Transmission Dynamics of *mecC* Resistance Among Staphylococcal Species From Hedgehogs and Ticks

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Abstract

The European hedgehog has been proposed as a reservoir for *mecC* positive methicillin resistant *Staphylococci*, although the mechanism of how this methicillin resistance determinant spilled over into other wildlife, livestock and humans is not fully understood. Ticks are well known vectors of tick-borne diseases and parasitise livestock and wild animals, including hedgehogs. Furthermore, within their diverse microbiome they also harbour including *Staphylococcaceae*. This research aimed to characterise the presence of *mecC Staphylococci* and *Mammaliicocci* in ticks removed from hedgehogs and compare these with samples directly from hedgehogs to determine associations between the two. Presumptive *Staphylococci* were isolated from homogenised tick samples, hedgehog faecal samples and hedgehog skin samples. These isolates were screened for methicillin resistance and resistant isolates were subject to PCR analysis for the presence of *mecA* and *mecC* genes. Isolates were then identified using biochemical analysis. In total, 203 ticks from 21 hedgehogs, 254 field collected questing ticks, 17 faecal samples and six skin samples were collected and screened. A total of 54 isolates were obtained and 18 of these were identified to species level which were predominantly obtained from ticks. The most abundantly identified species was *Staphylococcus xylosus* (n=7), followed by *Mammaliicoccus sciuri* (n=3) and *Mammaliicoccus lentus* (n=3). A single *mecC* positive isolate was obtained from a tick sample; a 718bp *mecC* amplicon shared 100% sequence identity with the reference strain of *mecC*-MRSA (*Staphylococcus aureus* M10/0061). The *Staphylococcaceae* identified are frequently present in the environment and on the skin of mammals, but often overlooked as a potential reservoir of resistance genes. Similarly, *Staphylococcaceae* in ticks are not considered as significant. The finding of *mecC* in ticks but not hedgehogs suggests a potential role for ticks in the transmission of *mecC* that necessitates further research and consideration of ticks as a reservoir for antimicrobial resistance.

1 CONTENTS

1. CHAPTER ONE: BACKGROUND AND AIMS

1.1. Introduction

Staphylococcaceae are Gram positive cocci bacteria that grow in clusters and consists of 9 genera: *Abyssicoccus, Aliicoccus, Corticicoccus, Jeotgalicoccus, Macrococcus, Mammaliicoccus, Nosocomiicoccus, Salinicoccus* and *Staphylococcus* (Dobritsa & Samadpour, 2020; Madhaiyan et al., 2020). Of these, *Staphylococci* and *Mammaliicocci* are of particular clinical interest due to the ability of some species in these genera to cause disease in human and animals and/or carry beta-lactam resistance genes (Murray et al., 2022; Nemeghaire et al., 2014). *Staphylococcus aureus* is one of the most studied species of this family due to its clinical significance in humans and livestock, causing diseases such as soft tissue infections, toxic shock syndrome (TSS) and meningitis (Aguilar et al., 2010; Baude et al., 2019; Berger et al., 2019). The World Health Organisation (WHO) lists antimicrobial resistance (AMR) as one of the top ten threats to global health with 4.95 million deaths associated with AMR and 1.27 million deaths directly ascribed to AMR; *S. aureus* is the second leading pathogen attributed to these figures (Murray et al., 2022) highlighting the importance of AMR in this opportunistic pathogen. The clinical importance of *S. aureus* is undebated, but with research indicating *Mammaliicocci* as the probable origin of the AMR gene, *mecA* (Shang Wei Wu et al., 2001; Tsubakishita et al., 2010) it is imperative to not overlook *Mammaliicocci* and other *Staphylococci* species when investigating mechanisms of AMR, whether the research aim is to investigate the origins of these genes or their prevalence and distribution.

1.2. *Staphylococci* and *Mammaliicocci*

The genus *Staphylococci* has more than 55 published species (Cuny et al., 2024; Munson & Carroll, 2023), including multiple well described species of clinical importance, for example *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus, Staphylococcus hominis, Staphylococcus haemolyticus and Staphylococcus capitis* as they have the capability to be pathogenic, causing infections such as bacteraemia and infective endocarditis in humans and bovine mastitis in cattle, among other pathologies (García-Álvarez et al., 2011b; Petti et al., 2008; Spanu et al., 2003); but can also be a part of the commensal microbiota of humans and animals (Bieber & Kahlmeter, 2010; Olsen et al., 2013; Rall et al., 2014; Severn et al., 2022; Zheng et al., 2022).

Mammaliicocci includes five species which were previously designated as *Staphylococci*: *Mammaliicoccus sciuri, Mammaliicoccus stepanovicii, Mammaliicoccus lentus, Mammaliicoccus fleurettii* and *Mammaliicoccus vitulinus.* These species, like other non-aureus *Staphylococci* are not as well described as *S. aureus*. Phylogenomic analysis of the 72 core gene sequences of 95 type strains of *Staphylococcaceae* revealed that these species were as distantly related to other *Staphylococci* as species from the *Macrococcus* genus; *Mammaliicocci* species shared a mean amino acid identity (AAI) of 86% with each other but shared only an average AAI of 65% with other *Staphylococci; Macroccocci* shared a mean AAI of only 62.2% with other *Staphylococci*. These findings required the redesignation of these species (Madhaiyan et al., 2020). *Mammaliicocci* are known to be prevalent among healthy and diseased animal populations and is frequently isolated from poultry, cattle, camelids and wildlife (Belhout et al., 2023; Loncaric et al., 2013; Nemeghaire et al., 2014).

There is a lack of research regarding the prevalence of *Mammaliicocci* colonisation in healthy humans, but one study tested 20,806 clinical isolates of coagulase negative

Staphylococci (CoNS) to species level over a 24-month period and it was found that only 0.5% of the isolates were *Mammaliicoccus sciuri* (Elamin et al., 2015). Despite the apparent low frequency of Mammaliicoccal infections in humans, *Mammaliicocci* are of increasing clinical interest as they serve as reservoirs of a diverse collection of AMR genes, including beta-lactam, phenicol, macrolide, aminoglycoside, lincosamides and tetracycline resistance genes (Belhout et al., 2023; Lienen et al., 2022). This genus has also been shown to possess a variety of virulence genes, particularly *M. sciuri*; research has shown the presence of genes encoding enterotoxins, biofilm production, capsule, adhesion proteins and leukocidin (Nemeghaire et al., 2014; Park et al., 2011; Stepanović et al., 2001), indicating that some *Mammaliicocci* may be potential reservoirs of virulence genes.

1.3. Beta-lactam Resistance

Resistance to beta-lactams such as penicilllins and cephalosporins in *Mammaliicocci* and *Staphylococci* is mediated by two main mechanisms such as the expression of the *blaZ* gene (Belhout et al., 2023; Vesterholm-Nielsen et al., 1999), producing beta-lactamases that hydrolyse the beta-lactam ring, inactivating the antibiotic. The other mechanism is through modification of penicillin binding proteins (PBPs) with a lower binding affinity for beta-lactams such as PBP2a and $PBP2_{LGA}$, preventing PBP inhibition (Hartman & Tomasz, 1984). This confers broad spectrum resistance against beta-lactams, except for 5^{th} generation cephalosporins such as ceftaroline (Chen et al., 2014) and is encoded by methicillin resistance genes, *mecA* and the recently discovered *mecC* (Bitrus et al., 2017; García-Álvarez et al., 2011a; Matsuhashi, Song, et al., 1986). Recently, another potential mechanism for beta-lactam resistance in *Staphylococci* has been identified in methicillin resistant isolates lacking *mec* (MRLMs); these are isolates that show phenotypic resistance but do not have the resistance-associated *mec* genes (Sommer et al., 2021). It is thought that MRLM resistance is mediated by mutations in both *pbp4* and *gdpP* genes, with one study showed that 83.78% (total 185) of MRLMs carried mutations related to *gdpP* (encoding GGDEF-domain protein phosphodiesterase) and *pbp4* (encoding Penicillin Binding Protein-4) and these mutations are thought to work synergistically with each other to confer a resistant phenotype compatible to that of MRSA (Lai et al., 2024), the exact mechanism is yet to be understood.

1.4. *mecA*

PBPs are a group of enzymes that have an essential role in the synthesis of peptidoglycan that is found in the bacterial cell wall (Goffin & Ghuysen, 1998) and also serve as the targets for the beta-lactam class of antibiotics, inhibiting their activity (Hartman & Tomasz, 1984; Łeski & Tomasz, 2005), therefore impeding cell wall synthesis which eventually leads to cell death. Methicillin resistant strains of *Staphylococci* and *Mammaliicocci* (MRSM), including Methicillin Resistant *S. aureus* (MRSA), possess an additional gene, *mecA* (Belhout et al., 2023), that encodes for an alternative PBP2, termed PBP2a, that is not present in methicillin susceptible strains but can induce resistance when inserted into susceptible strains (Beck et al., 1986; Matsuhashi, et al., 1986). PBP2a confers methicillin resistance via a lower binding affinity for methicillin (Hartman & Tomasz, 1984).

The *mecA* gene is a part of a larger, mobile genetic complex termed the SCC*mec* (Staphylococcal Cassette Chromosome *mec*), which also contains cassette chromosome recombinase (*ccr*) genes which are responsible for the excision and integration of this mobile element (Ito et al., 2003; Katayama et al., 2000). Further investigation of the SCC*mec* revealed that it is also the location of other antimicrobial resistance genes such as *ermA*, *tetM* and *qaqA* genes (Kuroda et al., 2001). Diversity within SCC*mec* (Ito et al.,

2001) has been used to establish a typing scheme and typing is based on the *ccr* genes allotypes and the organisation of the *mec* gene complex; the *mec* gene complex is composed of the *mecA* gene and its associated regulatory genes (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), 2009).

mecA has since been observed in other *Staphylococci* of clinical significance, including *S. haemolyticus, S. epidermidis, S. saprophyticus, S. hominis, S. capitis* and *S. lugdunensis* (Medis et al., 2022). *mecA* and its allotype *mecA1*, is also frequently identified in *Mammaliicocci*, although this does not always result in phenotypic resistance (Belhout et al., 2023; Fungwithaya et al., 2022; Nemeghaire et al., 2014; Silva et al., 2021).

The origins of the SCC*mec* are thought to be in *Mammaliicocci* species, namely *M. sciuri*, *M. vitulinus* and *M. fleurettii*; with these three species contributing to the evolution of the SCC*mec* complex, which then transferred to *Staphylococcal* species, including *S. aureus* (Rolo et al., 2017).

1.5. *mecC*

Methicillin resistance was thought to be primarily through *mecA*, until in 2011 during a study on antibiotic susceptibility among *S. aureus* isolates obtained from milk samples from cows with bovine mastitis, when a novel *mecA* homologue was discovered in *S. aureus* LGA251, termed $mecA^{LGA251}$ by the authors (García-Álvarez et al., 2011b). This isolate showed phenotypic methicillin-resistance that was not mediated by the presence of *blaZ* and tested negative for *mecA* using PCR; similarly, latex agglutination tests for PBP2a were also negative. Whole genome sequencing of the isolate was used to characterise its SCC*mec* element and compared with other SCC elements, resulting in the discovery of this homologue now been designated *mecC* (Ito et al., 2012a). The *mecC* sequence of LGA251 showed 70% nucleotide identity with previously described *mecA* sequences and 63% identity on the amino acid level (García-Álvarez et al., 2011a; Ito et al., 2003). Similarly to *mecA*, *mecC* is also located within the SCC*mec* element, however this type of SCC*mec* had not previously been seen but is the predominant SCC*mec* type associated with *mecC* (Larsen et al., 2022). The discovery of this homologue had considerable implications on the screening for MRSA as this homologue was not detectable by routine *mecA* PCR nor PBP2a agglutination assays (García-Álvarez et al., 2011a) which were a part of previous testing guidelines for MRSA (Brown et al., 2005), which have since been updated (Public Health England, 2020). This led to further research, trying to identify reservoirs of *mecC* carrying *Staphylococcaceae* and its prevalence.

1.6. Distribution of *mecC*

The *mecC* gene has since been detected in multiple species of *Staphylococci* and *Mammaliicocci* since its initial discovery, mostly from animal origin, including wildlife. Shortly after the initial detection of *mecC*, researchers were examining small intestinal tissue samples of European brown hares that were emaciated with pyogranulomatous lesions in numerous tissues or necro-suppurative inflammation, isolated three MRSA strains with the *mecC* gene; another *mecC* positive MRSA strain was isolated from a European hedgehog (Loncaric et al., 2013). Another study of 4299 wild and domestic animal samples analysed for *mecC Staphylococci*/*Mammaliicocci* yielded 2809 *Staphylococci*, of which 698 were methicillin resistant; in total 15 strains of five *Staphylococcaceae* species were identified to be positive for *mecC*: *M. stepanovicii*, *M. sciuri*, *S. xylosus*, *S. caprae* and *S. warneri* (Loncaric et al., 2019a). These strains were isolated from a variety of animals including the Eurasion lynx, European otter, Red fox, beaver, cattle, sheep, goats and alpacas, showing that the *mecC* gene is widely disseminated among

Staphylococci/*Mammaliicocci* from a variety of animals. *mecC* resistance is geographically widespread too, with detection in multiple European countries, Algeria, Japan and Australia (García-Álvarez et al., 2011a; Rasmussen et al., 2019; Sekizuka et al., 2020; Venla et al., 2023; Worthing et al., 2016). This low prevalence of *mecC*-*Staphylococci/Mammaliicocci* (*mecC*-SM) is also reflected in human derived samples; in 2011, Denmark, *mecC*-MRSA made up only 2.8% of all MRSA cases (Petersen et al., 2013) and in Germany out of 12,691 clinical MRSA isolates, only 11 were *mecC* positive (Cuny et al., 2011).

The European hedgehog (*Erinaceus europaeus)* has been implicated as a potential reservoir for *mecC*-SM. A Swedish study found a high prevalence of *mecC*-MRSA among wild European hedgehogs; 55 hedgehogs were sampled from 5 different areas of Sweden, and of these 35/55 hedgehogs (64%) were colonised by *mecC*-MRSA (Bengtsson et al., 2017). Another study sampled 188 hedgehogs from various regions of Denmark and found that 61% (114/188) of the hedgehogs harboured *mecC*-MRSA but no *mecA*-MRSA isolates were obtained, supporting the hypothesis that hedgehogs may be a reservoir of *mecC*-SM (Rasmussen et al., 2019). Early UK studies reported a prevalence of *mecC*-MRSA of 66% (81) of hedgehogs were colonised out of a sample size of 123 (Larsen et al., 2022). Not all studies of *mecC*-MRSA prevalence among European hedgehogs have obtained similar results; for example, in Finland for example, only 11 out of 115 sampled hedgehogs were positive for MRSA (prevalence of only 3.5%) (Venla et al., 2023). In Hungary MRSA was recovered from only two out of 200 hedgehogs screened, one of which was *mecA* and the other *mecC* positive (Sahin-Tóth et al., 2022). Both of the studies that found lower prevalence rates did not isolate *S. aureus* from many hedgehogs; the Hungarian study only isolated *S. aureus* from 13 hedgehogs and the Finnish study isolated *S. aureus* from only 11 hedgehogs. This low frequency of *S. aureus* isolation from hedgehogs compared to above mentioned research and that from 1965 (Smith, 1965), might reflect geographical differences. It is also important to note that these studies investigating *mecC* in hedgehogs solely focussed upon MRSA, so the diversity and prevalence of the Staphylococcal/Mammaliicoccal species that harbour this resistance gene could be larger than previously established.

1.7. Origins of *mecC*

The origins of *mecC*-SM are currently unknown as are the evolutionary history that led to the *mecA* homologue. The unusually high prevalence of *mecC* in hedgehog populations have led to researchers theorising that hedgehogs may be the origin and natural reservoirs of methicillin resistant *mecC*-SM (Dube et al., 2021; Larsen et al., 2022) however the idea that hedgehogs may be a reservoir for methicillin resistant *Staphylococci*/*Mammaliicocci* is not new (Smith & Marples, 1964, 1965). Hedgehogs have been hypothesised as a source of resistance as they are frequently infected with *Trichophyton erinacei* (Čmoková et al., 2022; Smith & Marples, 1965), a dermatophyte that has been shown to produce penicillins such as penicillin G and benzylpenicillin that are effective against beta-lactamase and *mecC* deficient *Staphylococci* (Dube et al., 2021; Larsen et al., 2022), thus creating a microenvironment under selective pressure due to the presence of beta-lactams, giving *Staphylococci*/*Mammaliicocci* with beta-lactam resistance mechanisms an advantage over susceptible strains. For this reason, combined with the increased prevalence of *mecC*-MRSA that it is theorised that hedgehogs may be the natural origin of *mecC*.

