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Epidemiological and molecular evidence of a monophyletic infection with *Staphylococcus aureus* causing a purulent dermatitis in a dairy farmer and multiple cases of mastitis in his cows

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SUMMARY

An epidemiological and molecular investigation of a cutaneous suppurative infection with *Staphylococcus aureus* in a dairy farmer, occurring concurrently with an outbreak of clinical mastitis in his herd, was carried out. A common aetiology for the diseases in the farmer and his cows was established by combining clinical evidence with a molecular genomic analysis of the bacterial isolates using pulsed field gel electrophoresis of DNA macro-restriction fragments. This case indicates the possibility of the emergence and circulation of anthropozoonotic clones of *S. aureus* in dairy herds. It also provides further evidence of the severe impact of infection with highly virulent clones on dairy lactating cattle.

INTRODUCTION

Bacteria belonging to the species *Staphylococcus aureus* are important causative agents of nosocomial and community-acquired infections in human beings worldwide. New infections, or endogenous organisms colonizing skin and mucous membranes, cause skin infections as well as respiratory, bone, joint and internal-organ infections, endovascular disorders, septicaemia and toxic shock syndrome [1, 2]. Skin infections with *S. aureus* are common in ambulatory routines, and the importance of nasal carriage of these bacteria for development of cutaneous lesions has been confirmed [3, 4]. In cattle, *S. aureus* is one of the most important causes of clinical and subclinical mastitis worldwide.

In the dairy farm econiche, the close physical bovine–human interaction, coupled with a high likelihood of skin cuts and abrasions may theoretically facilitate the cycling of pathogenic clones of *S. aureus* between man and cattle. A number of studies support this hypothesis and the general notion of human–animal strain cycling, whereas others have found that humans and cattle sharing the same environment were colonized with genetically distinct subpopulations of *S. aureus* and suggested that this clone cycling is not likely to occur [5–11]. Be that as it may, infection with *S. aureus* is not currently regarded as an occupational disease of milkers, while the presence of infected stock-handlers on the farms is not generally considered a risk factor for bovine mastitis.

We report the results of an investigation of an outbreak of bovine mastitis caused by a *S. aureus* clone also responsible of a purulent cutaneous infection in the farmer. The possible routes of transmission and the potential medical and epidemiological implications are discussed.

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CASE BACKGROUND

Between April 2001 and December 2002, an owner of a New Zealand dairy herd of 160 lactating cows suffered from repeated episodes of cutaneous infections in a variety of sites in his body. According to his medical records, the farmer visited his physician (a co-author of this paper) at irregular intervals on at least seven different occasions during that period, for cutaneous lesions appearing on the soft tissue in the nose and upper lip, boils on the axilla, hip and forearm and paronychias. The conditions were treated with antibiotic creams and disinfectant baths, but full courses of oral amoxicillin/clavulanic acid, flucloxacillin and clindamycin were prescribed on three different occasions. According to the farmer, while casual knocks and grazes tended to heal spontaneously, these lesions seemed to appear with no connection with skin trauma and heal during the antimicrobial courses, with new lesions appearing after drug withdrawal. The farmer had been dairy farming for approximately 35 years and, prior to this period, generally enjoyed good health. He managed the farm with the help of one part-time worker, and his occupation required an intensive daily physical interaction with his cows, mainly during milking. Due to the seasonal nature of the herd, each year all the cows were dried off during June and July.

On one occasion (in April 2001), a blood biochemistry panel, complete blood cell count and a bacteriological analysis for *S. aureus* from a swab taken from the anterior nares were performed. Haematological values were within the normal range, except for a moderate hypercholesterolaemia. A 'heavy growth' of *S. aureus* was reported but the bacterial isolate was discarded.

In early March 2002, the farmer's veterinarian (a co-author of this paper) contacted a staff member at Massey University, to discuss the possibility of the condition being transmitted to, or from his cows, because according to the farmer, there was an increased number of cases of clinical mastitis in the herd, which he associated with his skin disease. This hypothesis could not be corroborated because bacterial isolates from the farmer's lesion and from cows with mastitis were not available.

