting with various clinical signs, from geographically sympatric areas of Central NSW. An additional group of 11 *C. pecorum* samples from Australian sheep that were previously typed were also included (Jelocnik et al., 2013; Table 1). The overall sample collection included 29 ocular, two joint, six vaginal and 25 rectal swabs from the 35 sheep selected. These *C. pecorum* positive samples were selected based on criteria that: a) they originate from the same host/different anatomical sites; b) multiple hosts from the same flock were sampled; c) flocks/hosts are from sympatric region; and d) these samples cover a range of disease presentations observed in the affected sheep.

C. pecorum-specific MLST targeting seven *C. pecorum* house-keeping genes (*eno*A, *opp*A_3, gid*A*, *hem*N, *hfl*X, *fum*C and *gat*A) was performed on the 51 new ovine *C. pecorum* positive swabs identified in this study, as previously described (Jelocnik et al., 2013). The abbreviated names of *C. pecorum* strains refer to the geographical location of the sample/name of animal/site of infection (e.g. Narromine/Sheep No./ Ocular = Nar/S22/RE). Allele and ST assignation for the 51 Australian sheep *C. pecorum* MLST data set (Table S2) were determined at http://pubmlst.org/chlamydiales/ (Jolley and Maiden, 2010). The house-keeping gene sequences from the new 51 Australian ovine *C. pecorum* strains described here for the first time are available in Genbank (KJ145423-KJ145723 and KJ584475-KJ584530). Unfortunately, MLST could not be applied to many samples due to insufficient levels of *C. pecorum* DNA (Table 1, Table S1).

2.5 C. pecorum sequence, phylogenetic and cluster analyses

Individual house-keeping gene fragments, as well as the concatenated gene fragment sequences, were analysed using Geneious Pro 7.0 software package (Biomatters, 2013), as previously described (Jelocnik et al., 2013). The level of individual and concatenated sequence polymorphisms were determined by calculating the number of synonymous (d_s) and non-synonymous (d_n) substitutions per site, with Jukes-Cantor correction. The number of polymorphic (segregating) sites (Δnt), haplotypes and haplotype diversity (H) was analysed using DnaSP 5.0 (Librado and Rozas, 2009) (Table S3).

To construct the optimal phylogenetic tree for our seven house-keeping gene fragments concatenated data sets, best fit nucleotide substitution models were estimated using jModelTest v.2.1.1 (Darriba et al., 2012). A mid-point rooted Bayesian phylogenetic tree for the concatenated data set, composed of 62 Australian sheep *C. pecorum* STs was generated with the GTR+I+G model, predicted by the Akaike information criterion (AIC) and Bayesian information criterion (BIC) in jModelTest, using MrBayes (Huelsenbeck and Ronquist, 2001) (Figure 1). House-keeping gene sequences of the polyarthritis *C. pecorum* isolate IPA (Page and Cuttlip, 1968) were used as an out-group in the phylogenetic analyses due to its sequence dissimilarity to the Australian ovine *C. pecorum* population sequences (Table S2). Run parameters for this tree included four Markov Chain Monte Carlo (MCMC) chains with a million generations, sampled every 100 generations and the first 10,000 trees discarded as burn-in.

Neighbour-Joining (NJ) phylogenetic tree constructed from the concatenated *C. pecorum* house-keeping gene sequences of a subset of 29 paired strains (eye, vaginal and/or rectal)

from 12 animals from the present study was generated with the HKY model, with 1000 bootstrap repetitions (Figure S1).

To alternatively assess the patterns of evolutionary descendancy of *C. pecorum* STs used in this study, the goeBURST algorithm for MLST data, available from http://goeburst.phyloviz.net/ (Francisco et al., 2009), was used. Briefly, *C. pecorum* MLST profiles were divided into clonal complexes (CCs) under a user-defined threshold level of identity. In this study, we used a stringent single locus variant (SLV) level; where CCs were defined as groups of STs, which share six out of seven loci (alleles) with at least one other ST in the group, while singletons were defined as STs that differ by at least two loci (alleles) from all other STs (Figure 2). A "putative progenitor" in CCs is a ST that has the most SLV links to other STs. In larger CCs, a ST (besides the predicted primary progenitor) that has a number of SLV links of their own is considered a "putative progenitor" of a subgroup.