The *mecC* gene has been found primarily on a type XI SCC*mec* element (Dierikx et al., 2023; Loncaric et al., 2013, 2019a), the same type as originally documented (García-Álvarez et al., 2011a). This suggests that *mecC* may have been disseminated from a common source, independently of *mecA*. However, recent research has shown that *mecC* is

also found not infrequently on a hybrid *mecA-mecC* SCC*mec* element that contains the associated regulatory genes of both *mecA* and *mecC* on a type VII SCC*mec* (Belhout et al., 2023; Harrison et al., 2014a; Loncaric et al., 2019b), this SCC*mec* type is a known type that harbours the *mecA* gene (Deurenberg & Stobberingh, 2008); in all of these cases this hybrid element was located within *Mammaliicocci* species (*M. sciuri, M. lentus*, *M. stepanovicii*) but the significance of this remains to be elucidated.

1.8. Ticks as Vectors of Disease

Hedgehogs are known to be parasitised by hard ticks (Gern et al., 1997); ticks are ectoparasites that feed strictly on blood and therefore are haematophagous; there are two main families of ticks, *Ixodidae*, which are also known as hard ticks, and *Argasidae* which are known as soft ticks (Boulanger et al., 2019). Ticks of the *Ixodidae* family are known to parasitise a range of hosts such as cattle and other livestock, birds, deer and hedgehogs across Europe and have the potential to transmit a variety of tick-borne infections to both humans and animals (Kahl & Gray, 2023; Keyte et al., 2021; Layzell et al., 2018; Pettersson et al., 2017).

Ixodes ricinus and *Ixodes hexagonus* are the two most prevalent tick species in the UK (*HPA - Tick Surveillance*, 2014) and are found in woodlands and suburban parks with dense vegetation (Kahl & Gray, 2023) such as Richmond Park, London (Greenfield & Butt, 2011). The hard tick life cycle consists of 4 stages, the egg stage followed by three life stages which are larvae, nymph and adult. Once the eggs hatch, larvae attempt to acquire a host by climbing vegetation and wait for a host to pass by, this is called questing; once on a host the tick attaches itself, it has a very large bloodmeal over the course of 2-4 days before detaching from the host. The six-legged larvae undergo development and moulting to the next stage, which is the nymphal stage. Nymphs have eight legs and are larger than larvae; during this life stage, nymphs also quest, looking for a host for a blood meal. Once the blood meal is complete (approximately 3-5 days feeding), the tick will detach itself from the host again and fall to the vegetation where they develop and moult into either adult male or female ticks. Male ticks do not feed much, whereas females will quest, looking for a host; an adult mated female may feed for around 7-10 days before detaching and falling to the vegetation, where she will lay thousands of eggs and die (Kahl & Gray, 2023).

Hard ticks are known vectors of many infectious diseases, most famously they are vectors of the causative agent of Lyme disease, *Borrelia burgdorferi* (Barbour & Benach, 2019; Benach et al., 1983; Steere et al., 1983). *Ixodidae* are also known vectors of other diseases including viral tick-borne encephalitis caused by a *Flavivirus*, human granulocytic anaplasmosis caused by *Anaplasma phagocytophilum* and Babesioses caused by various *Babesia* species. (Boulanger et al., 2019). Tick-borne diseases are also of clinical importance for animal health as infections with *A. phagocytophilum* and *Borrelia spp.* have been reported in domestic cats and dogs (Shaw et al., 2005). Louping ill virus is another example of a tick-borne pathogen causing fever that can progress on to encephalitis in livestock and can also cause disease in humans, although this is rare (Folly et al., 2022). These infectious agents, such as *Borrelia*, can be ingested by the tick from an infected host, which then enables the tick to transmit the infection on to other hosts (Gern et al., 1997). However, an infected host is not always required for some of these pathogens such as *Borrelia miyamotoi*, as transovarial transmission allows these pathogens to be passed from adult female ticks to their offspring via eggs (Lynn et al., 2022), allowing the persistence of these pathogens in the ecosystem; this is evidenced by the presence of *Rickettsia spp.* and *A. phagocytophilum* in 39/50 *I. ricinus* larval nests and *Borrelia spp.* detected in 3/50 nests under field conditions (Hauck et al., 2020).

Due to the range of pathogens that hard ticks transmit to both human and animals, the microbiome for *I. ricinus* has been of particular interest; evidence has shown a diverse presence of bacteria, including *Corynbacterium*, *Mycobacterium*, *Cutibacterium* and *Staphylococcus* (Alafaci et al., 2021; Rousseau et al., 2021). Evidence of *Staphylococcaceae* in ticks has also been published in other studies (Gil et al., 2020; Guizzo et al., 2022; Namina et al., 2023), however only a limited number of these studies characterise these beyond family or genus level (Rousseau et al., 2021) therefore the species of *Staphylococci* and *Mammaliicocci* present in ticks are still yet to be fully revealed, which would also give a better idea if there is any significance to their presence. A recent study in China investigating AMR genes in wild ticks found evidence of 100 different AMR genes and 20 different mobile genetic elements (MGEs), including two genes linked to macrolide, lincosamides and streptogramin B resistance, *ermB* and *ermQ* which were found to be strongly associated with *Staphylococci* (Wei et al., 2022). The research also found evidence of insertion sequence IS256 (Wei et al., 2022), which has been associated with methicillin resistance (Casagrande et al., 2012; Maki & Murakami, 1997).

1.9. Aims

mecC-SM has primarily been observed among livestock and wildlife with hedgehogs incriminated as a reservoir for *mecC*-MRSA, however the transmission dynamics of how this resistance mechanism made its way to livestock and humans has yet to be discovered. Although hedgehogs have been identified as a reservoir for *mecC*-MRSA, there is little literature regarding other hedgehog-derived *Staphylococci* and *Mammaliicocci* species that harbour the *mecC* gene, despite evidence showing that *Mammaliicocci* and CoNS species are important reservoirs of resistance and virulence genes. Therefore, one of the aims of this research is to isolate and identify *mecC* and *mecA* carrying *Staphylococcaceae* from hedgehog samples.

Ticks are known ectoparasites of European hedgehogs but literature concerning the species of *Staphylococcaceae* and their methicillin resistance profile in these ticks are scarce and so the potential role of ticks in the transmission dynamics of methicillin resistance genes is yet to be explored. This research therefore aims to isolate and characterise methicillin resistant *Staphylococcaceae* derived from ticks removed from hedgehogs and compare them to *mecC* positive isolates from hedgehog samples, which would enable us to understand whether *mecC* resistance could have spilled over from hedgehogs to livestock/other animals via ticks. The final aim of this investigation is to screen unfed, questing ticks that were not removed from hedgehogs for methicillin resistant *Staphylococcaceae*, which will serve as the hedgehog independent control group.

Establishing whether *mecC* positive *Staphylococcaceae* constitute a part of the tick microbiome is the first step towards clarifying whether ticks have a potential role in AMR gene spread. Ticks are known to be vectors of numerous infections but they have not yet been considered to have a role in the spread of AMR bacteria, despite the fact that they are also known to parasitise livestock and hedgehogs which are known reservoirs of AMR bacteria such as MRSA. The aims of this research use the ‘One Health’ approach to consider these factors when investigating the role of ticks as vectors of AMR spread which may have implications on the understanding of the origins and mechanism of *mecC* spread and potentially other AMR genes.

2. CHAPTER TWO: *MECC* PRIMERS REDESIGN

Initially *mecC* primers that were designed previously by Stegger *et al* (2012) were used (See chapter 3, Table 3.2) for screening the *mecC* gene in phenotypically methicillin resistant isolates, along with *mecA* specific primers from the same research article. Many isolates were negative for both *mecA* and *mecC* which prompted an investigation into the suitability of these primers. This work concluded that a primer redesign was required, which will be detailed in this chapter and the findings discussed.

2.1. Methods

2.1.1. Selection and Analysis of *mecC* Sequences

A GenBank search for the *mecC* gene was carried out using the term '*mecC*' and from this search, a reference sequence for *mecC* was obtained from the Reference Gene Catalogue (accessed on 13th June 2024) (accession numbers: NG_047955, FR823292). The sequence found under the NG_047955 accession number contained a 2198bp segment of the *mecC* gene (from this point onwards this *mecC* fragment will be referred to as the *mecC* reference sequence); the sequence under FR823292 was a 33,042bp sequence of Staphylococcal Cassette Chromosome *mec* element XI (SCC*mec* XI); both of these sequences were obtained from *S. aureus* strain M10/0061, which was isolated from an Irish patient (Shore et al., 2011).

BLAST® was then used to obtain similar and divergent sequences; a BLAST® search was carried out using three different program selections available: megablast, discontiguous megablast and blastn. From these results *mecC* gene sequences from various Staphylococcal/Mammaliicoccal species were imported to MEGA 11 where they were aligned with the *mecC* reference sequence, which can be seen in Figures 2.1, 2.2, 2.3 and 2.4 below. The sequence for the first *mecC*-MRSA described, LGA251 (García-Álvarez et al., 2011b), was also added to the alignment. A complete list of GenBank accession numbers used in this study can be seen in Appendix A. Phylogenetic analysis was performed via the construction of a maximum likelihood phylogenetic tree with MEGA 11.

2.1.2. Alignment of Sequences and Primer Design

Sequences were aligned via MUSCLE and consensus regions were identified in the sequences, and these regions were used as primer location parameters when designing primers using Primer-BLAST (NCBI), see Figure 2.1. The list of primer pairs that were generated was then narrowed down based on the following parameters: GC% content was between 40-60% for both forward and reverse primers; the Tm of the primers were between 55°C-65°C and that the Tm of the forward and reverse primers were within 4°C of each other; runs of a single base did not exceed 4 residues; primer Self complementarity and primer Self 3' complementarity scores were as low as possible. Specificity of the primers was also checked via BLAST.

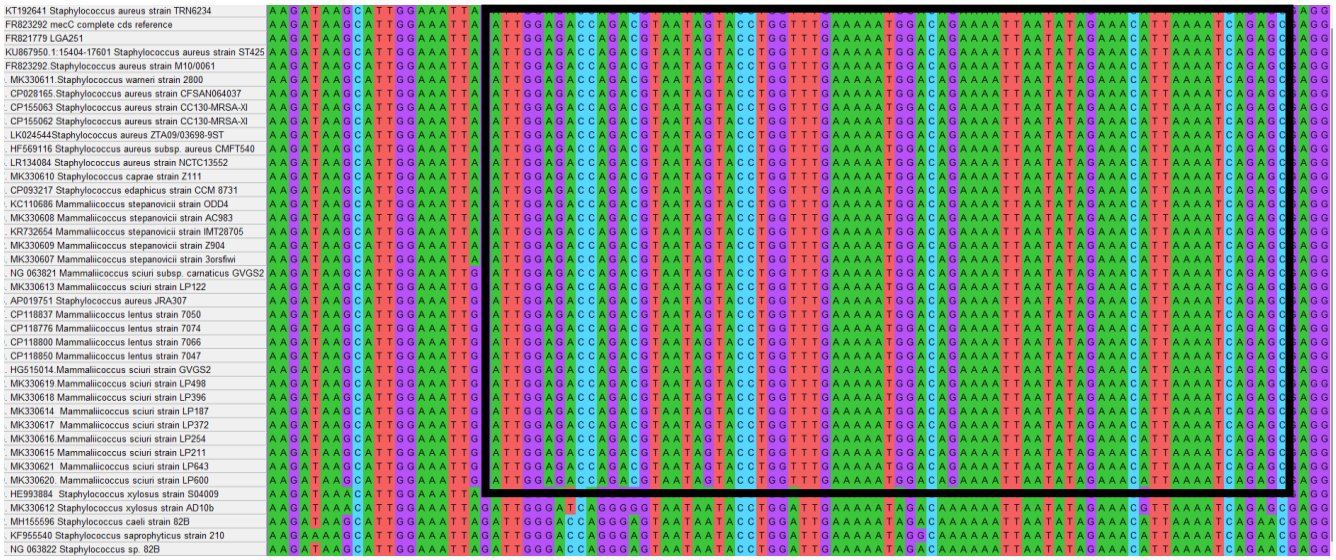

Figure 2-1 Example of a region of sequence identity. mecC allotypes, mecC1, mecC2 and mecC3 were excluded due to sequence variability.

2.1.3. Primer Testing

From the generated primer pairs, 4 primers that were within the acceptable parameters that are mentioned previously were selected for testing (Table 2.1). Primers were tested with a *mec*C positive control and negative control was DNA free water.

Table 2.1 Selected Primers and Nucleotide Sequences

Primers	Primer Sequence (5'->3')	Expected Amplicon Size
mecCP1 Forward	GGTATTGTCCCTAACAAAACACCC	~ 701
mecCP1 Reverse	TGGGTTGAACCTGGTGATGT	
mecCP3 Forward	CTACATCACCAGGTTCAACCCA	~ 377
mecCP3 Reverse	CTCGCCTTGGCCATATCCTG	
mecCP4 Forward	CATCACCAGGTTCAACCCAA	~ 375
mecCP4 Reverse	TCTCGCCTTGGCCATATCCT	
mecCP5 Forward	GGTATTGTCCCTAACAAAACACCC	~ 976bp
MecCP5 Reverse	GATAATCACTCGGGATATTTTCACC	

All PCR reaction mixes were made with Invitrogen *Taq* DNA polymerase, recombinant kit (Invitrogen, Thermo Fisher Scientific). For *mecC*P1, *mec*CP3 and *mecC*P4 primers a 25µL PCR mastermix containing the following was prepared for each set: x1 PCR buffer, 0.2mM of each dNTP (Invitrogen), 0.4µM of each primer, 2mM of $MgCl_2$, 1U *Taq* and 2µL of template DNA. *mecC*P5 PCR reaction mix was prepared with 0.4mM of each dNTP due to the longer amplicon length. For *mecC*P1 primers, the concentration of primers was subsequently adjusted to 0.2µM each to prevent dimer formation. Thermocycling conditions for *mecC*P1 and *mecC*P5 were identical; 10-minute denaturation at 94°C followed by 35 cycles of 94°C for 30 s; 60°C for 60 s; 72°C for 60 s; a final extension step at 72°C for 8 minutes. The thermocycling steps for *mecC*P3 and *mecC*P4 were the same; 94°C for 10 minutes followed by 35 cycles of 94°C for 30 s; 63°C for 60 s; 72°C for 45 s and a final extension of 72°C for 8 minutes. All products were resolved on 1.8% agarose (Sigma-Aldrich) stained with SYBR™ Safe (Invitrogen) run at 100V for 90 minutes in 1 x Tris Acetate EDTA (TAE) buffer (Thermo Scientific). Amplified products were

purified with QIAquick PCR Purification Kit (Qiagen) following manufacturers instruction, eluted in 30µL nuclease free H_20 (Invitrogen) and sent for Sanger sequencing at DBS Genomics (Durham University).

Received DNA sequences were processed using MEGA 11 (version 11) checked against the alignment that was created when designing the primers to ensure that the correct amplicons had been produced.

2.2. Results

2.2.1. Alignments in MEGA 11

The *mecC* primers (*mecA*$_{LGA251}$ primers as seen in Chapter 3, Table 3.2) described by Stegger *et al* (2012) that were used in this research were searched and analysed on the *mecC* sequence alignment created on MEGA 11. The forward primers used in the multiplex and the *mecC* singleplex are identical and can be seen highlighted in yellow, with nucleotide polymorphisms in the 8th and 11th bases of the primer (Figure 2.2). The differences are more pronounced in the multiplex reverse primer, where there are nucleotide polymorphisms in 3 locations (Figure 2.3). The reverse primer of the *mecC* singleplex from the same study covered the sequences of all nearly all strains included in the alignment, with the exception of *mecC* allotypes *mecC1, mecC2* and *mecC3* (Figure 2.4).