In the last week of August 2002, the farmer presented again with a round, circumscribed and bulging lesion of approximately 3 cm in diameter on his arm, filled with a purulent, watery-creamy exudate. In the clinic, pus was aseptically extracted from the abscess and a swab was sent to a commercial human laboratory for bacteriological analysis. The microbiological analysis of the pus resulted in a growth of *S. aureus*, sensitive, according to the standard disk diffusion test, to flucloxacillin, erythromycin, tetracycline, and sulphamethoxazole–trimethoprim. No other antimicrobials were tested. The isolate was shipped to the microbiology laboratory at Massey University, for future reference. One week later, the farmer recognized three new cases of clinical mastitis in his cows. He collected composite milk samples from two of the affected cows before culling them and retained the third cow in the herd for milk sampling by a third person. Individual quarter milk samples were taken from the third cow by one of the authors after cleaning and disinfecting the teat and teat orifice with ethanol-impregnated wipes and eliminating the first milk streaks. The two composite milk samples and four single-quarter samples from the third cow were sent to the veterinary diagnostic laboratory at Massey University. Pure cultures of *S. aureus* grew from three of the four single-quarter samples and from both composite milk samples. One isolate from each cow was conserved for future reference. At this point, the farmer decided to start culling cows showing signs of mastitis, but he was asked to collect composite milk samples before culling them and to send the samples for bacteriological analysis. Until February 2003, seven new isolates of *S. aureus* were collected from different affected cows on the farm. In total, 10 confirmed cases of clinical mastitis caused by *S. aureus* were diagnosed on the farm between September 2002 and February 2003, at an approximate incidence rate of 10 cases/900 cow-months. In March 2003, the farmer was found to be positive again for nasal carriage of *S. aureus* and one isolate from the nasal swab was submitted to Massey University for future reference.

MATERIAL AND METHODS

Bacterial strains

The isolate from the farmer's skin abscess and the 10 isolates from his cows were plated on 5% sheep blood agar plates and incubated at 37 °C in aerobic conditions, for 24 h. One colony, morphologically identified as *S. aureus*, was picked and streaked on a new 5% sheep blood agar plate and incubated as before. Subsequently, heavy bacterial inoculum were suspended in cryovials containing nutrient broth with

15% (v/v) glycerol and suspensions frozen at −70 °C until analysed.

In March 2003, the isolates were genotyped by pulsed-field gel electrophoresis (PFGE) of DNA macro-restriction fragments, a highly discriminatory typing method widely used for epidemiological tracking of *S. aureus* [12–17]. Fifteen epidemiologically unrelated *S. aureus* isolates were included in the analysis as a quality control measure, as recommended by Tenover et al. [18]. These isolates were randomly selected from a collection of strains isolated by a diagnostic laboratory from cows in different herds, but from the same district and milking season. Before genotyping, all the isolates were re-identified by phenotypic tests, comprising subculture on 5% sheep blood agar plates aerobically for 24 h at 37 °C, assessment of haemolysis and pigment production, a Gram stain, a catalase test, a tube rabbit plasma coagulase test and sensitivity to novobiocin and polymyxin B by the disk diffusion tests, as described previously [19]. The human isolate and the three isolates from cows isolated a week later were tested for resistance to staphylococcal β-lactamase-stable penicillins by the oxacillin disk diffusion test, according to the New Zealand guidelines for the control of methicillin-resistant *S. aureus* [2]. Following that, the 26 isolates were analysed by PFGE of DNA macro-restriction fragments. The nasal isolate reached Massey's facilities afterwards and it was therefore treated by the same methods, separately.