3. Results

3.1 Selection of C. pecorum positive sheep for MLST investigations

On-farm field examinations of affected sheep within Central NSW demonstrated different manifestations and severities of clinical disease including: (a) mild to severe conjunctivitis (n=20 sheep) - Clinical signs of conjunctivitis included epiphora, ocular discharge ranging from serous to purulent, conjunctival oedema, crusting and photosensitivity ; (b) mild to severe polyarthritis - Clinical signs of arthritis included lameness, stiffness, swollen joints, and elevated body temperatures (n=23 sheep) ; and (c) conjunctivitis with polyarthritis (n=17 sheep). At the time of sampling, a number of animals with conjunctivitis had been previously treated with antibiotics (n=24). Despite the treatment, some of these animals continued to

express mild degrees of conjunctivitis (e.g. crusting and serous ocular discharge) and others resolved with treatment (Table 1).

C. pecorum shedding at each anatomical site in assessed sheep is outlined in Table S1. Overall, the highest *C. pecorum* positivity rates of 73.8% and 72.2% were detected in the eyes (n= 65/88) and rectum (n= 57/79), respectively of sheep, both with and without chlamydiosis (Table 1 and Table S1). The *C. pecorum* infectious loads found in the eyes (av.1.8 $x10^3$) were significantly higher than those found in the vagina (p=0.004) and rectum (p=0.006). The lowest PCR positivity rates were found in the vaginal swabs (27/46; 58.7%) of sheep, with the shedding observed to be significantly lower than that found at rectal sites (p=0.001) (Table S1). Notably, seven animals with NCD were found to be shedding *C. pecorum* from the rectum.

To gain a snapshot of the *C. pecorum* strain genetic diversity associated with diseases in sheep sampled in this study, we applied our previously described *C. pecorum* MLST to a subset of 51 *C. pecorum* positive swabs from 26 sheep from the four affected properties (Nar, Cam, Hey and Cur) (Table 1). An additional set of 11 *C. pecorum* previously analysed STs detected in nine sheep from five different flocks: For, Nyn, Eug, Mer and Dub (Jelocnik et al., 2013) was also included in this analysis (Table 1).

3.2 Sequence analyses of C. pecorum STs used in this study

The level of polymorphisms in individual, as well as concatenated house-keeping gene sequences of the 62 sheep *C. pecorum* STs was limited, with four loci being informative. *gid*A was the most polymorphic locus in this sequence set, with six allelic variants and the highest haplotype diversity of 0.709 (Table S3). Overall, we observed 13 distinct

haplotypes/sequence types with distinct allelic profiles, indicating a high haplotype diversity of 0.788. Eight of the STs identified in this study were not previously described and following submission to the PubMLST database were assigned STs 62, 63, 78, 79, 80, 81, 82 and 83, respectively (Table S2). The remaining STs detected (23, 69, 71, 72 and 74) were previously described in Australian sheep and cattle (Jelocnik et al., 2013), as observed in the PubMLST database.

3.3 A range of C. pecorum *STs are associated with diseases of sheep*

Upon typing our 62 *C. pecorum* positive samples, an analysis of the samples collected from the diseased or affected site (ocular or joint) compared to the ones from clinically unaffected sites revealed a number of interesting observations about the relationship between different *C. pecorum* STs and different disease manifestations (Table 2).

Examining the samples collected from the eyes and joints of sheep with different disease presentations, we observed five distinct *C. pecorum* STs (ST 23, 69, 72, 74 and 82) shed from the eyes of 11 sheep with conjunctivitis (Table 2). Two of these STs (ST 23, ST 69) were also found in the eyes of eight sheep with conjunctivitis and polyarthritis at ocular sites, with ST 23 being detected more frequently than ST 69 in 6/7 (86%) ocular swabs, the latter detected only in a single case of conjunctivitis with polyarthritis. No other ST (e.g. 72, 74, and 82) was found in this second cohort of animals. In 12 animals with polyarthritis alone, ST 23 was the only ST detected at 10 ocular and two joint sites. Notably, the latter ST (23) was also detected in the eyes of a single sheep with NCD that was *C. pecorum* PCR positive (Table 2). Of the 31 *C. pecorum* swabs screened from the eyes and joints of sheep from each disease cohort (conjunctivitis, conjunctivitis with polyarthritis and polyarthritis alone), ST 23 was the

most common (21/31; 68%), followed by ST 69 (7/31; 22.5%), ST 72, 74 and 82 (all three detected 1/31, 3%) (Table 2).