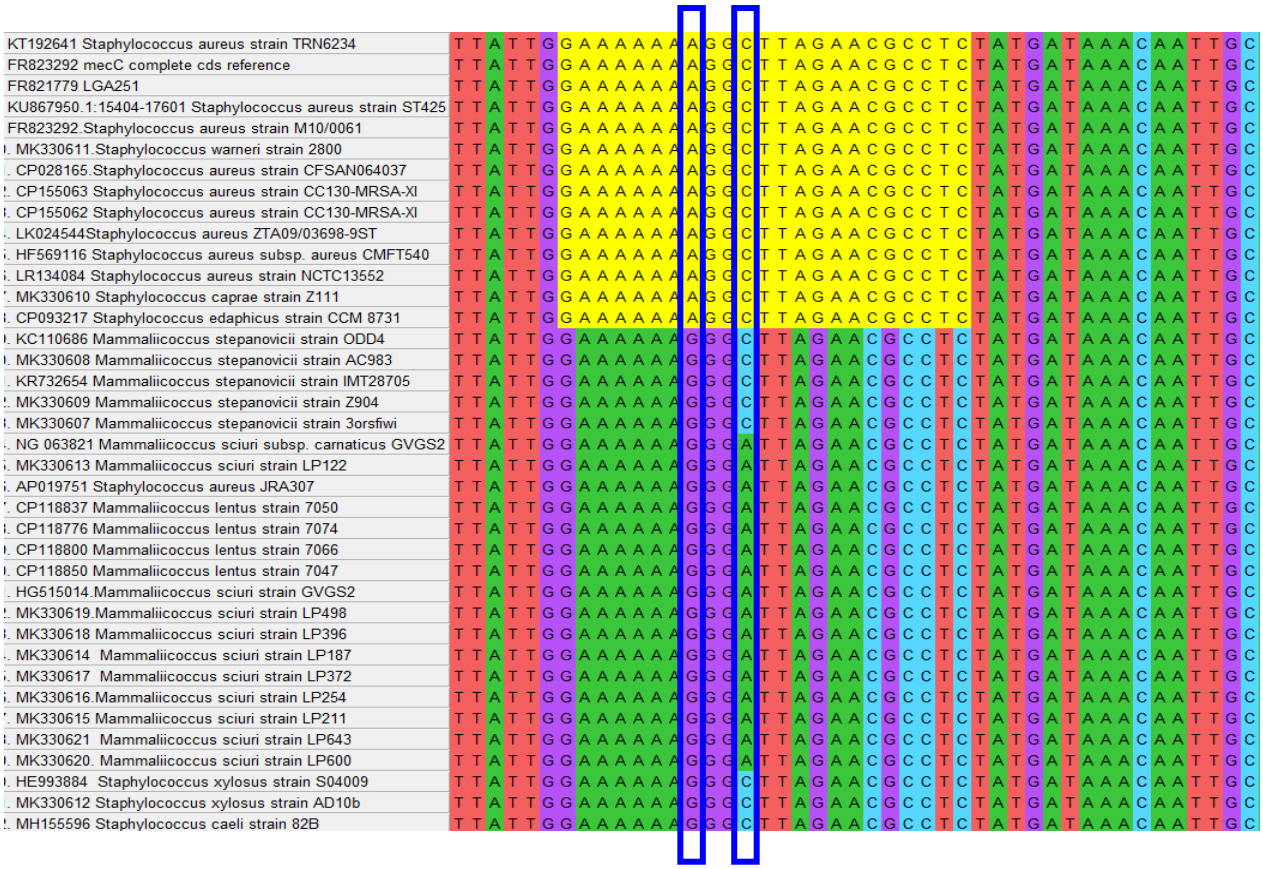

Figure 2-2 Alignment of mecC gene sequences; the mecC singleplex forward primer sequence (Stegger et al., 2012) is highlighted in yellow; nucleotide polymorphisms are encircled in blue. The majority of the mecC strains that had this nucleotide difference were from Mammaliicocci.

Figure 2-3 Multiplex mecC reverse primer location on the alignment. Nucleotide polymorphisms are circled in blue.

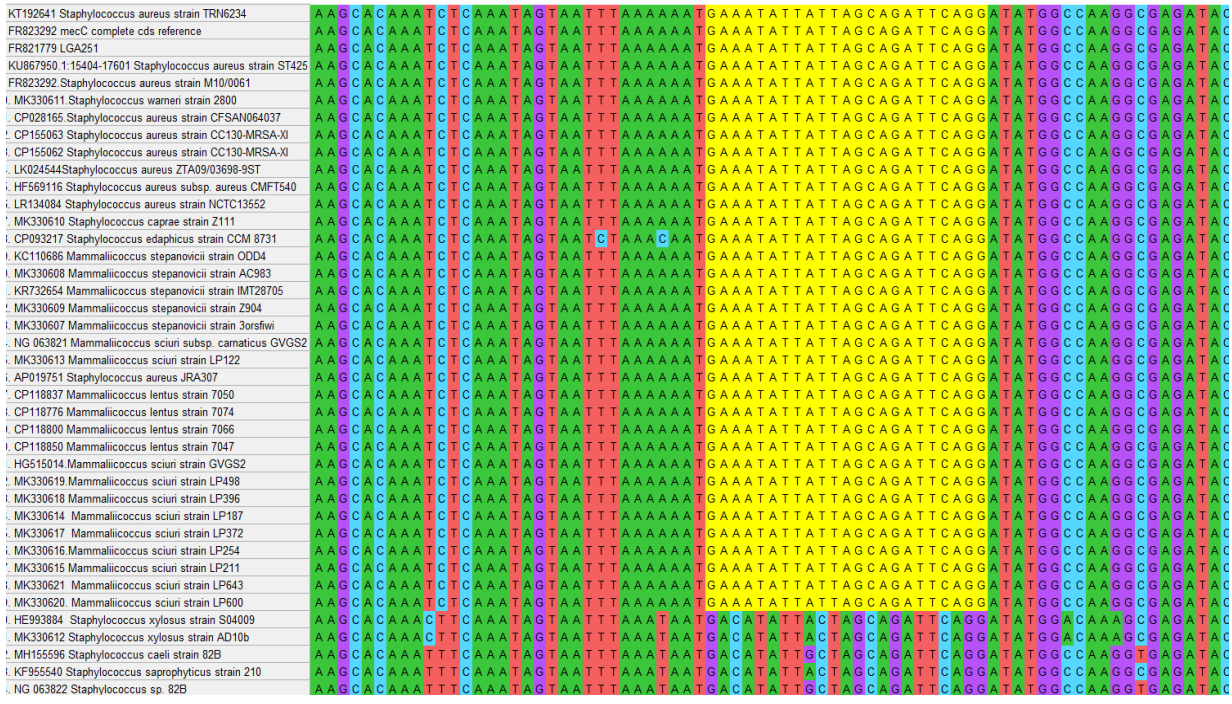

Figure 2-4 Alignment of the mecC gene showing mecC singleplex reverse primer coverage (highlighted in yellow).

2.2.2. Genetic Diversity of *mecC*

Nucleotide polymorphisms were seen in many regions of the *mecC* gene, including the binding site for the *mecA*$_{LGA251}$ primers (Figures 2.2, 2.3, 2.4). Distinct differences in *mecC* sequences derived from *Staphylococci* and *Mammaliicocci* strains were evident, with the exception of *M. stepanovicii* (Figure 2.5); the sequences circled in Figure 6 all share >99.0% sequence identity with each other over 2918bp. One exception to this is *S. aureus* strain JRA307 which was isolated from a wound on a racehorse and underwent WGS; the sequence of the *mecC* gene from this strain was 100% identical to *mecC* from *Mammaliicocci lentus* strain 7047; this dissertation will refer to this as *Mammaliicocci mecC* (M*mecC*). M*mecC* shares approximately 96% nucleotide identity with the *mecC* isolated from MRSA strain LGA$_{251}$ and the reference *mecC* gene.

The alignments seen in Figures 2.5 and 2.6 reveal that the *mecC* sequences of *Staphylococcus xylosus* S04009, *S. xylosus* AD10b, *Staphylococcus caeli* strain 82B, *Staphylococcus saprophyticus* strain 210 contain more distinct nucleotide polymorphisms from the other *mecC* sequences observed and have been designated as *mecC* allotypes according to guidelines for typing novel *mecA* homologues (Ito et al., 2012b). These are *mecC*1, belonging to *S. xylosus* strains AD10b and S04009; *mecC2* from *S. saprophyticus* 210; *mecC3* from *S. caeli* 82B. BLAST analysis of these sequences can be seen in the table below.

Table 2.2 Comparison of nucleotide identity between mecC allotypes over 2918bp.

	Reference *mecC* sequence	*mecC1* *S, xylosus* S04009	*mecC2* *S. saprophyticus* 210	*mecC3* *S. caeli* 82B
mecC1 *S, xylosus* S04009	92.95%			
mecC2 *S. saprophyticus* 210	92.35%	94.49%		
mecC3 *S. Caeli* 82B	91.59%	92.58%	93.94%	
M*mecC* *M. lentus* 7047	96%	90.50%	91.0%	91.32%

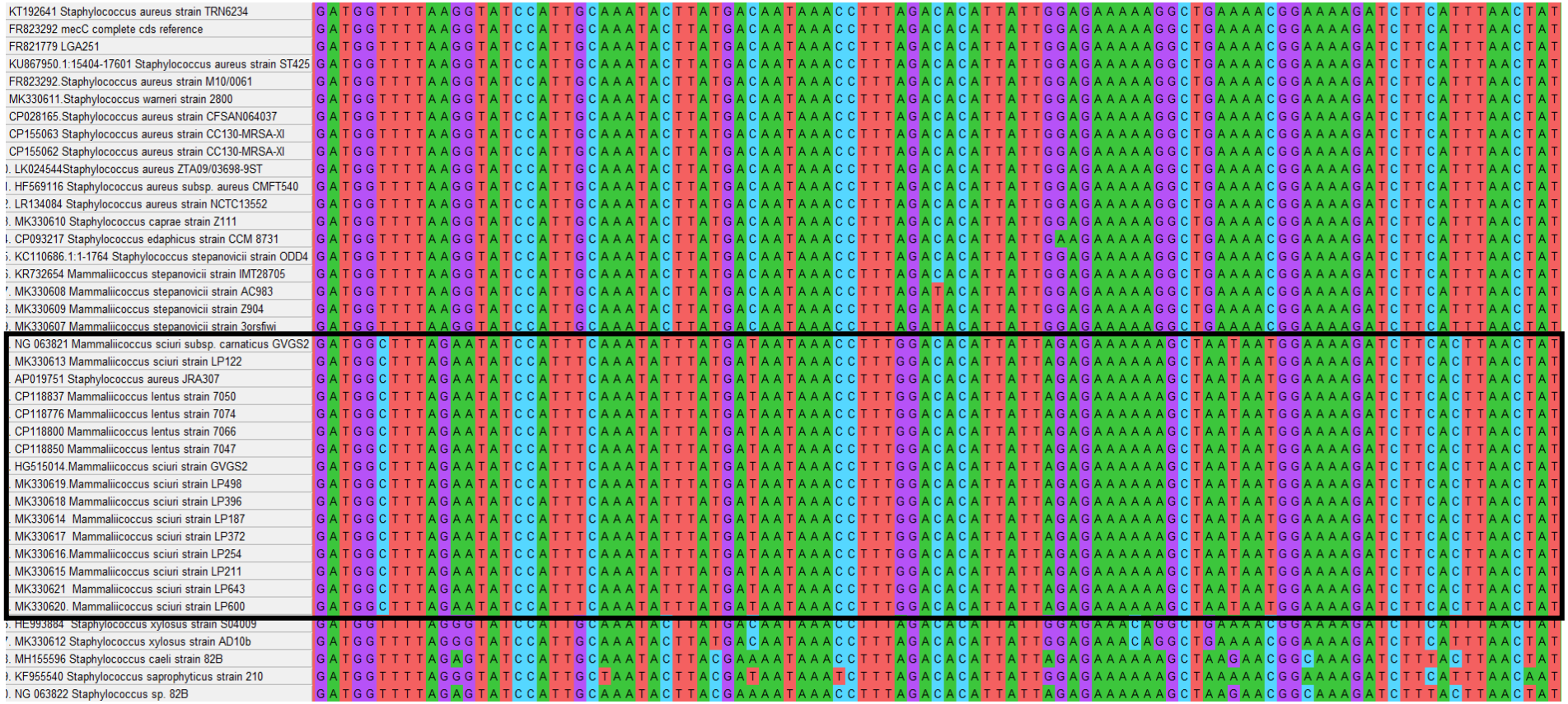

Figure 2-5 Some regions of the mecC gene showed far less homology between Staphylococci mecC and Mammaliicoci mecC genes, for example this figure shows a region of the mecC gene 52bp downstream of the $mecA_{LGA251}$*MultiFP, the forward primer for both the multiplex and singleplex mecC as seen in Chapter 3, Table 3.2. The figure above shows 14 nucleotide substitutions in an approximately 100bp section. Circled in black are the Mammaliicoci sequences.*

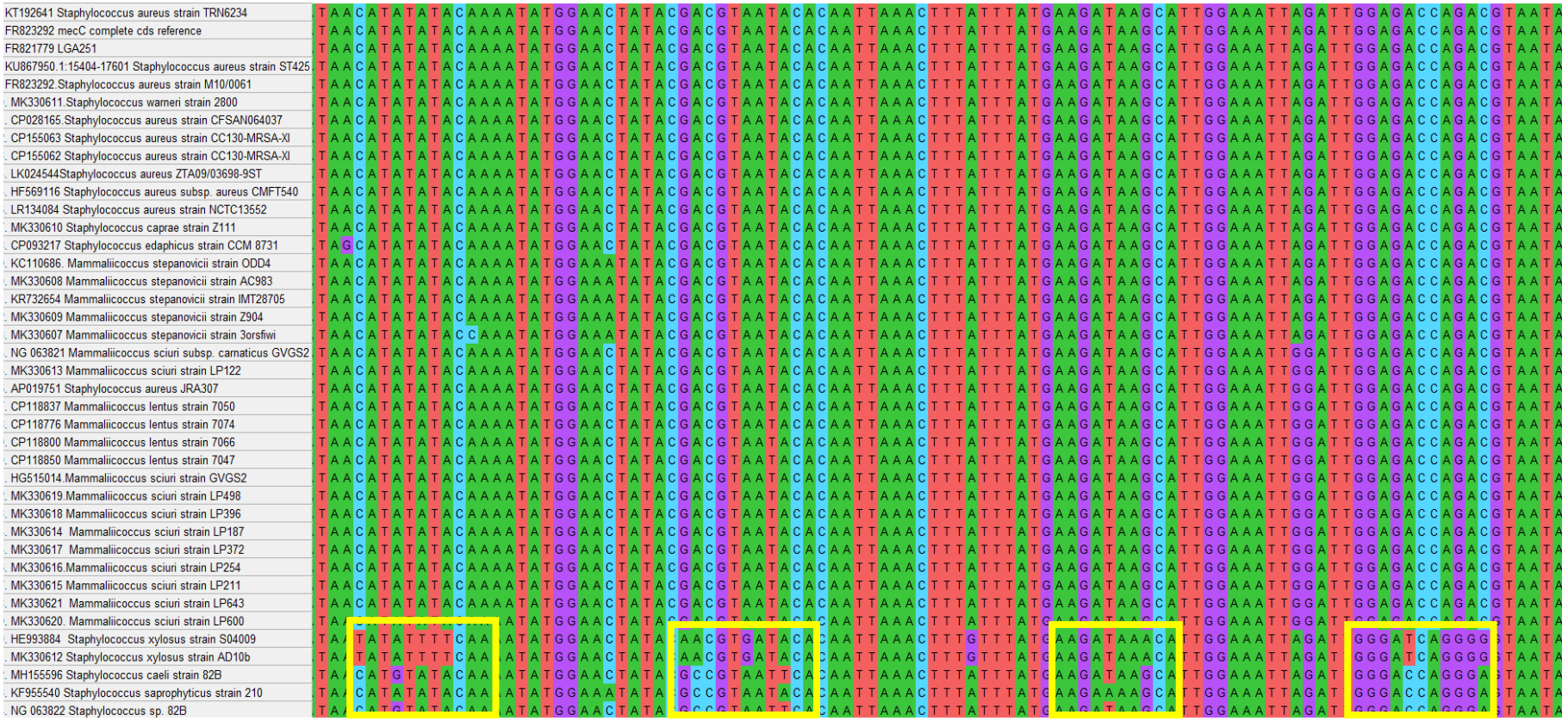

Figure 2-6 The mecC sequences alignment in MEGA 11 showing nucleotide polymorphisms in the mecC allotypes mecC1, mecC2 and mecC3. Differences in nucleotide sequences can be observed in the allotypes even when there is sequence homology between MmecC and SmecC.

