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SHORT COMMUNICATION

Random amplified polymorphic DNA analysis reveals no clear link between Staphylococcus epidermidis and acute mastitis

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Mastitis is commonly experienced by breastfeeding women. While *Staphylococcus aureus* is usually implicated in infectious mastitis, coagulase-negative staphylococci (CoNS) are a possible alternative pathogen. This case-control study examined the role of CoNS in mastitis using isolates cultured from breast milk of 20 women with mastitis and 16 women without mastitis. Gene sequencing determined bacterial species, and random amplified polymorphic DNA (RAPD) analysis investigated strain-level variation. The majority of CoNS isolates were *Staphylococcus epidermidis* (182/199; 91%). RAPD analysis identified 33 unique *S. epidermidis* profiles, with no specific profile associated with mastitis cases.

KEYWORDS

mastitis, *Staphylococcus epidermidis*, coagulase-negative staphylococci, case-control study

INTRODUCTION

Mastitis affects approximately 20% of breastfeeding women,[1] and is the most common postpartum maternal condition.[2] *Staphylococcus aureus* is the most common pathogen isolated in infectious mastitis.[3,4]

Coagulase-negative staphylococci (CoNS), particularly *Staphylococcus epidermidis,* are found in 70–100% of healthy breastmilk samples, and are regarded as part of the normal milk or skin microbiota.[3,5] However, they can also be opportunistic pathogens,[6] and studies have reported *S. epidermidis* was more prevalent in the milk of women with mastitis than *S. aureus*,[7]

suggesting a causative role.[8] These findings are in contrast with studies that found CoNS unrelated to mastitis.[3,5] The implication that *S. epidermidis* could be a pathogen in mastitis has consequences for the antibiotic treatment of the condition – as they are likely to be resistant to flucloxacillin, the usual first-line treatment for mastitis.[9]

This study was a nested case-control design examining the role of *S. epidermidis* in mastitis by investigating strain variation through random amplified polymorphic DNA (RAPD) analysis, in isolates cultured from breastmilk of both healthy women and those with mastitis.

MATERIALS AND METHODS

This was a sub-study of the *Candida* and *Staphylococcus* Transmission: Longitudinal Evaluation (CASTLE) study.[10,11] Women were recruited in pregnancy from two hospitals in metropolitan Melbourne, and followed until eight weeks postpartum, with milk samples collected in hospital after the birth and then weekly for four weeks postpartum.[10,11] Mastitis was defined as at least two breast symptoms (pain, redness or lump) and at least one of fever or flu-like symptoms.[1] Cases were women (n = 20) who had mastitis as defined in the first four weeks postpartum. Controls (n = 16) were matched in time and by recruitment site by selecting the next participant without symptoms of mastitis up to eight weeks postpartum from the same recruitment site as the case.

An average of five isolates from each participant were selected on the basis of morphology to obtain a diversity of isolates and minimise the overrepresentation of individual clonal types. Case isolates were chosen from milk of the breast affected by mastitis within one to two days of mastitis onset. Control isolates were selected to represent milk from both breasts.

Study inclusion/exclusion criteria and milk collection protocols have been previously described.[10] Milk samples were inoculated onto MacConkey agar (Thermo Fisher Scientific). After overnight incubation at 37°C, one well-isolated colony was sub-cultured onto mannitol salt agar (Thermo Fisher Scientific). Any mannitol-fermenting isolates (tentative *S. aureus*) were subject to a coagulase test (latex agglutination) prior to being considered tentative CoNS. DNA was extracted from tentative CoNS isolates using the MagNA Pure 96 system (Roche Applied Science). Isolates were identified through polymerase chain reaction (PCR) amplification of either the *S. epidermidis tuf* gene or 16S ribosomal RNA gene in cases where *tuf* gene PCR failed to produce the correct size amplicon, followed by Sanger DNA sequencing (Australian Genome Research Facility Ltd., Melbourne). Bacteria were identified to the species level by comparing sequences to those on the National Center for Biotechnology Information Genbank database using Basic Local Alignment Search Tool, using a minimum 98% similarity cut-off. Cycling conditions for the RAPD PCR mixtures were adapted from Mahenthiralingam et al.[12] DNA bands on RAPD gels were sized manually, comparing to HyperLadder IV DNA ladder standards (Bioline); bands judged to be of lower intensity than the 100 bp band were excluded from analysis.