In the same animals, *C. pecorum*-specific MLST revealed considerable genetic variation in *C. pecorum* strains being shed from rectal and vaginal sites of animals with and without chlamydial disease (Table 2). A total of 11 distinct STs were determined across all four disease cohorts, with STs 23 (5/31; 16%), 63 (5/31; 16%) and 78 (5/31; 16%) being the most common amongst those detected. The other STs were much less common (Table 2). Three of these STs (23, 69, 74) were also found at ocular and/or joint sites. The remaining STs were exclusively found in the gastrointestinal and genital tract samples of animals from all cohorts (Table 2). Overall, ST 23 was the most common ST found in 23/62 (42%) of all typed swabs , followed by the ST 69 (9/62, 14.5%) and ST 74 (6/62, 9.7%).

3.4 Phylogenetic analyses of C. pecorum *strains associated with various diseases in sheep*

In order to evaluate the phylogenetic relationships of the *C. pecorum* STs detected, a Bayesian phylogenetic tree was constructed using their concatenated house-keeping gene fragments sequences (Figure 1).

The mid-point rooted phylogenetic tree resolved the 62 ovine *C. pecorum* STs into six well supported clades. A clade, consisting of 26 Australian sheep *C. pecorum* sequences, all denoted with ST 23, except For/Ovi5/Eye, was resolved. These STs could be found at the clinically affected site of the infected animal (eyes and/or joints) and associated with diseases such as conjunctivitis with polyarthritis, polyarthritis only and conjunctivitis; as well as shed from hosts with no evidence of clinical disease (Figure 1).

The next distinct clade was comprised of *C. pecorum* STs, denoted with ST 69 and ST 82, found at ocular and/or rectal sites from sheep with conjunctivitis only, with the exception of two *C. pecorum* genotypes Nar/S44/LE and Rec, which were detected in a single case of conjunctivitis with polyarthritis (Figure 1).

In a third phylogenetic clade, we observed five rectal *C. pecorum* STs from various diseases, and one ocular ST (Nyn/Ovi4/Eye), found in case of conjunctivitis (Figure 1). The identical sequences were represented by ST 74.

The remaining clades consisted of a diverse mix of *C. pecorum* STs (black colour coded) shed from the rectum and/or vagina of sheep with chlamydial disease and/or NCD. Genetic diversity was the highest in this clade, with 19 *C. pecorum* strains represented by eight distinct STs (Figure 1).

C. pecorum phylogeny of the 62 *C. pecorum*-positive samples hinted at an otherwise complex on-farm epidemiology, including the observation that multiple *C. pecorum* STs could be observed (a) circulating in the same flock (e.g. Flock Nar: STs 23, 63, 69, 74, 81 and 82); and (b) in a single host at different anatomical sites, utilising paired strains from our study, as observed in Figure S1 (e.g. Cam/S192/Eye: ST 23 and Cam/S192/Rec: ST 79; Nar/S22/RE: ST 69, Nar/S22/Vag: ST 23 and Nar/S22/Rec: ST; Hey/S130/Eye: ST 23 while Hey/ S130/Vag and Rec: ST 78). Furthermore, we also observed that the same ST can be found at two different sites in a single host (e.g. Cur/L236/LE and Vag both ST 23; Nar/S43/RE and Rec both ST 69) (Figure 1, Figure S1; Table S2).

3.5 Relationship of Australian sheep C. pecorum *STs with STs found in other hosts*

With the identification of 13 distinct STs from 35 animals in the present study, we also wanted to assess the relationships of these STs to those described in other hosts that can be infected with *C. pecorum* (Figure 2A). Our identified sheep STs were compared to the STs of *C. pecorum* strains previously reported in the koala as well as other ovine and bovine hosts from Australia. The latter sequences were made available from the *Chlamydiales* PubMLST database. While the majority of sheep *C. pecorum* STs described in this study are unique to Australian sheep (STs 62, 63, 71, 72, 74, 78, 79, 80, 81, 82 and 83), this analysis also revealed several examples of *C. pecorum* STs that (a) were shared between Australian sheep and koalas (ST 69); and (b) a widely distributed *C. pecorum* ST (23) associated with infections in both sheep and cattle in Australia as well as globally.