2.2.3. Phylogenetic Analysis

Phylogenetic analysis using maximum likelihood can be seen in Figure 2.7 below. This revealed stronger evolutionary relationship with *mecC* genes that have been found in *M. sciuri* and *M. lentus* compared to *mecC* genes from *S. aureus* isolates. It also suggests that the relationship between *mecC* genes from *S. aureus, M. lentus, M. sciuri* have a closer evolutionary link than to the *mecC* allotypes obtained from the following strains: *S. xylosus* S04009, *S. xylosus* AD10b, *S. caeli* strain 82B, *S. saprophyticus* strain 210 (See Appendix A for accession numbers).

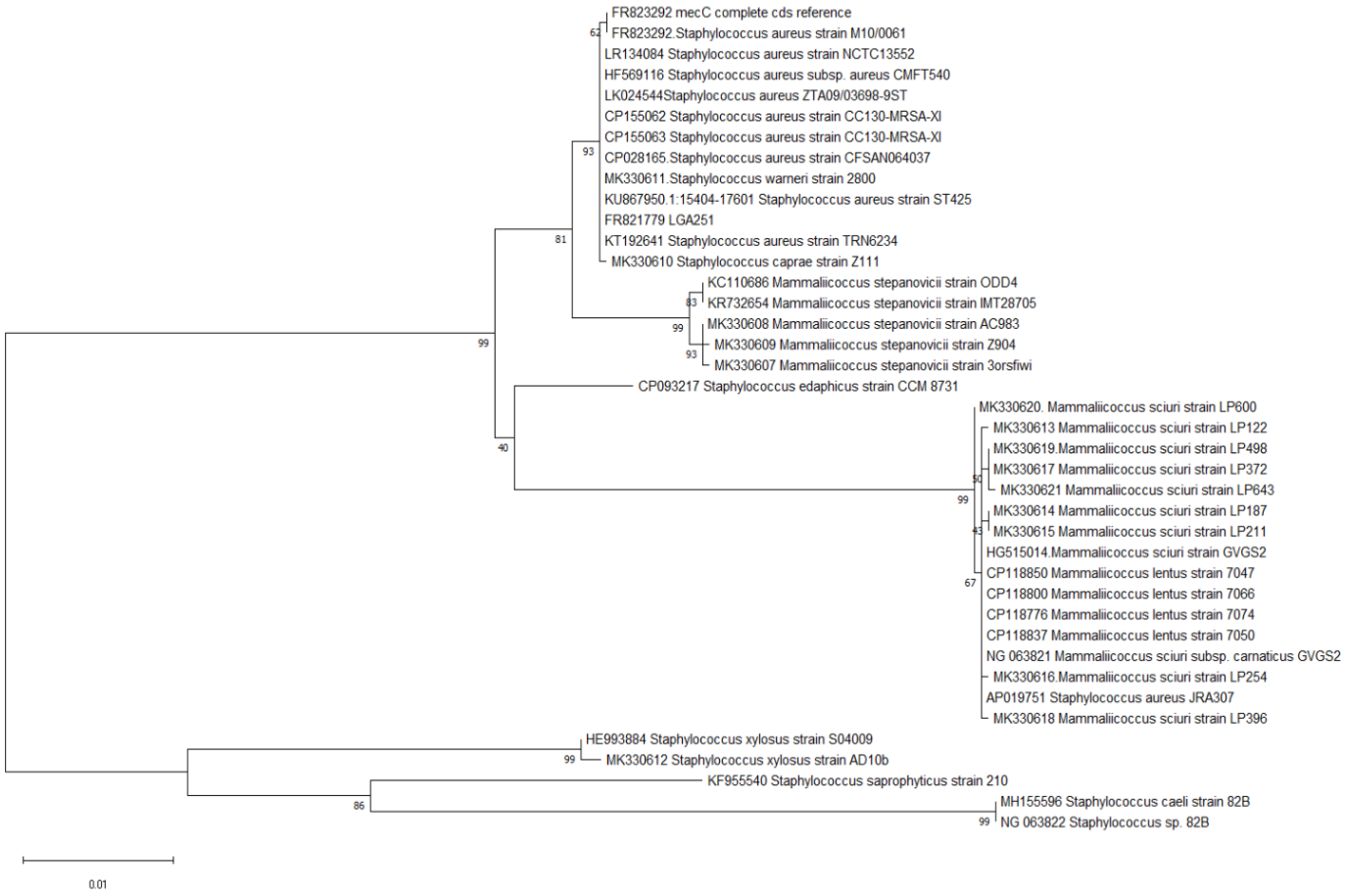

Figure 2-7 Maximum likelihood phylogenetic tree with bootstrap values set to 500 of the mecC gene from previous literature. MEGA 11 was used to construct this phylogenetic.

2.3. Discussion

A total of 33 methicillin-resistant isolates were obtained from various tick and hedgehog faecal samples, which this thesis will detail further in the following chapter. These isolates were screened for *mecA* and *mecC* genes using previously described primers (Stegger et al., 2012). Most of these isolates failed to yield amplicons prompting investigation into the suitability of these primers for the detection of *mecA* and *mecC* across all *Staphylococcaceae*. A multiple alignment of *mecC* gene sequences obtained from GenBank (as of June 2024) was used to evaluate nucleotide polymorphisms, particularly at primer binding regions. It was found that the

target site for the reverse primers of the *mecC* PCR (*mecA*$_{LGA251}$RP and *mecA*$_{LGA251}$MultiRP (Stegger et al., 2012)) were located in a region of nucleotide sequence variability, specifically in *mecC* sequences derived from *Mammaliicocci*, the importance of which will be discussed here.

Furthermore, a distinction could be drawn between *mecC* sequences derived from *Mammaliicocci* (M*mecC*) strains and *Staphylococci* (S*mecC*) strains due to nucleotide polymorphisms that were identical between various *Mammaliicocci* strains that were not present in S*mecC* (Figures 2.2, 2.3 and 2.5). The sequence identity between these two groups was 96%, just above the 95% cut-off for these sequences to be designated *mecC* allotypes, according to guidelines on reporting novel homologues (Ito et al., 2012b) and existing literature does not make a distinction between the two, designating them as both '*mecC' allotypes*. Although the sequence identity between M*mecC* and the reference *mecC* sequence is above the new allotype designation threshold, it bears a similarity with the *mecA* allotype, *mecA1*, in that this sequence type is frequently found in *Mammaliicocci* species according to BLAST searches carried out using *mecA1* sequences (accession numbers: *M. sciuri* K11, Y13094.1; *M. sciuri* ATCC 29062, NG 047949.1; *M. sciuri* SCBM1NG 047950.1) and according to previous literature (Couto et al., 1996a; Rolo et al., 2014). It is also thought *mecA1* might represent the ancestral form of *mecA* (Rolo et al., 2017;Miragaia, 2018) so it could be hypothesised that M*mecC* might represent an evolutionary marker for *Staphylococcal mecC*.

The *mecC* primers designed in this research was shown to amplify S*mecC* as bands of the expected size were observed following DNA gel electrophoresis of the *mecC* positive control PCR products and the absence of a band for the negative controls which was *S. aureus* ATCC 9144 and a DNA template free control (blank control) . This was further confirmed via sequencing of the PCR products and comparing them to previously deposited *mecC* sequences in GenBank. However, confirmation as to whether they can amplify M*mecC* awaits as there were no appropriate controls to test this with.

Previous studies on *mecC* are subject to isolate selection bias, as these primarily were focussing upon (solely/mostly) MRSA that may be carrying this gene (Bengtsson et al., 2017; Paterson et al., 2014a; Paterson et al., 2014b), which has contributed to the limitations that currently surround our knowledge on the origin of *mecC* mediated resistance. For example, our understanding of *mecA* origins have mostly come from studies investigating the genome of non-aureus *Staphylococci* and *Mammaliicocci* (Miragaia, 2018; Rolo et al., 2017), yet the research on *mecA* negative, methicillin-resistance non-aureus *Staphylococcaceae* is limited. This bias also has potential implications on further *mecC* research, *mecC*/MRSA surveillance programmes and potentially diagnostics; *mecA* allotypes have been largely found in *Mammaliicocci* species (Miragaia, 2018) and to date nearly all *mecC* allotypes (*mecC1*, *mecC2* and *mecC3*) have also been discovered in non-aureus *Staphylococcaceae* (Harrison et al., 2013; MacFadyen, Harrison, et al., 2019a; Małyszko et al., 2014), with the exception of one M*mecC* carrying *S. aureus* isolate being recovered from a racehorse in Japan (Sekizuka et al., 2020). The aureus-centred approach slowed down laboratory efforts to isolate potential *mecC*-MRIs presented in this thesis as the initial set of *mecC* primers used were based on the *mecC* sequences derived from *S. aureus* strain LGA251(Stegger et al., 2012) and another mecC-MRSA isolate of the same clonal complex, CC130, (Cuny et al., 2011); these would have failed to amplify M*mecC* and also the previously described *mecC* allotypes due to mismatches in the primer and target sequences, which was why new primer sets were designed with the capacity to detect M*mecC* and S*mecC* in one assay. The functionality of these primers was tested using an existing *mecC* positive isolate that was derived from hedgehog faeces in a previous project which has been shown to be of S*mecC*; whether these primers amplified M*mecC* in practice was unable to be determined due to lack of M*mecC* positive isolates which is why further investigation with isolates known to carry M*mecC* is required. Despite these new primers

covering both M*mecC* and S*mecC*, an increased detection of *mecC* was not seen. This could be due to multiple factors, which this thesis will consider in Chapter 3, Section 3.

These primers were designed used an alignment of *mecC* sequences from GenBank. A total of 39 *mecC* sequences from six species of *Staphylococci* and three species of *Mammaliicocci*, with at least 90% coverage of the gene could be obtained from GenBank (Appendix B). The lack of sequenced *mecC* genes deposited into these databases limits primer design which may impact target detection during studies and/or surveillance efforts. It is therefore crucial to conduct further genomic studies on *mecC* isolates and *mecA*/*mecC* negative isolates that display phenotypic methicillin resistance. This would allow more thorough prevalence studies which would in the long term would greatly help us better understand the epidemiology *mecC* and its evolution. Of the 39 sequences used in the alignment in this project (See Appendix A), 20 of the sequences (51%) were submitted from just two studies (Belhout et al., 2023; Loncaric et al., 2019a). The characterisation study conducted by Loncaric *et al* (2019) involved screening 4299 samples from both wild and domestic animals for the presence of *mecC*; their methodology involved the use of two sets of primers previously described primers for the detection of *mecC* (Harrison et al., 2014b; Małyszko et al., 2014). Although the combination of primers used in Loncaric *et al* (2019) amplify M*mecC*, *mecC*, *mecC1* and *mecC2*, they would not have detected *mecC*3 that has been more recently described (MacFadyen et al., 2019a), whose prevalence remains unknown. These primers were designed based on *mecC* allotype sequences from isolates derived from a common source – bovine infections. This indicates that bovine populations may be a reservoir of diverse *mecC* allotypes not yet fully identified. Further studies are required involving whole genome sequencing of (WGS) both *mecC* positive and *mecA*/*mecC* negative *Staphylococcaceae* from diverse sources. The primers used by Belhout *et al* (2023) similarly only detects S*mecC*; M*mecC* was identified in this study only following WGS.

The *mecC*3 allotype was discovered in a novel *Staphylococcus* species, *Staphylococci caeli* strain 82B^{T} obtained from air sampling a commercial rabbit holding facility in Italy (MacFadyen et al., 2019a) and only shares 91% sequence homology with the reference *mecC* sequence and M*mecC*; 92% sequence identity with *mecC1* and 94% sequence identity with *mecC*2. Due to this variation, designing universal *mecC* primers that would also detect this allotype, whilst also maintaining amplicon length for sequencing purposes was challenging and could not be accomplished for this project. The allotype was originally discovered via WGS of the isolate. Phylogenetic analysis of this novel *Staphylococcal* species using core gene analysis revealed that the closest relatives were *S. edaphicus* and *S. saprophyticus* (MacFadyen et al., 2019). Phylogenetic analysis of the *mecC* genes shows that *mecC*3 allotype is most closely related to *mecC*2 that was sequenced from *S. saprophyticus* 210, indicating an evolutionary link.

From the analysis taken place in this chapter it can be concluded that further genome sequencing studies are required to elucidate the origins and true diversity of this methicillin-resistance gene, particularly of *mecC* and the associated obtained from species other than *S. aureus*. This will enable more thorough screening and surveillance programmes to detect and characterise *mecC* genes and the corresponding isolates, expanding our current understanding of the evolutionary development and dissemination of *mecC*.

3. CHAPTER THREE: *MECC STAPHYLOCOCCACEAE* FROM HEDGEHOGS AND TICKS

This chapter will detail the investigation and detection of *mecC* in ticks and hedgehog samples, including the experimental design, covering the isolation of presumptive *Staphylococci* and *Mammaliicocci*, antimicrobial susceptibility testing, screening for *mecA* and *mecC* resistance genes and the identification of the isolates retrieved in this study.

3.1. Material and Methods

3.1.1. Collection and Preparation of Samples

Ticks were obtained via three sources: South Essex Wildlife Hospital (Orsett, Grays, UK), Hornbeam Wood Hedgehog Sanctuary (Harpenden, Hertfordshire, UK) and Richmond Park (London, UK). Ticks obtained from the wildlife rescue hospitals were removed from hedgehogs by veterinary staff following their arrival at the hospitals for treatment and placed in sterile universal tubes. Where possible, faecal and skin scales samples were also collected. These ticks and other hedgehog samples were then sent via post to the University of East London (UEL, UK).

Questing ticks were collected from Richmond Park with permission from park managers as Richmond Park and this group was used as an unfed control group. Richmond Park is a known tick infested area driven by the deer population that act as hosts for *I. ricinus* ticks; ticks were collected by dragging a 1m x 1m white blanket over and through overgrown grass and bracken; ticks that attached to the blankets were removed and placed into universal containers with moistened paper towels and transported back to UEL the same day for counting and pooling of ticks; ticks were processed within four days of collection.

Ticks were identified morphologically under a microscope using x400 magnification and classified as *I. ricinus* and *I. hexagonus* based on the shape of the scutum and other distinctive features. The life stage of the tick was identified by counting the number of legs, size and presence of a genital opening. The ticks were pooled in varying numbers (detailed below) based on origin of ticks and life stage, as occasionally a large number of ticks were received from a single hedgehog from the wildlife hospital; larvae were pooled in groups of 23-33, nymphs were pooled in groups between 2-14 and adults were either processed individually or in pools of up to five adults (for further detail, please refer to Appendix B). Ticks collected from Richmond Park were pooled in groups of 10 larvae; nymphs into pools between 3-5; and adult ticks were processed individually. Tick pools were then washed in 70% ethanol for 30 seconds to decontaminate the exterior of the tick. This was followed by 2 rinses with sterile phosphate-buffered saline (PBS) to remove residual ethanol. All steps carried out were performed using aseptic technique. Ticks were then placed in a sterile 2mL screw-cap tube with 200µL of sterile PBS, homogenising mix consisting of Lysing Matrix A (Garnet) and Lysing Matrix 2 (MP Biomedical, Fisher) using the FastPrep-24 5G bead beating grinder and lysis system (Thermo Fisher Scientific, United Kingdom). Ticks were homogenised in 20 second intervals with a 5-minute rest in between each round.

Hedgehog faecal samples and skin scrapings samples were refrigerated at 4°C upon receipt and processed within five days as described in section 3.1.3.

3.1.2. Growth Media Used

Table 3.1 Growth media used in this research. Media were prepared according to manufacturer's instructions, except BHI that was additionally supplemented with 6.5% sodium chloride.