Data were analysed using PRIMER Version 6.1.14. The degree of homology between samples was calculated using a similarity presence/absence model and a Jaccard similarity coefficient for each band present in each sample. Cluster analysis multidimensional scaling (MDS) plots were generated to enable visualisation of clustering of case and control samples. An analysis of similarity (ANOSIM) was conducted to confirm results of the MDS plots. Further, RAPD cluster groups were defined, taking into account the RAPD profiles of both primers that underwent manual scoring by two individuals, and were analysed using Stata (v14. StataCorp) logistic regression taking into account the number of samples per participant.

Informed consent was obtained from all individual participants included in this study. This study was approved by the La Trobe University Human Ethics Committee (06–078); Human Research Ethics Committee of the Royal Women's Hospital (06/41); Human Research Ethics Committee of the University of Melbourne (1033949); and Medical Advisory Committee at Frances Perry House.

RESULTS

The mean age of women in the mastitis group was 31.5 years (range 27–38) and the controls 32 years (range 24–42) years; 80% (16/20) of cases and 82% (13/16) of controls were educated to tertiary level. Overall in the CASTLE study, 45% had a caesarean delivery:[11] cases 55% (11/20) and controls 44% (7/16). There were no differences in sociodemographic characteristics between cases and controls.[13]

A total of 104 bacterial isolates analysed from 16 women who did not develop mastitis consisted of *S. epidermidis* (n = 96, 92.3%), *S. hominis* (n = 5, 4.8%), *S. capitis* (n = 2, 1.0%), and *S. lugdunensis* (n = 1, 1.9%). A total of 110 isolates from 20 women with mastitis comprised *S. epidermidis* (n = 86, 90.5% of CoNS isolated), *S. hominis* (n = 5, 5.3% of CoNS isolated), *S. capitis* (n = 1, 1.1% of CoNS isolated), and *S. lugdunensis* (n = 3, 3.2% of CoNS isolated). A further 15 non-CoNS isolates were identified from this sample, mainly *S. aureus* (n = 10). These ten *S. aureus* isolates were from seven participants in the mastitis group. Non-CoNS bacteria were excluded from further analysis. Overall, 96 and 86 isolates of *S. epidermidis* from controls and cases respectively were analysed by RAPD PCR.

The 208-primer produced 1–9 bands per isolate, with 43% of samples returning more than four DNA bands. The 272-primer produced 1–7 bands per sample, with 34% of samples returning more than four DNA bands per sample. Multidimensional scaling plots comparing the RAPD profiles from the mastitis samples to those with no mastitis are shown for both the 272 and 208 primer (Fig. 1). There was no distinct clustering of cases or control samples, and overall no differences were observed