The goeBurst algorithm (24) was used to explore alternative descendancy relationships between koalas, ovine and bovine *C. pecorum* STs described in this study and previously reported. Based on a stringent fit where STs must share 6/7 alleles, the 18 *C. pecorum* STs clustered into 2 clonal complexes (CCs), with one singleton STs (Figure 2B). CC1 included STs 23 (highlighted in turquoise colour as a putative founder in Figure 2B), 72 and 82, all associated with chlamydial diseases of cattle and/or sheep and ST 69, associated with chlamydial disease in Australian sheep and koalas (highlighted in bright green in Figure 2B), as the predicted putative progenitor of the remaining koala disease-associated STs. Koala ST 70 was defined as a singleton. With the exception of ST 74, *C. pecorum* STs detected in association with ovine conjunctivitis, CC2 was dominated by *C. pecorum* STs detected in the vagina and rectum of sheep with NCD and/or with other chlamydial disease syndromes (Figure 2B). In CC2, faecal ST 63 (highlighted in bright green) was the predicted main putative progenitor, while the rectal/vaginal ST 78 (highlighted in olive green) was the putative progenitor of a sub-group which included STs 71, 79, 80 and 83.

4. Discussion

Despite the growing global recognition of *C. pecorum* infections as a problem in livestock, much still needs to be learnt about the genetic diversity and relationships between the diverse range of strains and the *C. pecorum* disease manifestations reported. In our attempt to elucidate which *C. pecorum* strains may be associated with chlamydial diseases in sheep, we have performed Bayesian phylogenetic and MLST cluster analyses on 62 *C. pecorum* positive swab samples taken from sheep presenting with a range of chlamydial diseases. Additionally, we have also compared the genetic networks of Australian sheep *C. pecorum* strains to those reported in koalas and sheep and cattle *C. pecorum* strains reported in the rest of the world. In doing so, we observed two distinct *C. pecorum* ST associated with disease, one represented by ST 23 found in association to sheep with polyarthritis and conjunctivitis and cattle with encephalomyelitis, and the other, represented by ST 69 and presently only described in Australian animals in association with ovine conjunctivitis as well as koala urogenital tract and ocular infections. The majority of vaginal and rectal *C. pecorum* STs from animals with NCD and/or clinically non-affected anatomical sites in diseased animals, mostly clustered together in a separate group, away from STs associated with disease by both analyses.

Cases of conjunctivitis investigated in the current study, included individual animals from the same or different flocks presenting with significant levels of ocular pathology (Table 1). Typing of the *C. pecorum* positive conjunctival, rectal and/or vaginal swabs from sheep with active conjunctivitis revealed five distinct *C. pecorum* STs in the eyes of sheep with conjunctivitis, two of which were also found in the vagina and/or rectum of infected animals in paired samples (ST 23 in Nar/S24/LE and Vag; and ST 69 in Nar/S43/RE and Rec). The most common of these was ST 69, a *C. pecorum* ST also isolated from koalas, followed by ST 23, a globally distributed ST profile associated with ovine and bovine diseases (Table

2A). The remaining *C. pecorum* STs implicated in conjunctivitis in sheep have not been described outside of Australia yet. Unfortunately, due to low infectious loads detected in cases of treated conjunctivitis, we could not type these samples; however it remains of interest to investigate the ST profile of the shed pathogen in order to compare with the other observations for sheep with conjunctivitis.

We also assessed a total of 20 sheep presenting with *C. pecorum* associated conjunctivitis with polyarthritis and polyarthritis alone. In these animals, *C. pecorum* ST 23 was the dominant ST detected in the *C. pecorum* positive ocular samples in contrast to the diversity identified in the conjunctivitis cases alone. We also found this same ST in the joints of the two sheep for which we were able to sample and detect *C. pecorum*. One exception to the detection of ST23 was *C. pecorum* ST 69, shed from the eyes and rectum of a single animal with conjunctivitis and polyarthritis. As no other conjunctivitis-associated STs were detected in the polyarthritis cohorts, the results of our *C. pecorum* MLST on this limited pool of samples from 10 properties would suggest that ST 23 appears to be the most common *C. pecorum* strain associated with arthritis-related diseases in Australian sheep, albeit more joint samples are needed from diseased sheep to confirm this. Interestingly, the observation that one chlamydial strain appears to be associated with joint and ocular pathology mirrors the recent observation that in *Chlamydia trachomatis*-associated arthritis cases are associated with ocular but not genital tract serovars (Gerard et al., 2010). The genetic determinants in these strains that may influence tropism for the joints and/or conjunctival mucosal membranes remain unclear for both *C. pecorum* and *C. trachomatis*.