Media	**Supplier**	**Purpose**
Brain-Heart Infusion (BHI) broth	Oxoid™, Thermo Fisher Scientific; Hampshire, United Kingdom	This medium was supplemented with 6.5% NaCl and was used to cultivate *Staphylococci* and *Mammaliicocci*
Columbia blood agar base	Oxoid™, Thermo Fisher Scientific; BD Difco™, Thermo Fisher Scientific	Cultivation and propagation of *Staphylococci* and *Mammaliicocci*
Horse blood	TCS biosciences, Buckingham, United Kingdom	Used with the above Columbia blood agar base to make blood agar for cultivation and propagation of *Staphylococci* and *Mammaliicocci*
Mannitol Salt Agar (MSA)	Oxoid™, Thermo Fisher Scientific; Hampshire, United Kingdom	Identify mannitol fermentation and select for growth of *Staphylococci*
Mueller-Hinton Agar (MH agar)	Oxoid™, Thermo Fisher Scientific; Hampshire, United Kingdom	Poured to 5mm thickness for disc diffusion susceptibility testing
Mueller-Hinton Broth (MH broth)	Oxoid™, Thermo Fisher Scientific; Hampshire, United Kingdom	Used for MIC determination

3.1.3. Cultivation of Methicillin-resistant *Mammaliicocci* and *Staphylococci*

Tick and Faecal Samples

20 µL of each tick homogenate was inoculated into 6mL Brain Heart Infusion (BHI) broth enriched with 6.5% NaCl (Rasmussen et al., 2019); 19.5g of NaCl was dissolved in 300ml of BHI broth and autoclaved. This is because *Staphylococci* and *Mammaliicocci* are halotolerant (Rodríguez et al., 1996; Scybert et al., 2003), it has a lower concentration of salt compard to MSA, which contains 7.5% NaCl; high salt concentrations can inhibit growth which is why a lower concentration of salt was used for the enrichment broth. Inoculated broths were incubated overnight (18-24 hours) at 37°CA loopful (20µL) of each faecal sample was inoculated into 6mL 6.5% NaCl BHI broth and incubated as above.

Skin Samples

Skin scraping samples (n= 5) were received as stainless-steel surgical blades that were scraped against the hedgehog skin, with the exception of two samples, one of which also contained flakes of skin (S1) and the other was three quills from a hedgehog (H9998S2) which also arrived with a surgical blade. These surgical blades were transported in sterile universal containers and were received without a date of collection.

For skin scraping and scales samples, the entire contents of the sample tube, including any surgical blades used to obtain the scrapings were rinsed with BHI into the enrichment broth

supplemented with additional salt as describe above. These were then incubated overnight as above.

Isolate Cultivation and Microscopy

Following 18-24 hours turbid BHI inoculated cultures were streaked onto 6% horse blood agar and MSA agar and incubated overnight (18-24 hours) at 37°C. Horse blood agar was used to check culture purity and morphology and MSA was used to detect mannitol fermentation as this is usually associated with *S. aureus*, however this is not always the case (dos Santos et al., 2015), therefore colonies resembling *Staphylococci* that did not show mannitol fermentation were not excluded. Plates were inspected for colonies morphologically resembling *Staphylococci*; colonies that were circular, slightly convex and either whitish/yellow/off-white/cream in colour were selected and subcultured onto 6% horse blood agar to obtain a pure culture.

Gram staining was then performed on fresh, pure cultures to confirm cultures were Gram positive cocci upon light microscopy (x1000 magnification).

Antibiotic Susceptibility Testing (AST)

Gram positive cocci with cultures morphologically presenting as *Staphylococci* were then screened for resistance using oxacillin and cefoxitin discs (1µg Oxoid™ Oxacillin antimicrobial susceptibility discs and 30µg Oxoid™ Cefoxitin antimicrobial susceptibility discs; Thermo Fisher Scientific, United Kingdom) according to EUCAST guidelines. MH agar was poured to 5mm thickness and inoculum was prepared to a 0.5 McFarland standard equivalent (1.5×10^8 CFU/mL) to obtain semi-confluent growth. A *mecC* positive isolate obtained from a hedgehog faecal sample from a previous project and clinical MRSA strains EMRSA and EMRSA15 were used as methicillin resistant controls and *S. aureus* ATCC 9144 strain was used as a susceptible control. MH agar was used as this is the recommendation of EUCAST guidelines for disc diffusion due to it being a standardised medium that is free of/low in inhibitors that may affect the activity of the antimicrobial that is being tested.

EUCAST breakpoint guidelines for cefoxitin state to use 22mm as a zone diameter breakpoint for *S. aureus* and coagulase negative *Staphylococci*, except for *Staphylococcus epidermidis* and *Staphylococcus lugdunensis*; the zone diameter breakpoint to be used for the latter two species should be 27mm; for unknown isolates the guidelines recommend using breakpoints of 25mm. For oxacillin, the zone diameter breakpoint was 20mm. At this stage, isolates were unknown so a zone of 25mm was used as a breakpoint for cefoxitin resistance and an ATU (area of technical uncertainty) of 26-27mm. Isolates resistant to both oxacillin and cefoxitin were selected for further analysis. Isolates that were resistant to oxacillin but had zone diameters within the cefoxitin ATU were also selected for further analysis.

Minimum Inhibitory Concentration (MIC) testing was performed on all isolates that displayed a methicillin resistant or oxacillin resistant phenotype via disc diffusion according to EUCAST guidelines using the broth microdilution technique, with the above strains/isolates used as controls. 5120µg/L stock solutions of both oxacillin (Sigma-Aldrich) and cefoxitin (ThermoScientific) were made; 64ug/mL was used as a starting concentration in the first columns and each well contained 5×10^5 CFU/mL. Incubation conditions for the disc diffusion method and MIC testing were 35°C for 20 hours. MH broth was used as it is low in inhibitors

that may affect the activity of the antimicrobial that is being tested and is recommended by EUCAST guidelines.

3.1.4. DNA Extraction

Ticks

DNA extraction from the homogenised ticks used the DNeasy® Blood & Tissue Kit (Qiagen®; Hilden, Germany). 30µL of tick homogenate was suspended in 180µL of ATL buffer and 20µL proteinase K and incubated overnight at 56°C; DNA extraction and elution was then completed according to the manufacturer's instruction; DNA was checked to ensure extraction and purity using NanoDrop 1000 (ThermoScientific) and the samples were promptly frozen at -20°C for storage.

Bacterial Cultures

Methicillin-resistant isolates (MRI) and oxacillin resistant isolates (ORI) were grown overnight on Columbia agar base (Oxoid™, Thermo Fisher Scientific) at 37°C and DNA extraction was performed using InstaGene™ Matrix (Bio-Rad) as cultures were less likely to contain PCR inhibitors and this gave an increased DNA yield. A loop-full of each culture was suspended in 100µL of matrix in a 1.5mL Eppendorf tube and vortexed at high speed for 15 seconds; the suspension was incubated at 56°C for one hour and then vortexed as above; the suspension was then incubated at 95°C for one hour and vortexed as above. The suspensions were then centrifuged at 13,200 rpm for 5 minutes; DNA was used for PCR from the supernatant; DNA was checked to ensure extraction and purity on the NanoDrop and frozen at -20°C for storage.

3.1.5. PCR Amplification for *mecC* Screening and Characterisation

All PCR reaction mixes were prepared using the Invitrogen *Taq* DNA polymerase, recombinant kit (Thermo Fisher Scientific, United Kingdom).

A 25µL PCR reaction mix for *mecC*P1 screening of isolate and tick DNA was prepared as described in Section 2.1.3. Isolate and tick DNA was initially screened with mecC-F and mecC-R (Table 3.2) according to a previously described protocol (Stegger et al., 2012) but this was discontinued as described in Chapter Two. DNA was extracted from a *mecC* positive isolate from a previous project was used as a *mecC* positive control; *S. aureus* ATCC 9144 was the negative control; DNA free water was included as a template free control.

A multiplex PCR for the detection of *mecA*, *mecC*, *spa* genes and the *PVL* gene was performed on isolate DNA and tick DNA as a multiplex using primers described previously (Table 3.2). This assay also included PVL specific primers to detect the virulence factor Panton-Valentin leucocidin. The primers specific for *mecA* and *mecC* respectively were to detect the presence of these specific resistance genes. A 25µL reaction mixture was prepared with x1 PCR buffer, 0.4µM of each primer, 0.2mM of each dNTP (Invitrogen), 2mM of $MgCl_2$, 0.75U of *Taq* DNA polymerase and 2µL of DNA template. Thermocycling conditions were as follows: 94°C for 15 minutes followed by x30 cycles of 94°C for 30 s; 54°C for 60 s; 72°C for 60 s and a final extension of 72°C for 10 minutes. Products were visualised on 2% agarose (Sigma-Aldrich) stained with SYBR™ Safe (Invitrogen®) according to the manufacturers instruction and run with the same conditions mentioned in Section 2.1.3. DNA was extracted from a *mecC* positive isolate from a previous project was used as a *mecC* positive control for *mecC*; EMSA15 (provided by UEL) was used as positive control for *mecA*; *S. aureus* ATCC 9144 DNA was used as a negative control for *mecA*/*mecC* and was also used as a positive control for *spa* and *PVL* genes; DNA free water included as a template free control.

A 976bp *mecC* amplicon was obtained by using *mecC*P5 primers (see Table 3.2) from any tick and isolates that were *mecC* positive by either the above multiplex or *mecC*P1 PCR. A 25µL PCR mix containing the following: x1 PCR buffer, 0.4mM of each dNTP (Invitrogen), 0.4µM of both forward and reverse primers, 2mM of $MgCl_2$, 1U *Taq* and 2µL of template DNA. Thermocycling conditions were the same as *mecC*P1, as above. *mecC*P5 *a*mplicons and a single *mecC* amplicon were purified with QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instruction; products were eluted in nuclease free, molecular grade water (Ambion, Invitrogen) and sent for Sanger sequencing at DBS Genomics (Durham University). DNA was extracted from a *mecC* positive isolate from a previous project was used as a *mecC* positive control; *S. aureus* ATCC 9144 was the negative control; DNA free water was included as a template free control.

Table 3.2 Primers Used in This Research

Table 3.2 Primers used in this study. Primers in the green shaded region were used together as a multiplex PCR, as described previously.

Primers	Nucleotide Sequence (5'-3')	DNA Samples That Were Tested	Amplicon length	Reference
*mecC*P1 Forward	GGTATTGTCCCTAACAAAACACCC	Tick DNA Bacterial Isolate DNA	~701bp	*mecC* Alignment
*mecC*P1 Reverse	TGGGTTGAACCTGGTGATGT			
*mecC*P5 Forward	GGTATTGTCCCTAACAAAACACCC	Tick DNA and Bacterial Isolate DNA that tested positive for *mecC* with *mecC*P1 primers	~976bp	*mecC* Alignment
*mecC*P5 Reverse	GATAATCACTCGGGATATTTTCACC			
spa-1113F Forward	TAAAGACGATCCTTCGGTGAGC	Tick DNA Bacterial Isolate DNA	Variable	(Stegger et al., 2012)
spa-1514R Reverse	CAGCAGTAGTGCCGTTTGCTT			
pvl-F Forward	GCTGGACAAAACTTCTTGGAATAT		~85bp	(Stegger et al., 2012)
pvl-R Reverse	GATAGGACACCAATAAATTCTGGATTG			
mecA P4F Forward	TCCAGATTACAACTTCACCAGG		~162bp	(Stegger et al., 2012)
mecA P7R Reverse	CCACTTCATATCTTGTAACG			
mecC-F (*mecA*$_{LGA251}$MultiFP)	GAAAAAAAGGCTTAGAACGCCTC		~ 138bp	(Stegger et al., 2012)
mecC-R (*mecA*$_{LGA251}$MultiRP)	GAAGATCTTTTCCGTTTTCAGC			
Tuf F (TStaG422)	GGCCGTGTTGAACGTGGTCAAATCA	Identification of any *mecC* positive bacterial isolates	370bp	(Martineau et al., 2001)
Tuf R (TStag765)	TIACCATTTCAGTACCTTCTGGTAA			

Gap F (GF-1) Gap R (GR-2)	ATGGTTTTGGTAGAATTGGTCGTTTA GACATTTCGTTATCATACCAAGCTG	Identification of any *mecC* positive bacterial isolates	933bp	(Yugueros et al., 2001)
mecC F (*mecA*$_{LGA251}$MultiFP) mecC R (*mecA*$_{LGA251}$RP)	GAAAAAAAGGCTTAGAACGCCTC CCTGAATCWGCTAATAATATTTC	Tick DNA Bacterial Isolate DNA	718bp	(Stegger et al., 2012)

3.1.6. Analysis of *mecC*P5 Amplicons

Sequence files were processed using MEGA 11 and the resulting sequencing analysed via nucleotide BLAST® to compare to matching sequences in GenBank.

3.1.7. Biochemical Identification of Isolates

Isolates were tested for catalase and a selection of catalase positive MRIs and ORIs derived mostly from ticks, one faecal isolate and the *mecC* positive control isolate were identified using the Thermo Scientific™ RapID™ STAPH PLUS System (Thermo Fisher Scientific) according to the manufacturer's instructions. Isolates were grown on Columbia agar base overnight at 37°C; inoculum was prepared to a 3.0 McFarland turbidity standard using Thermo Scientific™ RapID™ 2ml Inoculation Fluid (Thermo Fisher Scientific). Resulting micro codes generated were interpreted using ERIC software (Thermo Fisher Scientific). *S. aureus* ATCC 9144 was used as a known control for identification.

3.1.8. Molecular Identification of Isolates

PCR for the house keeping genes *gap* and *tuf* were also used to identify bacterial isolates (primers seen in Table 1) (Martineau et al., 2001; Yugueros et al., 2001). For *gap* PCR a 25µL reaction volume containing x1 PCR buffer, 0.4mM of each dNTP (Invitrogen), 0.8µM of each primer, 1.25U of *Taq* DNA polymerase, 2.5mM of $MgCl_2$ and 2µL of DNA template. Thermocycling conditions were as follows: 94°C for 2 minutes, followed by 40 cycles of 94°C for 20s; 60°C for 30s; 72°C for 40s and a final extension of 72°C for 5 minutes. For *tuf* PCR, a 25µL reaction volume containing x1 PCR buffer, 2.5mM $MgCl_2$, 0.2mM of each dNTP (Invitrogen), 0.2µM of each primer, 0.5U of *Taq* and 2µL of template DNA. Nuclease free, molecular grade H_20 (Ambion, Invitrogen) was used as a negative control and *S. aureus* ATCC 9144 was used as a positive control. *tuf* and *gap* amplicons were checked on 2% agarose gel (Sigma-Aldrich) stained with SYBR™ Safe as described earlier and run at 100V for 90 minutes in TAE buffer. Products were purified and sent for sequencing as above. Received sequences were then compared to published sequences to identify matches.

3.2. Results

3.2.1. Collected/Received Samples

A total of 203 ticks at various life stages were obtained from South Essex Wildlife Hospital (SEWH) and Hornbeam Wood Hedgehog Sanctuary (HWHS) from 21 hedgehogs (labelling of some sample tubes was insufficient to determine the hedgehog of origin for some ticks). All of the ticks received from the wildlife hospitals were identified by visual inspection as *Ixodes*

hexagonus. These were split into 27 pools of adult ticks, 11 pools of nymphs and 3 pools of larvae; three ticks in individual tubes that had released eggs into the tube were each treated separately from the ticks that they had originated from. The ticks were a mixture of live and deceased, engorged and not engorged (See Appendix B for further details on ticks received and pooling of samples). In total, 17 faecal samples and 6 skin scrapings/swabs were also received from the wildlife hospitals.

From Richmond Park, a total of 155 nymphs were collected, split into 36 pools; 97 larvae collected split into 10 pools; 2 adult female ticks were collected and processed individually (See Appendix B). All collected ticks were morphologically identified as *Ixodes ricinus*, commonly known as the 'deer' or 'castor bean' tick.