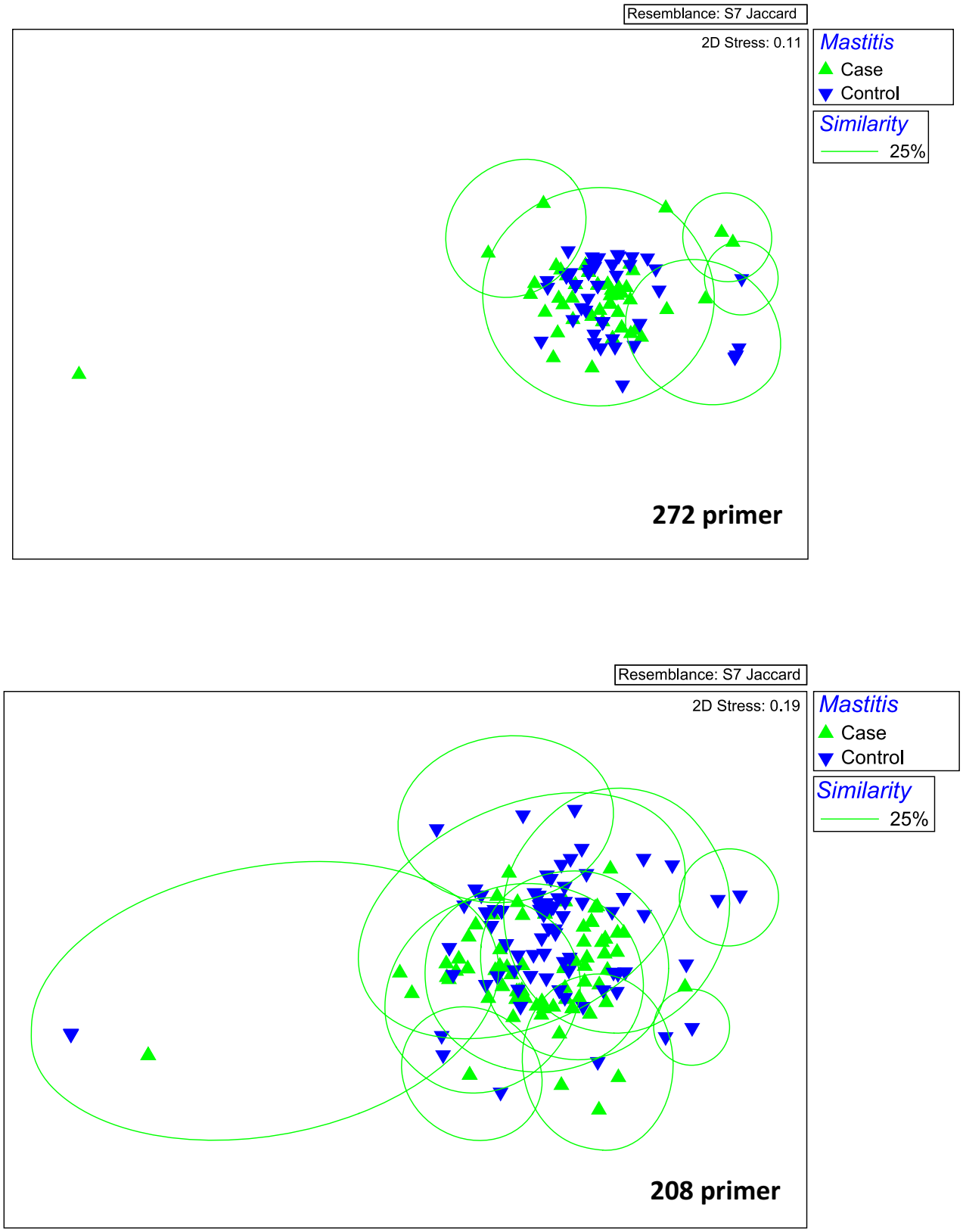

FIGURE 1 Multidimensional scaling plot of isolates based on random amplified polymorphic DNA (RAPD) profiles (272 and 208 primer) for all cases and controls.

between these two groups for either primer (208: ANOSIM *R* value = 0.036, 0.6% significance; 272: ANOSIM *R* value = 0.095, 0.1% significance).

Classification of these samples based on the combined RAPD results resulted in 33 RAPD profiles (Table 1). While there was no significant difference in the distribution of the majority of profiles

TABLE 1 *Staphylococcus epidermidis* RAPD profile types analysed by participant group