Molecular typing of rectal *C. pecorum* positive samples revealed a rich diversity of *C. pecorum* STs. With the exception of four cases where the same *C. pecorum* ST was shed from multiple sites of a diseased host (Nar/S43, Nar/S24, Nar/S44 and Cur/L236), most of the STs observed were uniquely found at vaginal and/or rectal sites. This observation raises

an interesting question about the transmission of the *C. pecorum* STs we found most commonly associated with infection in the eyes and joints of sheep with conjunctivitis and/or arthritis (e.g. ST 23 and ST 69). Faecal-oral transmission is currently considered to be the most likely route for transmission of *C. pecorum* in ruminants. If this is the case, why we did not detect these former *C. pecorum* STs in the gastrointestinal tract of the affected animals in place of these genetically diverse alternate strains? This is an interesting question that requires further investigation but one may speculate that the *C. pecorum* ST associated with disease may have been shed from these seemingly unaffected sites, but in such low infectious loads that our molecular typing only sampled the most predominant strain infecting the gastrointestinal tract. It also remains possible that the "harmless" rectal *C. pecorum* STs could spread via the circulatory system to other tissues as has been predicted in other chlamydial infection models (Carter et al., 2012; Rank and Yeruva, 2014).

Other factors will almost certainly influence the outcome of infection by these genetically distinct *C. pecorum* STs and should not be discounted. Examples of factors that need further study include the role of constant re-exposure to the pathogen, but also environmental and farm management factors associated with each flock (Entrican et al., 2012; Lenzko et al., 2011; Longbottom et al., 2013). The role of innate and adaptive immune responses will also play a role, a particularly important consideration in highly susceptible neonatal lambs which depend on maternal passive immunity (Balamurugan et al., 2012; Buchanan et al., 2013).

In the absence of experimental studies to confirm the pathogenic potential of strains described in this study, the molecular analyses described here nevertheless provide evidence for the existence of *C. pecorum* strains with different pathogenic potentials among Australian sheep. While our sampling and analysis was more comprehensive than that previously performed (Yousef Mohamad et al., 2008), these results would appear to support this earlier study despite the use of an alternative Multi Virulence Locus Sequence Typing (MVLST)

scheme. In both studies, rectal *C. pecorum* strains of diseased sheep from the present study, as well as those faecal livestock isolates from Europe and the USA (Yousef Mohamad et al., 2008), clustered away from samples collected at disease-affected sites, highlighting the robustness of this analysis and the global implications for the work.

The detection of *C. pecorum* ST69 in association with infection and disease of Australian koalas and sheep is of significant local interest as well. Our previous investigation identified *C. pecorum* ST 69 in koalas with chlamydiosis and in limited number of sheep with conjunctivitis (Jelocnik et al., 2013). Using an expanded set of samples for analysis, in the current study, ST 69 was predicted as the putative progenitor of the koala *C. pecorum* strains by the cluster analysis (Figure 2B). The detection of ST 69 in seven sheep presenting with conjunctivitis and conjunctivitis with polyarthritis from three geographically separated flocks suggests that this *C. pecorum* ST is widely distributed in this endemic region of NSW, a region that also overlaps with the koala's range and contains a number of koala populations that are currently threatened by *C. pecorum* infection (Devereaux et al., 2003; Jelocnik et al., 2013; Polkinghorne et al., 2013). Nevertheless, these findings reconfirm the possibility of ongoing cross-host transmission between Australian sheep and koalas, an important epidemiological observation that requires further urgent investigation including sympatric sampling of *Chlamydia*-infected populations of koalas adjacent to or in contact with *Chlamydia*-infected sheep and cattle.

Beyond the Australian setting, the results of this work provide the basis for significant advances, not only in our understanding of *C. pecorum* disease pathogenesis, but in the diagnosis of clinically relevant *C. pecorum* infections. Although the strains analysed in this study are mainly limited to one sympatric region of Australia, the ability to discriminate between *C. pecorum* strains associated with polyarthritis and conjunctivitis versus gastrointestinal tract colonisation provides the basis for improved diagnostics that may

simultaneously allow detection and intra-species discrimination. The fact that we could perform this work on dry swabs is also promising; particularly since the most clinically relevant *C. pecorum* STs could be detected in conjunctival swabs from sheep with conjunctivitis and/or polyarthritis. Alongside our ongoing efforts to further validate these molecular typing tools, a major focus of future research will be to perform detailed genomic comparisons of *C. pecorum* strains classified into these genetically distinct *C. pecorum* STs to begin to identify the genetic determinants that may be associated with tissue tropism and virulence in this animal pathogen. The genomics era has just begun for this pathogen (Sait et al., 2014) and will be further enhanced by recent advances in culture-independent genome sequencing of chlamydiae to address these key clinical questions (Putman et al., 2013).