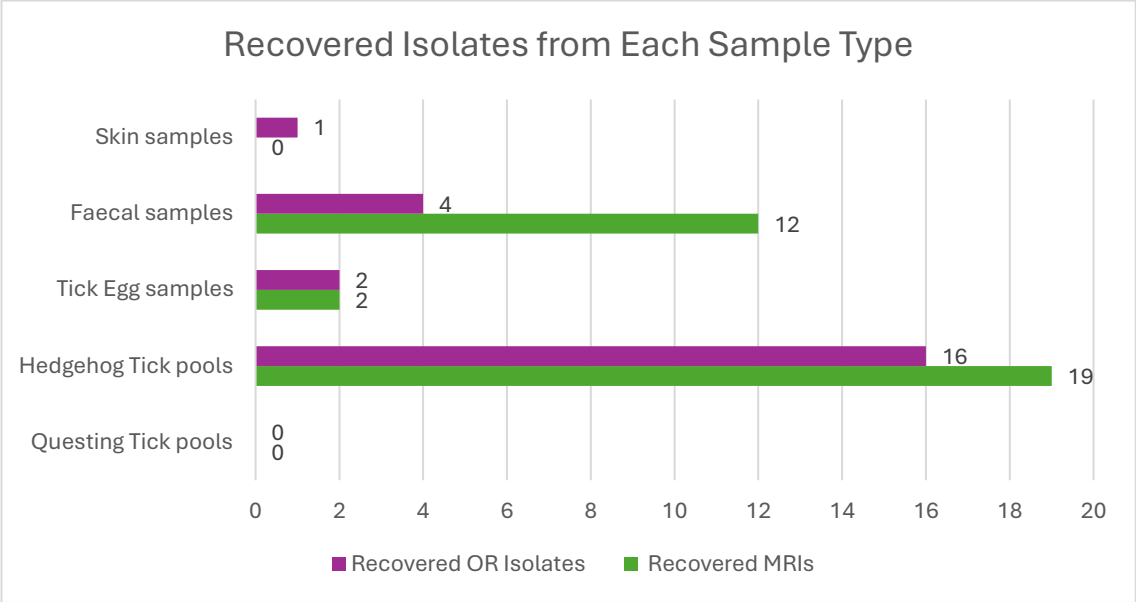

Figure 3-1 Recovered isolates from each sample type, broken down into OR and MRI isolates.

Bacterial Isolates recovered

A total of 33 Methicillin-Resistant Isolates (MRIs) were recovered. A further 21 isolates displaying resistance to oxacillin but not to cefoxitin/inhibition zone diameter within the cefoxitin disc ATU were recovered (Oxacillin Resistant/OR).

3.2.2. Hedgehog Skin and Faecal Samples

3.2.2.1. Bacteria

From the hedgehog faecal samples, 12 MRIs were isolated from eight faecal samples and four ORIs were isolated from four faecal samples. Only one ORI was isolated from the skin samples and no MRIs were obtained from this sample type.

3.2.2.2. PCR

None of the MRI and ORI isolates recovered from skin and faecal samples were positive for the *mecC* gene via PCR.

A further 21 isolates displaying resistance to oxacillin but not to cefoxitin/inhibition zone diameter within the cefoxitin disc ATU were recovered (Oxacillin Resistant/OR); 16 isolates

were obtained from 9 tick samples and 2 tick egg samples; 4 isolates obtained from 4 hedgehog faecal samples; 1 sample obtained from 1 hedgehog skin sample. No growth was observed from the pools of ticks collected from Richmond Park, thus no MRIs were isolated from that group.

No growth was observed from the pools of ticks collected from Richmond Park, thus no MRIs were isolated from this group.

3.2.3. Ticks from Hedgehogs

3.2.3.1. Bacteria

21 MRIs were isolated from 13 tick pools and two egg samples. 16 ORIs were isolated from nine tick pools and two egg samples.

3.2.3.2. PCR

In total, one isolate was recovered from tick that was positive for *mecC*, 9995Ad$_1$ via previously described *mecC* primers (Stegger et al., 2012); a 700bp amplicon was amplified from the isolate and sequenced. However, the *mecC* multiplex primers from the same research paper above did not amplify nor did the *mecC* primers designed for this research. This isolate was obtained from a pool of 4 adult female *I. hexagonus* ticks that were partially fed; they were removed from an adult male hedgehog that was being treated at the South Essex Wildlife Hospital. The MIC of this isolate was 4mg/L for both oxacillin and cefoxitin.

Another 11 isolates when tested on the multiplex showed bands between the sizes 100-200bp; *mecC* and *mecA* expected band sizes were 138bp and 162bp respectively. Subsequent PCR using the same *mecC*/*mecA* primers as a singleplex on these isolates yielded negative results for both targets. PCR with *mecC*P1 primers also yielded a negative result.

A total of three tick pools were positive for *mecC* using the *mecC*P1 primers: 9995Aa, 9995Ab and 9995Ad. All these ticks from these pools were removed from the same hedgehog. A 976bp amplicon from each of the *mecC* positive pools mentioned above was produced with *mecC*P5 primers and sequenced. The remaining 38 tick pools from the wildlife hospitals were negative.

3.2.4. Ticks from Richmond Park

3.2.4.1. Bacteria

No growth was observed in 6.5% NaCl BHI broth inoculated with the homogenate of questing ticks collected from Richmond Park, there for no isolates of any kind were obtained.

3.2.4.2. PCR

All field collected tick pools were negative for *mecC* via PCR.

Figure 3-2 Clustered bar chart of isolates recovered from various sample types. OR = Oxacillin resistant isolates; MRI = Methicillin resistant isolates.

3.2.5. Isolate Identification

A total of 19 isolates were identified using the Staph Plus system (results in Table 3.5), which includes the *mecC* positive isolate that was used as a positive control in this study; single isolate (9995Ad$_1$) was unable to be identified with the Staph Plus kit nor the *tuf*/*gap* PCR targets; one isolate tested ‘Not typeable’ when tested twice with the Staph Plus kit and due to time

considerations *tuf* and *gap* PCR was not attempted. In summary, the predominant genus identified in this study was *Staphylococci*, which made up 58% (n= 11) of identified isolates and included the following species: *S. aureus* (n= 3), *S. gallinarum* (n=1) and *S. xylosus* (n=7). This was followed by *Mammaliicocci*, which made up 37% of identified isolates (n= 7); this included the species *M. lentus* (n=3), *M. sciuri* (n=3), *M. vitulinus* (n=1). The remaining identified isolate was *Kocuria rosea*. Further identification of the remaining 37 isolates could not take place due to time considerations. Isolate 9995Ad$_1$ could not be identified with the Staph Plus kit; PCR amplification of *gap* did not yield any amplicons and PCR for the *tuf* yielded a 370bp amplicon that was not successfully sequenced and therefore could not be identified within the timeframe of this research.

Identified Isolates

Table 3.3 Isolates that were selected for identification and the results from AST testing and Staph Plus System. All isolates except 162Pb and the mecC positive control were obtained from ticks/tick eggs removed from hedgehogs. Red font indicates methicillin resistance; orange indicates an indeterminate result. Cell shading key: light blue shading shows bacterial isolates that are of hedgehog tick origin; green shading indicates bacterial isolates of hedgehog tick eggs origin; grey shading indicates bacterial isolates of hedgehog faces origin.

Isolate	Source	Identification (Staph Plus System)	Disc Diffusion (Inhibition zone mm)		OX MIC (mg/L)	FOX MIC (mg/L)
			OX	FOX		
T1a	Partially fed adult, female *Ixodes hexagonus,* SEWH	*S. xylosus*	15	25	1	2
T2b	Engorged adult, female *I. hexagonus,* SEWH	*M. sciuri*	11.5	23	2	4
T4b	Adult, female *I. hexagonus,* SEWH	*S. xylosus*	12	19	2	8
9998Aa	Adult, female *I. hexagonus,* SEWH	*S. xylosus*	13	29	1	2
9998Ab	Adult, female *I. hexagonus,* SEWH	*S. aureus*	16	28	0.5	2
HBT1c	Engorged adult, female *I. hexagonus,* HWHS	*S. xylosus*	15	25	1	2
HBT5a	Engorged adult, female *I. hexagonus,* HWHS	*M. sciuri*	11	23	1	2

HBT5b	Engorged adult, female *I. hexagonus,* HWHS	*S. xylosus*	15	24	1	2
HBT6a	Engorged adult, female *I. hexagonus,* HWHS	*M. lentus*	10	23	2	4
HBT6b	Engorged adult, female *I. hexagonus,* HWHS	*M. sciuri*	10	24	2	4
168T2a	Partially fed adult, female *I. hexagonus*, HWHS	*M. lentus*	9	23	2	4
168T3	Partially fed adult, female *I. hexagonus*, HWHS	*M. lentus*	10	23	2	4
168T4a	Partially fed adult, female *I. hexagonus*, HWHS	*S. xylosus*	15	27	1	2
168T4b	Partially fed adult, female *I. hexagonus*, HWHS	*M. vitulinus*	10	23	2	4
9995Ad$_1$	4 partially fed adult female *I. hexagonus* SEWH	Unidentified	0	15	4	4
162Tb	Engorged adult, female *I. hexagonus,* HWHS	Not typable	11	25	>64	4
162Na	Engorged *I. hexagonus* nymph, HWHS	*S. aureus*	16	27	0.5	4
HBE1c	Eggs from HBT6 (the same tick as above)	*S. xylosus*	18	29	0.5	2
H9998E	Eggs from *I. hexagonus*	*S. gallinarum*	13	28	1	2
162Pb	Hedgehog feaces	*Kocuria rosea*	0	0	>64	8
mecC Positive control isolate.	Hedgehog faecal sample from a previous project	*S. aureus*	-	-	-	-

3.2.6. Phylogenetic Analysis

BLAST analysis of the 700bp amplicon from 9995Ad$_1$ and the 976bp amplicons of 9995Aa, 9995Ab and 9995Ad showed a 100% nucleotide identity with sequences from *S. aureus* LGA$_{251}$, *S. aureus* CMFT540, *S. aureus* ST425, *S. warneri* 2800and *S. caprae* Z111.

A maximum likelihood phylogenetic tree was created using MEGA 11 based on the alignment used in the primer design, with the addition of the *mecC* amplicons from this study and can be seen in Figure 3.3. The *mecC* sequences obtained from the tick samples this study show greatest similarity to *mecC* sequences that have been found predominantly in MRSA strains.

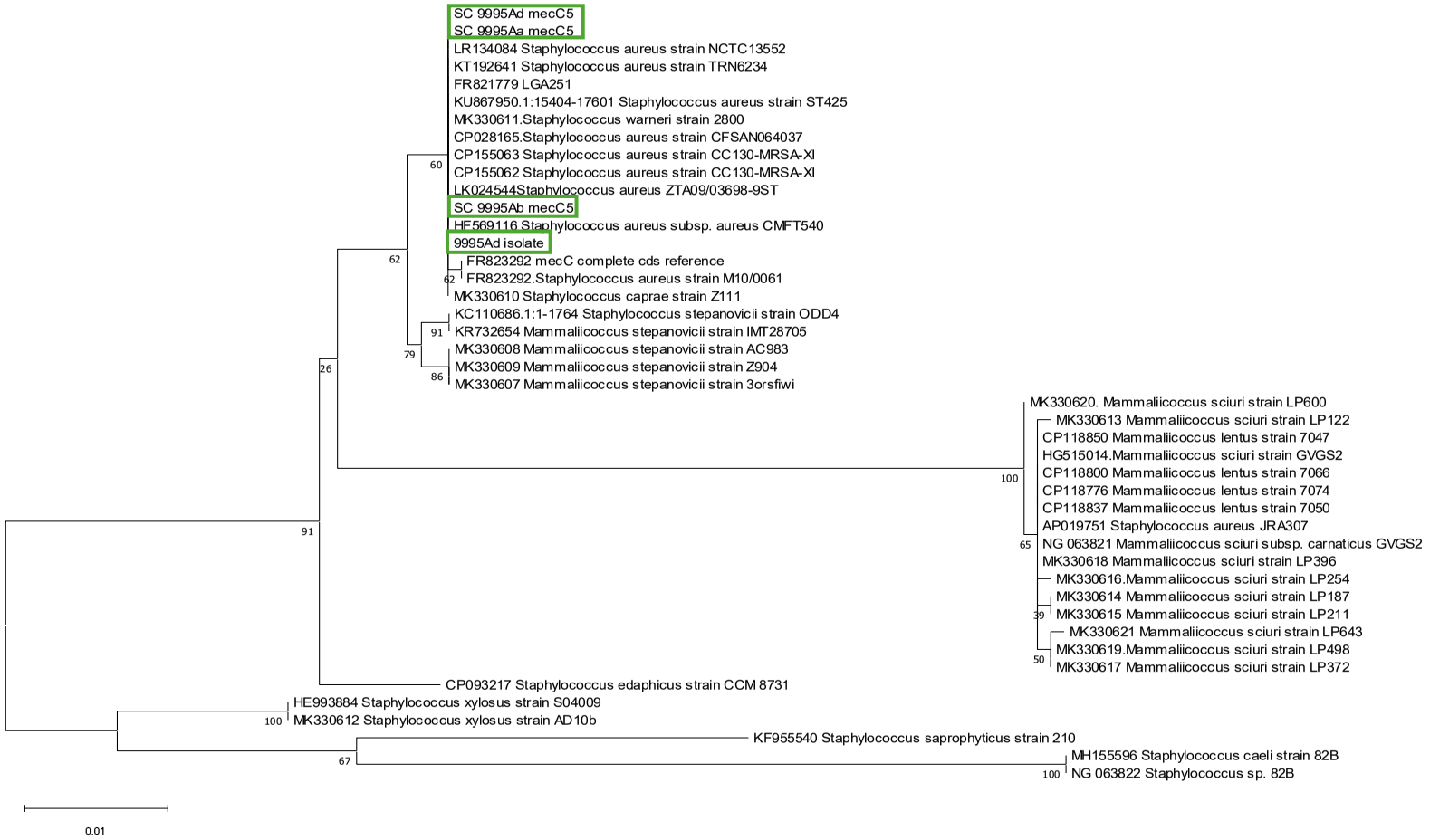

Figure 3-3 Maximum likelihood phylogenetic tree with bootstrap values set to 500 replicates showing the mecC gene fragments obtained from this research and previously published mecC sequences from various species and strains. Sequences from this study encircled in green. Sequence length ~976bp.

3.3. Discussion

The study aim was to explore the potential role of ticks and hedgehogs in the transmission dynamics of *mecC*/*mecA* carrying *Staphylococcaceae*. To do this, presumptive *Staphylococci/Mammaliicocci* were isolated from ticks and from hedgehog samples, evaluated for phenotypic resistance and presence of specific resistance genes confirmed by molecular methods. A total of 18 of the obtained isolates were also identified on a species level, however this could not be performed for all isolates due to budget and time restrictions.

In this study a total of 33 methicillin resistant isolates (MRIs) were obtained from *Ixodes hexagonus* ticks that were parasitising European hedgehogs (*Erinaceus europaeus*) of which 19 were identified. All but four of the identified isolates were obtained from either adult female ticks or from nymphs belonging to the *Ixodes hexagonus* species. The remaining identified isolates were obtained from hedgehog faecal samples (n=2) and from tick eggs that were obtained from *I. hexagonus* from the wildlife hospitals. Three MRIs were isolated from the ticks that the eggs were retrieved from, plus a further two oxacillin-only resistant isolates No isolates or any kind of growth was observed from *Ixodes ricinus* tick that were collected from Richmond Park.

3.3.1. *mecC* Isolate Detection

Of a total of 56 isolates obtained in this study only a single *mecC* positive isolate (9995Ad$_1$) was recovered from a pool of ticks removed from a hedgehog, although this result is inconclusive because when the DNA was re-extracted using the same methods after losing track of the original DNA extract, repeat testing for the *mecC* was negative, despite multiple attempts at extracting the DNA and testing with all the *mecC* specific primers used in this research. It was noted when subculturing for re-extraction that the growth of this isolate was poor, despite using appropriate growth media such as 6% horse blood agar. This weak growth could account for its failure to be identified via biochemical methods, but this does not explain why the *mecC* positive result could not be repeated. A previous study examining a *mecA* and *mecA1* positive methicillin resistant *M. sciuri* strain K3 in 2003 found that when subculturing this strain in the absence of methicillin, it would segregate into methicillin resistant (yellow coloured) and susceptible colonies (white colonies) (Couto et al., 2003). Further analysis of these susceptible colonies showed loss of *mecA2* and production of PBP2a ceased. The yellow colonies would still have the ability to express *mecA2* resistance but could continue to produce both yellow and white colonies, whereas the white colonies would only produce white colonies. Resistance could be induced in the white colonies with gradually increasing concentrations of methicillin, likely due to the presence of *mecA1*. This research suggests that acquired resistance may not be stable in the absence of selective pressure from antibiotics. The 9995Ad$_1$ isolate was not consistently exposed to oxacillin/cefoxitin when being subcultured consequently it is possible that a similar effect could have happened. Given hindsight, cultures could be stored both with or without cefoxitin to address this anomaly.