	Mastitis cases (n = 20)		Controls (n = 16)		
RAPD profile	**Number of participants**	**Number of samples**	**Number of participants**	**Number of samples**	***P*-value[†]**
1A	6 (30.0%)	**9 (10.6%)**	10 (62.5%)	**27 (28.4%)**	**0.013**
1B	9 (45.0%)	27 (31.8%)	11 (68.8%)	29 (30.5%)	0.903
1C	2 (10.0%)	2 (2.4%)	3 (18.8%)	5 (5.3%)	0.387
1D	-	ND	2 (12.5%)	5 (5.3%)	0.892
1E	2 (10.0%)	2 (2.4%)	4 (25.0%)	7 (7.4%)	0.203
1F	1 (5.0%)	1 (1.2%)	2 (12.5%)	3 (3.2%)	0.399
1G	3 (15.0%)	6 (7.1%)	1 (6.3%)	1 (1.1%)	0.122
1H	4 (20.0%)	10 (11.8%)	-	ND	0.576
1I	2 (10.0%)	2 (2.4%)	-	ND	0.744
2B	-	ND	3 (18.8%)	3 (3.2%)	0.847
2I	1 (5.0%)	2 (2.4%)	-	ND	0.744
3A	4 (20.0%)	6 (7.1%)	1 (6.3%)	1 (1.1%)	0.075
3B	2 (10.0%)	2 (2.4%)	2 (12.5%)	6 (6.3%)	0.263
4A	1 (5.0%)	1 (1.2%)	1 (6.3%)	2 (2.1%)	0.665
7H	1 (5.0%)	3 (3.5%)	-	ND	0.723
Rare types[‡]	13	12	5	6	
Total	20	85[§]	16	95[§]	

Bold indicates $P < 0.01$.

ND, not detected; RAPD, random amplified polymorphic DNA.
[†]Logistic regression taking into account multiple samples per participant.
[‡]Only detected once in this study.
[§]RAPD profile could not be obtained for one sample.

between the case and control samples, profile 1A was more frequently identified in control samples (P = 0.013). In addition, several profiles were found to only occur in mastitis cases (1H, 1I and 7H), although numbers were too low to show statistical significance. No differences were observed between the left and right breast samples of those women who did not develop mastitis (data not shown).

DISCUSSION

S. epidermidis was the primary CoNS identified in this study, found in both mastitis and non-mastitis samples. RAPD analysis showed no clear link between *S. epidermidis* types and acute mastitis, although it did reveal a level of intraspecies diversity in the *S. epidermidis* isolates typed. RAPD profile 1A, based on the combination of two RAPD patterns was found to be significantly associated with milk of women who did not develop mastitis. No RAPD pattern was found to be significantly associated with cases of mastitis, although three profiles were found to only occur in mastitis cases (numbers too low for significance).

Our results are in agreement with others suggesting that *S. epidermidis* is not associated with mastitis.[3,14] They also agree with a small-scale metagenomics study from Spain;[15] however, this Spanish study with five cases of acute mastitis contradicts earlier findings from the same group which suggested *S. epidermidis* has a pathogenic role in mastitis because they were more likely to be isolated than *S. aureus* in mastitic milk samples.[7,16] These latter two studies were either small scale (n = 20),[7] or did not include a control group.[7,16]

From a disease mechanism viewpoint, the role for *S. epidermidis* in mastitis is still unclear. Delgado and colleagues compared the potential virulence traits and resistance to antibiotics in isolates of *S. epidermidis* from milk of 12 healthy women and 30 women with mastitis, and found an association between strains isolated from mastitic milk, the biofilm-related *icaD* gene, and resistance to several antibiotics.[8] However, the relationship between biofilm production and mastitis is unclear. The dairy industry is active in mastitis research, yet there is no evidence that biofilm production occurs *in vivo* in mammary tissue, and a bovine study found over 75% of *S. aureus* and CoNS are able to build biofilms, indicating this may not be a key factor in virulence.[17] On the other hand, it has also been suggested that *S. epidermidis*, rather than causing mastitis, may play a protective role. One study found that other bacteria in milk, including *S. epidermidis* inhibit the growth of *S. aureus*.[18]

There were several limitations to our study. First, there are more defined genotyping techniques that could have been

explored for this work such as pulsed-field gel electrophoresis, multi-locus sequence typing or genomic sequencing,[7,8,19] although RAPD typing has previously been successfully used in this context.[20] The primary bacteria isolated from samples was *S. epidermidis,* and as such we cannot comment upon the association of other CoNS and mastitis. This being said, the protocol implemented for CoNS isolation should not favour *S. epidermidis* growth over other related species.

Overall this study did not find evidence of an association between *S. epidermidis* and mastitis; however, given the intraspecies diversity seen in *S. epidermidis* isolated from human breast milk, more research is warranted to determine whether particular strains may provide a possible protective effect or play a pathogenic role.

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