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Figure 1. Bayesian phylogenetic analysis of concatenated sequences of seven housekeeping gene fragments of 62 Australian ovine *C. pecorum* genotypes found in eyes, joints, vagina and rectum in association with various clinical presentations. Posterior probabilities > 0.75 are displayed on tree nodes. Ovine IPA isolate was included as an out-group. Host diseases are indicated by the colouring on the legend. STs in a clade are indicated by brackets.

Figure 2. Distribution and relationships of *C. pecorum* STs detected in different animal hosts described in this and previous studies, including (i) a Venn diagram of *C. pecorum* STs from Australian koalas, cattle and sheep (Panel A) and; (ii) Cluster analyses of *C. pecorum* STs from Australian koalas, cattle and sheep (Panel B). STs highlighted in bright green and turquoise (ST23, ST63 and ST69) represent putative progenitors in a CC, while STs highlighted in olive green (ST78) represent the progenitor of a sub-group.

- Molecular typing reveals a link between *Chlamydia pecorum* strains and disease
- ST23 ‘pathotype’ was found in association with diseases in sheep and cattle
- A second ‘pathotype’ was described only in Australian sheep but also in koalas
- Rectal and vaginal strains clustered into a separate ‘non-pathotypic’ group

Table 1. *C. pecorum* screening, disease presentations in a flock and *C. pecorum* positive samples analysed by MLST from the sheep flocks tested.

Flock	Clinical presentation	*C. pecorum* positive sheep/flock[6]	No. of *C. pecorum* positive samples analysed by MLST[7]			
			Eye	Vagina	Rectum	Joints
Nar (N=13)	**Conjunctivitis**[1]	9/11	5/11	3/5	5/10	NA[8]
	Conjunctivitis[3] **with polyarthritis**	2/2	2/2	NA	2/2	NA
Cam (N=10)	**Conjunctivitis**[3] **with polyarthritis**	8/8	4/8	0/5	4/8	NA
	Polyarthritis[2] **only**	2/2	1/2	NA	0/2	NA
Hey (N=6)	**Polyarthritis**[2] **only**	6/6	5/6	2/5	5/6	NA
Cur (N=21)	**Conjunctivitis**[3] **with polyarthritis**	9/9	0/9	0/1	1/3	NA
	Polyarthritis[2] **only**	7/7	4/6	1/1	2/6	0/0
	NCD[4]	5/5	1/1	NA	3/5	NA
Hol (N=24)	**Treated conjunctivitis**[5]	14/24	0/13	0/10	0/10	NA
Previously screened flocks: For, Nyn, Eug, Mer and Dub (N=17)	**Conjunctivitis**[1]	6/7	6/6	NA	1/1	NA
	Conjunctivitis[3] **with polyarthritis**	1/2	1/1	NA	1/1	NA
	Polyarthritis[2] **only**	3/6	NA	NA	0/1	2/2
	NCD[4]	2/2	NA	NA	1/2	NA
Total		**74/91**	**29/65**	**6/27**	**25/57**	**2/2**

[1]: Conjunctivitis: any presentation of ocular discharge, conjunctival oedema, epiphora, photosensitivity and crusting; [2]: Polyarthritis: any presentation of lameness, stiffness, swelling and heat involving more than one joint, lambs tucked in; [3]: Polyarthritis concurrent with conjunctivitis as above; [4]: NCD: no clinical disease; [5]:

Treated conjunctivitis: conjunctivitis or evidence of resolving disease; [6]: *C. pecorum* detected at any anatomical site tested. [7]: compared to total number of *C. pecorum* positive swabs detected at this anatomical site for each flock; [8]: NA = not available for testing.