The *mecC* primers designed here were shown to amplify product of the expected base pair lengths, which could be seen upon DNA gel electrophoresis whilst the negative controls and the blank controls did not show bands of any length. However, only amplification of S*mecC* could be confirmed as there were no controls available that had the M*mecC* type. Screening of all the isolates also did not reveal any strains carrying M*mecC*, which would have shown that the primers are capable of amplifying both types. The purified products of the in-house designed primers were also sequenced and analysed and showed similarity to previously deposited *mecC* sequences in GenBank.

Despite the redesign of the *mecC* primers used to detect the *mecC* gene, an increase in detection was not observed for *mecC*/*mecA* negative MRIs. This could be for a couple reasons, the most obvious being *mecC* positive *Staphylococcaceae* simply not being present. However, there are a few other potential causes for this too. As discussed previously (Chapter 2.3), research of *mecC Staphylococcaceae* has been biased, often focusing on *S. aureus* derived *mecC*. This bias is likely to have resulted from the fact that the first *mecC* positive strain isolated was in *S. aureus* (García-Álvarez et al., 2011a) and the direct clinical relevance of this species makes it of greater interest that other *Staphylococci* to researchers. This bias affects commonly used *mecC* detection methods such as PCR and bottlenecks primer design and target detection as the designing of primers relies on deposited gene sequences, which in the case of many *mecC* gene sequences are detected by primers designed based on the sequence of the original *mecC* strain. WGS of *mecA*/*mecC* isolates that are phenotypically methicillin resistant should be considered by future studies to gain better insight into the genetic mechanism of resistance in these isolates; for example, the *mecC*3 allotype was discovered in *S. caeli* 82B via WGS (MacFadyen et al., 2019b). The initial report of *mecC* MRSA by García-Álvarez *et al* is still a relatively new discovery so there is the potential that there are more novel *mecC* allotypes that are not detectable by previously described techniques are yet to be identified.

Another possibility as to why there were many *mecA*/*mecC* negative MRIs in this study could have arisen from the *mecA* primer design/selection. BLAST analysis of the *mecA* gene (*S. aureus* strain N315; accession number in Appendix A), shows it is predominantly found in MRSA. BLAST analysis of *mecA1* sequences (*M. sciuri* strains K11, ATCC 29062, ATCC 700061 and SCBM1; accession numbers listed in Appendix A) show that this allotype has been predominantly identified in *Mammaliicocci* species, as well as *mecA2* (*Mammaliicoccus vitulinus* Strain CSBO8; accession number AM048810.2). This is corroborated by studies that found *mecA* allotypes are quite frequent in *M. sciuri* (Couto et al., 1996b; Egyir et al., 2022; Rolo et al., 2017), particularly *mecA1*, but this does not necessarily confer a methicillin-resistant profile (Couto et al., 2000) which could be due to differences in the promoter region rather than the *mecA1* gene itself (Couto et al., 2003). For example, it was shown that insertion sequences such as IS256 can hybridise with promoter regions which can result in strong promoter activity, increasing the expression of *mecA1*, suggesting that gene regulation also affects resistance phenotype (Couto et al., 2003; Maki & Murakami, 1997).

The *mecA* primers used in this study were analysed with an alignment of *mecA* allotypes, where it was found that these primers only detect *mecA*, and not its allotypes which are *mecA1* and *mecA2*. All the *Mammaliicocci* identified in this study showed phenotypic methicillin resistance but were *mecA*; *Mammaliicocci* that carry the *mecA* are typically known to carry the *mecA1* allotype, therefore the suitability of the *mecA* primers used in this study was potentially compromised which could be why some of the MRIs showed phenotypic resistance but did not yield genotypic evidence for *mecA*.

3.3.2. Direct *mecC* Detection in Homogenised Tick Samples

All tick pools (n=41) were tested the presence of *mecC* via PCR. In total 3 tick pools (7.1%) tested positive for the *mecC* gene: 9995Aa, 9995Ac and 9995Ad. A *mecC* positive isolate was recovered only from 9995Ad. This shows that despite not being able to cultivate potential *mecC* isolates directly from the ticks, PCR was still able to detect this resistance gene in tick samples. 9995Aa and 9995Ac were pools of 3 *I. hexagonus* fed females and 9995Ad was a pool of 4 *I. hexagonus* fed females; all ticks were obtained from the same hedgehog (HH9995). The three *mecC* positive tick pools all shared identical *mecC* sequences when aligned in MEGA 11, indicating that these sequences originate from a common strain which was likely obtained from a common reservoir. The sequences were identical to the *mecC* sequence amplified from the

9995Ad$_1$ isolate and the positive control isolate; it belonged to the M*mecC* type. No *mecC* isolates were obtained from the faecal and skin samples from HH9995 suggesting that this may not have been the original source of the *mecC* isolate. However, not every site from the animal was sampled, consequently *mecC* isolates may have been present in sites such as the nostrils that were not sampled in this study. Hedgehog nasal site sampling has been used in other studies (Larsen et al., 2022), but is infrequently a site of tick attachment.

The possible reasons that *mecC* isolates were not able to be cultivated from 9995Aa and 9995Ac vary. The simplest explanation is that they were simply missed because it is not possible to visually discriminate between different species or strains of *Staphylococcaceae* and whether they carry resistance genes, so they could have been missed when selecting colonies to subculture and purify. One way of improving MRI cultivation would be through increasing the selectivity of the growth medium through the addition of antibiotics; the addition 3.5mg/L cefoxitin and 20mg/L aztreonam to 2.5% NaCl tryptic soy broth improved the MRSA detection sensitivity by 18% (Böcher et al., 2010). Future studies should consider this step after inoculating samples into the 6.5% NaCl enrichment broth. Conversely, this might reduce the recovery of isolates through subjecting them to overly stringent cultivation conditions.

Another reason for detection of *mecC* genes but not being able to cultivate the source isolate could be because the detected *mecC* genes are from non-cultivable cells. A previous study in Belgium investigating bacterial community of the *I. ricinus* midgut gathered 81 ticks and extracted the midgut; one half was used to culture bacteria and the other half DNA analysis was performed to assess the bacterial community. They found that despite *Staphylococci* being detected in 28% of ticks (23/81), *Staphylococci* was only cultivated from 3.7% of the ticks (3/81) (Guizzo et al., 2022). They also tried to manipulate the gut bacteriome of the ticks through capillary feeding the ticks with *Micrococcus luteus* originally isolated from tick midguts and found that it was completely eliminated within two hours, suggesting that there is a rapid clearance of bacteria from the midgut of unfed female *I. ricinus* through an unknown mechanism. This could contribute to why *mecC* as detected in multiple tick samples without detectable presence following cultivation. Further studies have shown the constitutive production of antimicrobial peptides (AMPs) in the midgut of *I. ricinus*, referred to as 'guard' AMPs; these have shown activity against *M. luteus*, *S. aureus* and *Staphylococcus epidermidis* (Guizzo et al., 2024). Literature on the production of AMPs by *I. hexagonus* ticks is scarce but production of similar AMPs is a likely possibility as they have also been identified in other *Ixodes* species such as in *Ixodes persulcatus* and *Ixodes holocyclus* (Cabezas-Cruz et al., 2019; Saito et al., 2009). The potential production of AMPs could have led to clearance of some host/environmental bacteria in some of the tick samples in this study. This could also explain the lack of *Staphylococcaceae* cultivated from questing *I. ricinus* ticks in this study.

There were a further two tick pools of 14 partially fed *I. hexagonus* nymphs and two more tick pools of *I. hexagonus* adult females removed from the same hedgehog; none of these yielded any *mecC* positive isolates or *mecC* sequences from the extracted tick DNA nor did the skin and faecal samples from HH9995. Collectively this suggests that ticks were not acquiring host derived *mecC* carrying bacteria, but that this might reside in the tick, possibly derived from elsewhere. Nymph ticks would have fed on a previous host, but given the recovery of *Staphylococci* from egg masses, this could have been transovarially transmitted. AMR genes other than *mecC* have been demonstrated to be transmissible by this route (Wei et al., 2022).

3.3.3. Hedgehog Samples

Hedgehog skin samples failed to yield cultivable isolates of *Staphylococci*. Research has shown that *S. aureus* is able to be detected on a dry stainless-steel surface 96 hours after contamination occurs when the contamination level was high, at lower contamination levels *S. aureus* could

not be detected 48 hours after contamination (Kusumaningrum et al., 2003). If the number viable cells of potential *Staphylococcaceae* on these blades was too low then there is a possibility that any *Staphylococci* and *Mammaliicocci* present would not have survived very well and potential isolates could have been below the detection limit by the time they reached the laboratory, especially considering the storage conditions and collection date of the samples were not known when they were received. Future research should request more details when obtaining samples, such as date of collection and dispatch; an alternative to stainless steel blades to sample skin should also be considered, such as the use of a cotton tip swab with transport medium.

These results are not consistent with other studies conducted in the UK, where the *mecC* MRSA prevalence among 295 hedgehogs in England and Wales was 61.7% *(Larsen et al., 2022)*. *mecC Staphylococci* have been isolated from the faeces of small mammals such as the wood mouse (*Apodemus sylvaticus*) and common vole (*Microtus arvalis*) (Gómez et al., 2014), where *mecC* positive *Staphylococci* were detected in 13% of faecal samples (13/101). In this study, a total of there were 17 skin and faecal samples taken from hedgehogs and no *mecC* isolates were obtained from any of them. This is consistent with the 3.5% carriage rate among European hedgehogs in Finland (Venla et al., 2023) and of less than 1% in Hungary (Sahin-Tóth et al., 2022); using sample sizes of 115 hedgehogs and 200 hedgehogs respectively. These are larger scale studies that replicate the findings of this research in that *mecC* resistant *Staphylococcaceae* was not common among samples from hedgehogs. This is in sharp contrast to research in other countries investigating the prevalence of *mecC*-MRSA in hedgehogs such as in Denmark where 188 Hedgehogs were sampled and found a prevalence rate of 61% (Rasmussen et al., 2019). Another study in Sweden screened 55 hedgehogs for *mecC*-MRSA and found a carriage rate of 64% (Bengtsson et al., 2017). These differences may also be influenced by geographical differences.

No *mecC* isolates were obtained from hedgehog faeces in this study, although 37% of MRIs were isolated from the faecal samples (n=12). The above studies all use nasal and/or buccal cavity sampling as a part of their sample collection whilst the hedgehog samples used in this study were faecal or skin samples which may have impacted upon detection of *mecC*; studies on the nasal vs intestinal colonisation of *Staphylococcaceae* in European Hedgehogs are lacking so the exact role the sampling site has on the outcome is unknown. Future research should look to include nasal swabbing of the hedgehogs to make the findings more comparable to existing studies. This would also give better insight into whether there is a difference in *Mammaliicocci* and *Staphylococci* colonisation between the two sampling sites. The hedgehog sample numbers in this study were low which makes it difficult to interpret these results in a prevalence context. Another explanation for the differences in prevalence could also be the geographical location; but a larger scale study will be needed to determine this.

3.3.4. Tick Derived Isolates

In total, 56 methicillin resistant/oxacillin resistant *Staphylococcaceae* were isolated from *I. hexagonus* ticks that were removed from hedgehogs and aside from isolate 9995Ad$_1$, which was discussed above in Section 3.3.1, no *mecC* isolates were obtained from ticks removed from hedgehogs. Whether *I. hexagonus* ticks are carriers of *mecC* positive *Staphylococcaceae* has not been investigated in previous literature. No isolates were recovered from the *I. ricinus* control group.

Currently there is limited literature investigating the microbiome of *I. hexagonus* ticks despite this species of hard tick being the second most prevalent in the UK, after *I. ricinus* (*HPA - Tick Surveillance*, 2014). Existing literature generally is focussed upon established tick-borne pathogens such as *Borrelia spp, Anaplasma spp* and *Rickettsia spp*; there is however research

investigating the microbiome of other *Ixodes* tick species. Isolation and identification of a *Staphylococcal* species from *I. hexagonus* currently represents a knowledge gap. Previous studies employing molecular biology and bioinformatic techniques have shown that *I. ricinus* ticks are hosts to a range of bacterial species, mostly of the *Proteobacteria* phyla and made up 78% of the detected phyla; this includes *Sphingomonas spp, Anaplasma spp, Rickettsia, Borrelia, Klebsiella*, and others. Among these, evidence of *Staphylococci* was also reported, with a much lower abundance (Gil et al., 2020; Wiesinger et al., 2023). These microbiome studies do not often characterise the bacteria that is present further than the genus level, so identification to a species level had not been performed but it has been presumed that they would likely be *Staphylococcaceae* species that were present on the skin of the host, or derived from the bloodmeal from a vertebrate host, or acquired from the surrounding environment, such as the soil (Tóth et al., 2023).

In this study, identification of 21 isolates was attempted via a commercial kit that uses biochemical analysis, although only 19 were successfully identified as two isolates were not able to be typed. Here, 19 *Staphylococcaceae* isolates were cultivated from *I. hexagonus* ticks of which have been identified to a species level. Future research should incorporate alternative/additional methods of identification, such as utilising MALDI-TOF, WGS and other molecular methods including the amplification and analysis of housekeeping genes.

Seven isolates from this study were identified as *S. xylosus* and were all obtained from *I. hexagonus* ticks, except for one isolate, which was cultured from *I. hexagonus* tick eggs; this corroborates with a previous study where the most abundantly cultured bacteria from ticks was *S. xylosus* (Rousseau et al., 2021). This species has been isolated from a range of hosts, including wild animals, livestock, domestic animals and humans (Kloos, Zimmerman, et al., 1976; Nagase et al., 2002; Štempelová et al., 2022). This species is also very frequently associated with fermented foods such as sausages, cheeses and fermented soybean foods (Coton et al., 2010; Kong et al., 2022; Leroy et al., 2010; Sánchez Mainar et al., 2017). The isolation of *S. xylosus* from healthy human and animal populations (Kloos et al., 1976; Nagase et al., 2002) indicates that this species of *Staphylococci* may have a role as a commensal bacterium, which could explain why this species was most frequently isolated from the ticks in this study. The cultivation of this species corroborates with another study that cultivated various *Staphylococci* form *I. ricinus* ticks in Belgium (Rousseau et al., 2021).

Staphylococcus xylosus was also recovered from *I. hexagonus* eggs in this study which indicates that this species is not only picked up from the surrounding environment and hosts, but also that there may be transovarial transmission of this *Staphylococci* and potentially other species. This vertical transmission of *Staphylococci* is significant as it shows that the presence of *Staphylococcaceae* in ticks may not always exclusively linked to the surrounding environment and host(s). Further research on whether *Staphylococcaceae* persist after hatching and moulting are needed to fully understand the persistence of *Staphylococcaceae* in the *I. hexagonus* lifecycle.

Another species of *Staphylococci* was cultivated from *I. hexagonus* eggs, *Staphylococci gallinarum*; this species was first described in in 1983 and was isolated from poultry (Devriese et al., 1983) however they have been implicated in infections of humans, albeit rarely (Kaur et al., 2019; Morfin-Otero et al., 2012; Tibra et al., 2010). *Staphylococcus gallinarum* has most recently been shown to be present in all the life stages of the cowpea weevil (*Callosobruchus maculatus*) and analysis of its genome and metabolic pathways suggest the production of B vitamins, inferring this species may have a symbiotic relationship with this beetle by contributing to its nutritional ecology (Berasategui et al., 2021), indicating that this beetle may be a natural reservoir for this *Staphylococci*. There is a possibility that *S. xylosus* and *S.*

gallinarum may also have symbiotic relationship with *I. hexagonus*; this is considering that *S. xylosus* is a producer of extracellular proteases (Wang et al., 2024) with blood being rich in proteins and as mentioned above, *S. gallinarum* may be a producer of vitamins. Further studies are required to elucidate the interactions of these *Staphylococci* with ticks and if there is significance to their presence.

Three *M. lentus* isolates were also isolated from three adult female *I. hexagonus* ticks removed from 2 different hedgehogs and all three were MRIs. *M. lentus* is an animal associated *Mammaliicocci*, frequently isolated from livestock and their environment (Alexyuk et al., 2023; Belhout et al., 2023; Reydams et al., 2023). They are infrequent human pathogens but when it has been detected in clinical cases, there has also been a link to livestock/animals (Rivera et al., 2014). *M. lentus* is a commensal bacterium of many animals but it is also known to cause disease in livestock and also carry the *mecA* and *mecA* genes (Belhout et al., 2023; Kalai et al., 2021) however the isolates in this study did not test positive for either of these genes, although this could be due to primer selection, as mentioned in a previous chapter (Chapter 2).