Table 2. Distribution of *C. pecorum* STs detected in ocular/joint and vaginal/rectal swabs collected from sheep with different disease presentation

Disease presentation	**Typed *C. pecorum* PCR positive swabs**	**STs detected**												
		ST 23	**ST 62[a]**	**ST 63[a]**	ST 69	ST 71	ST 72	ST 74	**ST 78[a]**	**ST 79[a]**	**ST 80[a]**	**ST 81[a]**	**ST 82[a]**	**ST 83[a]**
Conjunctivitis (11 sheep)	**Ocular** (n=11)	2/11 (18%)	-	-	6/11 (55%)	-	1/11 (9%)	1/11 (9%)	-	-	-	-	1/11 (9%)	-
	Vaginal/Rectal (n=9)	3/9 (34%)	-	2/9 (22%)	1/9 (11%)	-	-	2/9 (22%)	-	-	-	1/9 (11%)	-	-
Conjunctivitis with polyarthritis (8 sheep)	**Ocular** (n=7)	6/7 (86%)	-	-	1/7 (14%)	-	-	-	-	-	-	-	-	-
	Rectal (n=8)	-	-	-	1/8 (12.5%)	1/8 (12.5%)	-	2/8 (25%)	1/8 (12.5%)	2/8 (25%)	1/8 (12.5%)	-	-	-
Polyarthritis (12 sheep)	**Ocular/Joint** (n=12)	12/12 (100%)	-	-	-	-	-	-	-	-	-	-	-	-
	Vaginal/Rectal (n=10)	1/10 (10%)	1/10 (10%)	3/10 (30%)	-	-	-	1/10 (10%)	4/10 (40%)	-	-	-	-	-
NCD (4 sheep)	**Ocular** (n=1)	1/1 (100%)	-	-	-	-	-	-	-	-	-	-	-	-
	Rectal (n=4)	1/4 (25%)	-	-	-	-	-	-	-	-	2/4 (50%)	-	-	1/4 (25%)
Total:	**62**	26/62 **(42%)**	1/62 (1.6%)	5/62 (8%)	9/62 **(14.5%)**	1/62 (1.6%)	1/62 (1.6%)	6/62 **(9.7%)**	5/62 (8%)	2/62 (3.2%)	3/62 (5%)	1/62 (1.6%)	1/62 (1.6%)	1/62 (1.6%)

[a]: novel ST (denoted in bold).