Another *Mammaliicocci* species. *M. vitulinus* (168T4b) was also isolated from the same tick (168T4) as an *S. xylosus* isolate (168T4a) and was methicillin-resistant but was also *mecA*/*mecC* negative. Like *S. xylosus*, this has also been isolated from processed meats and fermented foods (Fettahoğlu et al., 2023; Looman et al., 2020; Nam et al., 2012) with one isolate from ground beef even carrying the *mecA2* gene. This species is also a commensal of animals and has been isolated from healthy livestock and birds (Szczuka et al., 2023; Wesołowska & Szczuka, 2023). *mecA* carrying *M. vitulinus* has also been recorded from human nasal samples, thus also able to colonise humans (Ayodo et al., 2023).

Three isolates were identified as *M. sciuri* and were obtained from female adult *I. hexagonus* ticks that were all engorged and one of which had laid eggs in the tube prior to arrival (HBT6). All isolates were methicillin resistant according to EUCAST disc diffusion method for *Staphylococci* and were not positive for *mecA* or *mecC* via PCR. *Mammaliicoccus sciuri* is known to be found in both healthy and diseased livestock, poultry and wildlife (Kloos, Schleifer, et al., 1976; Loncaric et al., 2019a; Nemeghaire et al., 2014; Ruiz-Ripa et al., 2020). *Mammaliicoccus sciuri* has also been isolated from cases of human disease, albeit rarely (Meservey et al., 2020). *Mammaliicoccus sciuri* is a potential reservoir for virulence genes such as *lukS*, encoding leukocidin; *fnbA, bbp, ebps* which encode adhesion factors; *isaB* encoding immune-evasion factor (Nemeghaire et al., 2014) and so further investigation on whether the tick derived *Mammaliicocci* also carry these and/or other virulence genes are required and whether ticks also be a reservoir for the exchange of virulence factors between *Staphylococcaceae* such as clinically important species such as *S. aureus*.

Finally, two *S. aureus* isolates were also recovered from 2 *I. hexagonus* ticks; *S. aureus* is an established opportunistic pathogen with the ability to cause severe disease (Murray et al., 2022) and is known to possess virulence factors that are associated with mobile genetic elements (MGEs) (Lindsay, 2014). *Staphylococcus aureus* has also been known to colonise many animals, including humans and importantly, hedgehogs (Olsen et al., 2013; Smith & Marples, 1964) which suggests that this species may also be from the tick host. Both strains isolated in this study only demonstrated resistance to oxacillin only however and were both negative for *mecA*/*mecC*, indicating that this resistance may be due to alternative resistance mechanisms such as the presence of beta-lactamases.

Many of these isolates are thought to have come from the surrounding environment and host that the ticks parasitise; this is especially considering that only the ticks removed from the hedgehogs showed growth upon inoculation, whereas the questing *I. ricinus* ticks showed no growth. It is unclear if there is a difference in the microbiome of *I. hexagonus* and *I. ricinus*.

Further studies investigating the presence of *Staphylococcaceae* in unfed *I. hexagonus* ticks should be considered.

Mammaliicocci were identified in ticks, which are known reservoir of virulence and AMR genes, including *mecA1, mecA2* and also M*mecC*. These genes, including virulence genes, are usually located on mobile genetic elements (MGEs) which are transferrable between *Staphylococcaceae* (Novick, 2003). This genus is of particular importance as it is thought methicillin resistance has emerged from the species belonging to this genus on multiple occasions, which eventually made their way to *Staphylococcal* species (Rolo et al., 2017). *Staphylococcus aureus* was also isolated from the ticks and the hypothetical concurrent transmission of these *Staphylococcaceae* may lead to an immune response, providing some of the conditions required for *Staphylococcal* transformation as research has recently shown that reactive oxygen species from the host immune system can upregulate competence genes which are linked to transformation (Cordero et al., 2022).

The lack of *mecA*/*mecC* detection in this study may also indicate an alternative resistance mechanism. Recently it was found that mutations in the genes encoding PBP4 and gdpP can confer a methicillin resistant phenotype, as has been found in MRLMs (Lai et al., 2024; Sommer et al., 2021). To investigate this possibility, presence of the SCC*mec* should be excluded via testing for *ccr* genes, followed by analysis of the *pbp4* and *gdpP* genes.

4. Conclusions

The findings of this study indicate that *I. hexagonus* are reservoirs of methicillin resistant *Staphylococcaceae*, including *mecC* mediated resistance. This is not only because of the ticks that were positive for *mecC* but because nearly all the MRIs were negative for *mecA* and *mecC*, implying alternative mechanisms of resistance or divergent sequence types not detected by the primers used. However, when the evidence in this thesis is combined with previous literature it indicates that *mecA*/*mecC* allotypes may be responsible but only further inspection of these isolates will clarify this; this can be done via alternative primers that are able to detect the allotypes. Alternatively, this could be achieved through the use of WGS of isolates that are not positive for *mecA* or *mecC*.

This thesis establishes *I. hexagonus* ticks as carriers of methicillin resistant *Staphylococcaceae* which raises the possibility that ticks can be vectors of AMR genes. The potential for ticks to disseminate AMR genes suggests that we need to redefine our definition of tick-borne pathogens. There is a possibility that they can be transmitted from the wild and into livestock and human populations as ticks are known to parasitise wild mammals, livestock and humans. However, before this can be concluded it is vital to definitively understand whether the *Staphylococcaceae* isolated from the ticks in this study are a part of the ‘native’ microbiome, whether they are acquired and whether they can be transmitted through blood feeding.

These findings highlight the importance of the One Health approach, particularly as research regarding AMR tends to focus on the human and livestock aspect rather than a holistic awareness of the interconnection between humans, animals and the shared environment.

To conclude, the microbiome of *I. hexagonus* includes *Staphylococci* and *Mammaliicocci* of clinical importance. Furthermore, they can possess AMR and virulence genes, including their evolutionary pre-cursors. Further research to characterise these isolates and their resistance mechanisms is required in order to determine their role in the ecology of AMR gene flow in a One Health setting.

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3 APPENDICES

Appendix A: Table of Accession Numbers Used in This Thesis

Accession number	Strain/Isolate	Source	*mecC* Allotype
KU867950	*Staphylococcus aureus* strain ST425	Domestic cat, Australia	*mecC*
FR821779	*Staphylococcus aureus* subsp. aureus LGA251	Cattle; bovine mastitis	*mecC*
KC110686	*Mammaliicoccus stepanovicii* strain ODD4	Bank vole (*Myodes glareolus*), Germany	*mecC*
MK330608	*Mammaliicoccus stepanovicii* AC983	Red fox (*Vulpes vulpes*), Austria	*mecC*
KR732654	*Mammaliicoccus stepanovicii* strain IMT28705	Faecal sample from Bank Vole (*Myodes glareolus*)	*mecC*
MK330609	*Mammaliicoccus stepanovicii* Z904	European otter (*Lutra lutra*), Austria	*mecC*
MK330607	*Mammaliicoccus stepanovicii*	Eurasian lynx (*Lynx lynx*)	*mecC*
KT192641	*Staphylococcus aureus* TRN6234	Human wound infection, Denmark	*mecC*
FR823292	*Staphylococcus aureus* strain M10/0061	85-year-old male inpatient, South East Irland	*mecC*
MK330611	*Staphylococcus warneri* strain 2800	Cat	*mecC*
CP028165	*Staphylococcus aureus* CFSAN064037	Clinical Sample	*mecC*
CP155063	*Staphylococcus aureus* strain CC130 -MRSA-XI	Diagnostic sample, Germany	*mecC*

CP155062	*Staphylococcus aureus* strain CC130-MRSA-XI	Diagnostic sample, Germany	*mecC*
LK024544	*Staphylococcus aureus* strain ZTA09/03698-9ST	River water, Wild boar and Fallow deer game estate, Spain	*mecC*
HF569116	*Staphylococcus aureus subsp. aureus* CMFT540	Clinical sample, England	*mecC*
MK330610	*Staphylococcus caprae* strain Z111	Beaver (*Castor fiber*)	*mecC*
LR134084	*Staphylococcus aureus* NCTC13552	Bulk milk tank, United Kingdom	*mecC*
CP093217	*Staphylococcus edaphicus* strain CCM 8731	Sandy soil sample, James Ross Island, Antarctica	*mecC*
NG_063821	*Mammaliicoccus sciuri* strain GVGS2	Skin infection, Cattle, England	*mecC* (*mecC*m)
MK330613	*Mammaliicoccus sciuri* strain LP122	Calf, Austria	*mecC* (*mecC*m)
AP019751	*Staphylococcus aureus* JRA307	Wound, race horse, Japan	*mecC* (*mecC*m)
CP118837	*Mammaliicoccus lentus* strain 7050	Nasal swabs from heslthy Dromedaries (*Camelus dromedarius*), Algeria	*mecC* (*mecC*m)
CP118776	*Mammaliicoccus lentus* strain 7074	Nasal swabs from healthy Dromedaries (*Camelus dromedarius*), Algeria	*mecC* (*mecC*m)
CP118800	*Mammaliicoccus lentus* strain 7066	Nasal swabs from heslthy	*mecC* (*mecC*m)

		Dromedaries (*Camelus dromedarius*), Algeria	
CP118850	*Mammaliicoccus lentus* strain 7047	Nasal swabs from heslthy Dromedaries (*Camelus dromedarius*), Algeria	*mecC* (*mecC*m)
CP118856	*Mammaliicoccus lentus* strain 7046	Nasal swabs from heslthy Dromedaries (*Camelus dromedarius*), Algeria	*mecC* (*mecC*m)
HG515014	*Mammaliicoccus sciuri* GVGS2		*mecC* (*mecC*m)
MK330619	*Mammaliicoccus sciuri* LP498	Calf, Austria	*mecC* (*mecC*m)
MK330618	*Mammaliicoccus sciuri* LP396	Cattle, Austria	*mecC* (*mecC*m)
MK330617	*Mammaliicoccus sciuri* LP372	Goat Austria	*mecC* (*mecC*m)
MK330616	*Mammaliicoccus sciuri* LP254	Alpaca, Austria	*mecC* (*mecC*m)
MK330615	*Mammaliicoccus sciuri* LP211	Goat, Austria	*mecC* (*mecC*m)
MK330614	*Mammaliicoccus sciuri* strain LP187	Goat, Austria	*mecC* (*mecC*m)
MK330621	*Mammaliicoccus sciuri* strain LP643	Sheep, Austria	*mecC* (*mecC*m)
MK330620	*Mammaliicoccus sciuri* strain LP600	Alpaca, Austria	*mecC* (*mecC*m)
HE993884	*Staphylococcus xylosus* strain S04009	Bovine mastitis	*mecC*1

MK330612	*Staphylococcus* xylosus strain AD10b	Brown rat (*Rattus norvegicus*)	*mecC*1
MH155596	*Staphylococcus caeli* strain 82B	Air sampling of commercial rabbit holding	*mecC*3
KF955540	*Staphylococcus saprophyticus* strain 210	Common shrew (*Sorex araneus* L.)	*mecC*2
NG_047949	*Mammaliicoccus sciuri subsp. sciuri ATCC 29062*	N/A	*mecA1*
Y13094	*Mammaliicoccus sciuri* strain K11	Sliced veal leg	*mecA1*
AM048810	*Mammaliicoccus vitulinus* strain CSBO8	Surgical wound of a horse	*mecA2*
NG_047938	*Staphylococcus aureus* strain N315	Clinical sample	*mecA*
NG_047950	*Mammaliicoccus sciuri* SCBM1	Dairy cow	*mecA1*
AB547235	*Mammaliicoccus sciuri* ATCC 700061	Rat	*mecA1*

Appendix B: Tick Details and Pooling

Tick pool ID	Pool Contents Ix hex = *Ixodes hexagonus* Ix ric = *Ixodes ricinus*	Origin
T1	Single adult female ix hex, not engorged; alive	South Essex
T2	Single adult engorged female ix hex, alive	South Essex

T3	Single adult female not engorged ix hex; dark in appearance alive	South Essex
T4	Ix hex adult female; alive	South Essex
T5	2 ix hex nymphs; dead; tube 3	South Essex
T6	22 larvae all dead and desiccated; tube 3	South Essex
T7	32 tick larvae all dead and desiccated; tube 3	South Essex
T8	23 tick larvae all dead and desiccated; tube 3	South Essex
H9998N	2 ix hex nymphs, dead	South Essex
H9998E	Eggs from H9998A	South Essex
H9998A	Ix hex adult F	South Essex
H9913A	1 desiccated ix hex adult female	South Essex
H9913E	Eggs from H9913A	South Essex
H9995Na	14 ix hex fed nymphs, dead	South Essex
H9995Nb	14 ix hex fed nymphs, dead	South Essex
H9995Aa	3 adult ix hex female fed adults	South Essex
H9995Ab	3 adult ix hex female fed adults, dead	South Essex
H9995Ac	3 adult ix hex female fed adults, dead	South Essex
H9995Ad	4 adult ix hex fed female adults, dead	South Essex
H9995A	4 ix hex female adults, dead	South Essex
H10009Aa	3 ix hex female adults, engorged, dead	South Essex
H10009Ab	3 ix hex female adults, engorged, dead	South Essex
H10009Ac	2 ix hex female adults, engorged, dead	South Essex
H10009Na	10 ix hex nymphs, dead	South Essex
H10009Nb	10 ix hex nymphs, dead	South Essex
H10009Nc	10 ix hex nymphs, dead	South Essex
H10009Nd	10 ix hex nymphs, dead	South Essex
H10009Ne	5 ix hex nymphs, dead	South Essex
HBT1 (Hedgehog No. 2408)	Ix hex, adult female fed/engorged, alive	Hornbeam
HBT2 (Hedgehog No. 2408)	Ix hex adult female fed/engorged dead	Hornbeam
HBT3	Adult female ix hex engorged dead	Hornbeam
HBT4	Adult female ix hex engorged dead	Hornbeam
HBT5	Adult female ix hex engorged dead	Hornbeam
HBT6	Adult female ix hex engorged dead with eggs	Hornbeam

HBE1	Eggs from HBT6	Hornbeam
HBN1	Ix hex nymph dead	Hornbeam
168T1	Ix hex adult female, alive, partially fed	
168T2	Ix hex adult female, alive, partially fed	Hornbeam
168T3	Ix hex adult female, alive, partially fed	Hornbeam
168T4	Ix hex adult female, alive, partially fed	Hornbeam
162T	Adult ix hex female, engorged alive	Hornbeam
162N	Ix hex nymph alive engorged	Hornbeam
115T	Engorged ix hex adult female dead	Hornbeam
L1	7 ix ric larvae, questing	Field collected from Richmond Park 25/05/2024
AT1	Ix ric adult female, questing	Field collected from Richmond Park 25/05/2024
N1	4 ix ric nymph, questing, dead upon arrival to lab	Field collected from Richmond Park 25/05/2024
N2	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N3	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N4	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N5	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N6	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N7	of 4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N8	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N9	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N10	ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N11	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N12	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N13	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N14	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N15	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024

N16	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N17	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N18	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N19	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N20	4 ix ric nymph,questing	Field collected from Richmond Park 25/05/2024
N21	4 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N22	3 ix ric nymph, questing	Field collected from Richmond Park 25/05/2024
N23	3 ix ric nymph, questing , dead upon arrival to lab	Field collected from Richmond Park 25/05/2024
RN1	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN2	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN3	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN4	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN5	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN6	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN7	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN8	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN9	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN10	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN11	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN12	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RN13	5 ix ric nymph, questing	Field collected from Richmond Park 19/07/2024
RL1	10 ix ric larvae, questing	Field collected from Richmond Park 19/07/2024
RL2	10 ix ric larvae, questing	Field collected from Richmond Park 19/07/2024
RL3	10 ix ric larvae, questing	Field collected from Richmond Park 19/07/2024
RL4	10 ix ric larvae, questing	Field collected from Richmond Park 19/07/2024
RL5	10 ix ric larvae, questing	Field collected from Richmond Park 19/07/2024
RL6	10 ix ric larvae, questing	Field collected from Richmond Park 19/07/2024

RL7	10 ix ric larvae, questing	Field collected from Richmond Park 19/07/2024
RL8	10 ix ric larvae, questing	Field collected from Richmond Park 19/07/2024
RL9	10 ix ric larvae, questing	Field collected from Richmond Park 19/07/2024
RT1	Ix ric adult female questing	Field collected from Richmond Park 19/07/2024