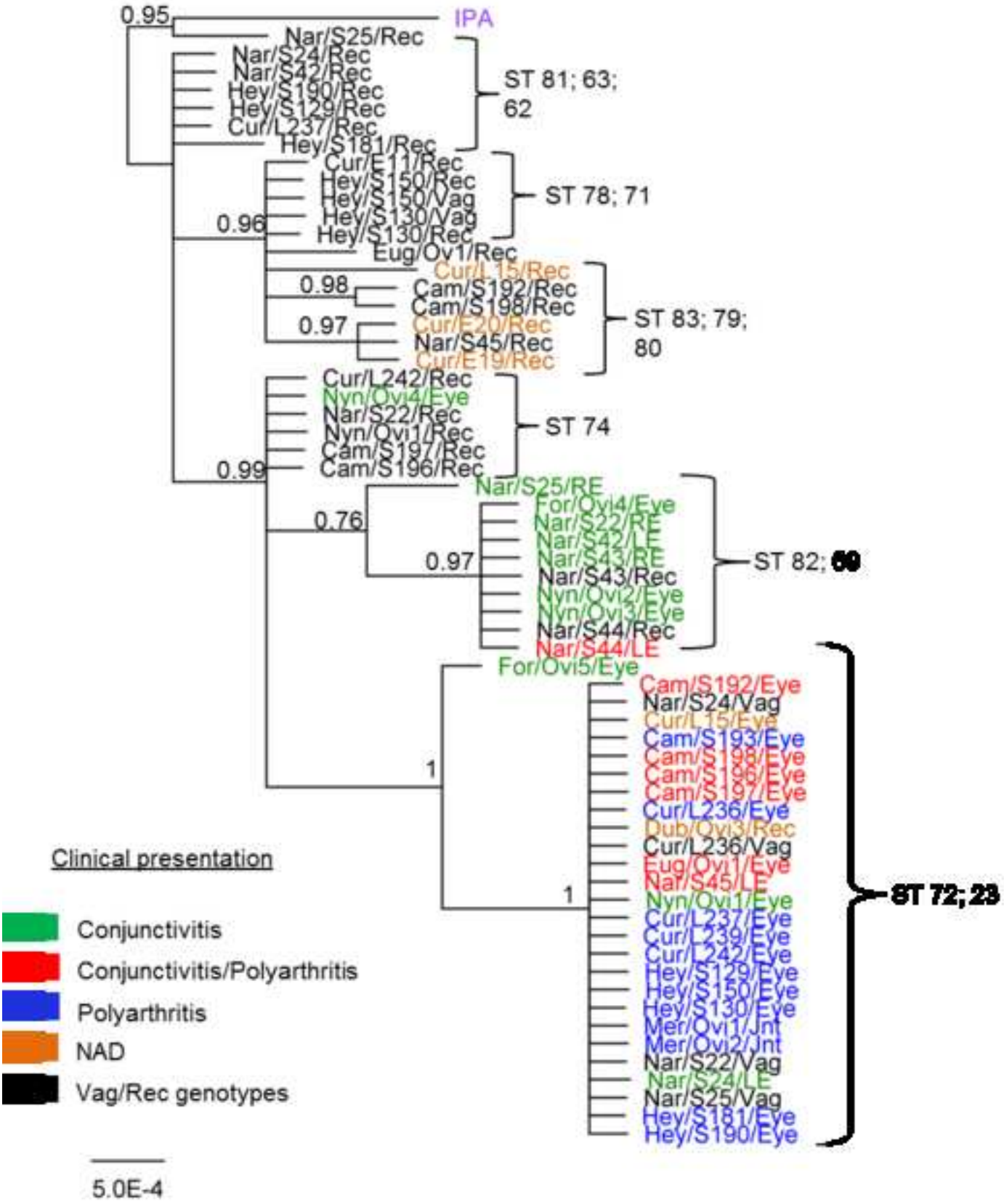
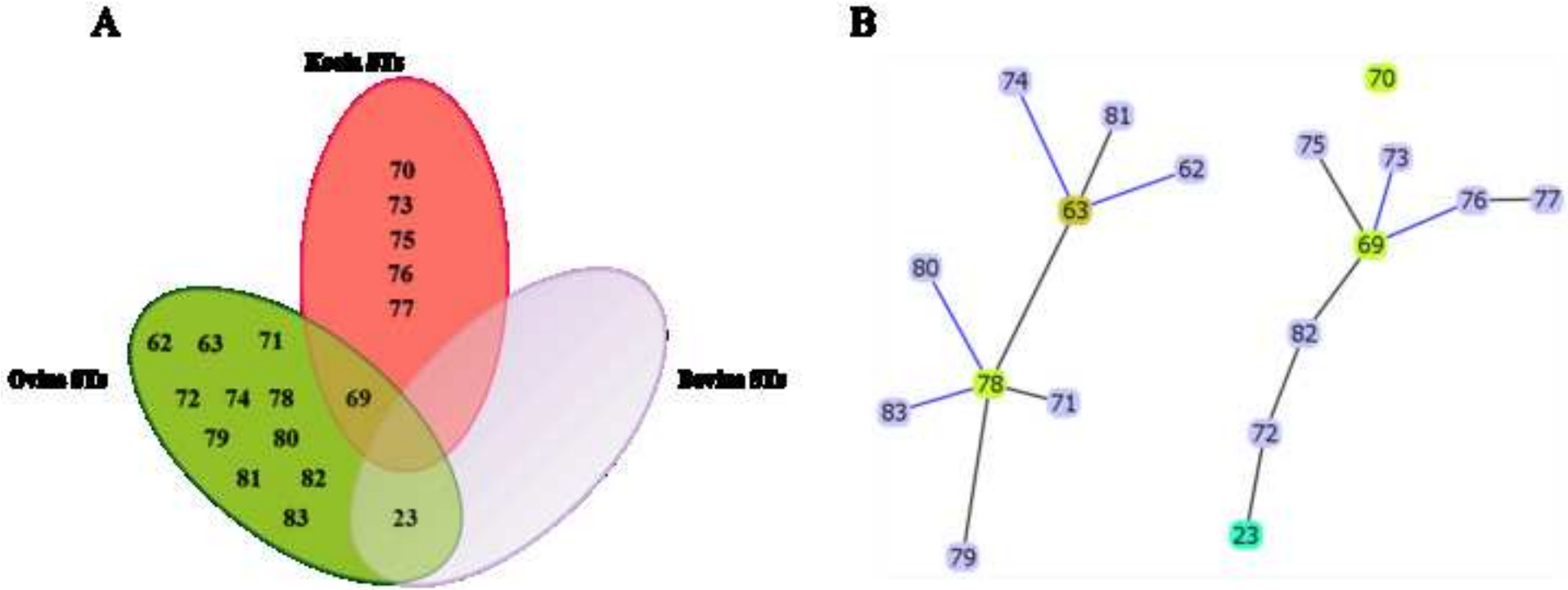


Figure 2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