DNA extraction and digestion, bands resolution with PFGE and gel analysis

DNA was prepared according to the methods of Smith and Cantor [20] and Vanderlinde et al. [21] with a number of modifications. The *S. aureus* strains were grown overnight on 5% sheep blood agar at 37 °C. The cells were harvested into 3 ml brain heart infusion broth and the optical density of the broth was measured and adjusted to 1·4 at 610 nm. A 150 μl aliquot of cells was pelleted by centrifugation at 13 000 rpm (12 000 ***g***) for 5 min. Cells were washed once with 150 μl of Pett IV buffer [1 M NaCl, 10 mM Tris–HCl (pH 8·0), 10 mM EDTA (pH 8·0)] then centrifuged and resuspended in 50 μl Pett IV buffer. The bacterial suspension was mixed with 100 μl of 1% low melt preparative grade agarose (Bio-Rad Laboratories, Hercules, CA, USA) and was dispensed into plug moulds. After being solidified for 1 h on ice, the plugs were incubated overnight at 37 °C in a lysis buffer solution [1 M NaCl, 100 mM EDTA (pH 8·0), 6 mM Tris–HCl (pH 8·0), 0·2% sodium deoxycholate, 0·5% sodium lauroyl sarcosine] containing 1 mg of lysozyme (Roche Diagnostics, Indianapolis, IN, USA) and 50 μg lysostaphin (Sigma, St. Louis, MO, USA) per ml. The plugs were transferred into 1 ml ESP buffer (0·5 M EDTA, 1% sodium lauroyl sarcosine) containing 1 mg proteinase K (Roche Diagnostics) per ml and were incubated overnight at 56 °C. Following lysis, the plugs were washed five times for 1 h each time with 10 ml of TE buffer (10 mM Tris–HCl, 1 mM EDTA), on ice. A 3 mm slice of each plug was equilibrated in 100 μl of 1·2× restriction buffer (New England Biolabs, Beverly, MA, USA) for 45 min on ice. The restriction buffer was removed and replaced with 100 μl of fresh 1× restriction buffer containing 30 U of *Sma*I (New England Biolabs). The plug slices were held on ice for an additional 45 min before an overnight incubation at 25 °C. The macro-restriction fragments were separated by PFGE at 6 V/cm, with ramped pulse times of 0·5–5 s for 12 h and 10–20 s for 10 h, on a CHEF-DRII system in a 1% pulsed field certified agarose gel (Bio-Rad Laboratories) immersed in 0·5× TBE buffer [45 mM Tris, 45 mM boric acid, 1 mM EDTA (pH 8·0)] at 14 °C. Lambda ladder PFG marker and low-range PFG marker (New England Biolabs) were included as molecular size standards. Gels were stained with ethidium bromide and images were captured under UV illumination by the Gel Doc 2000 (Bio-Rad Laboratories) (Fig. 1). The DNA banding patterns were analysed using Diversity Database software (Bio-Rad Laboratories), with a maximum position tolerance of 1% for the calculation of Dice correlation coefficient. Cluster analysis was performed with the unweighted pair group method with arithmetic mean (UPGMA) using the similarity matrix of Dice correlation coefficients generated by the software. During the human–software interaction a blinding procedure was applied, so the investigator was not aware which isolate belonged to each lane on the gel, except the human isolate.

The nasal isolate reached the laboratory after completion of the above-mentioned phases and it was analysed by the same method, but in a separate analysis.

RESULTS

For convenience, the terms indistinguishable, closely related (up to 3-band difference), possibly related

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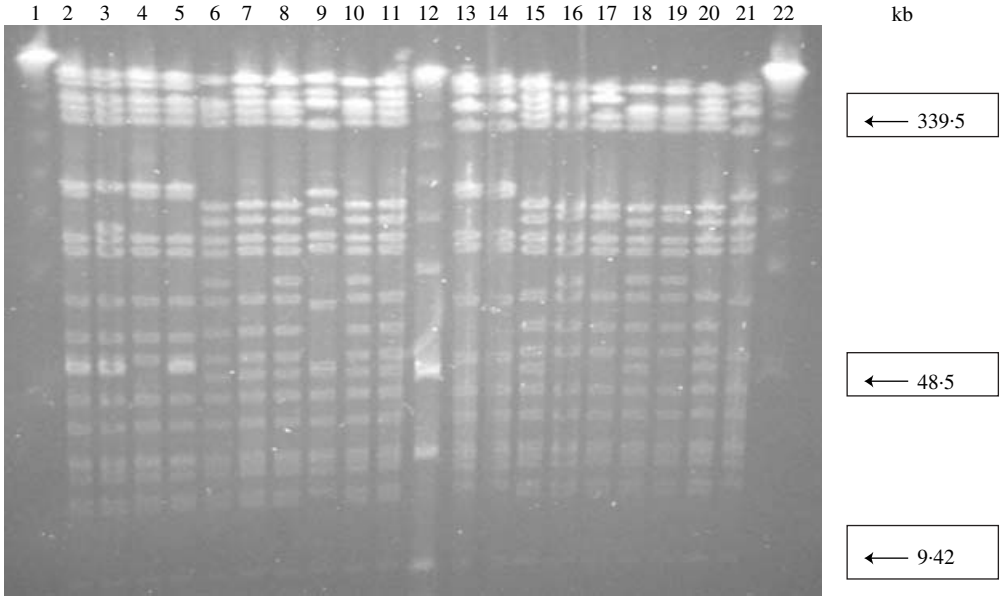

Fig. 1. PFGE banding patterns of *Sma*I digested genomic DNA from the 25 *S. aureus* isolates. Lane 2, the banding pattern of the human isolate; lanes 3–5, banding patterns of the bovine isolates from the same farm (lane 5 is indistinguishable from the human isolate); lanes 6–11 and 13–21, the 15 control isolates; lanes 1 and 22, lambda ladder PFG markers; lane 12, low-range PFG marker. Arrows indicate fragment sizes in kilobase (kb) pairs.

(4- to 6-band difference) and unrelated (7- or more band difference) will be used, in accordance with the guidelines for bacteria strain typing according to the restriction patterns produced by PFGE, formulated by Tenover et al. [18].

All the isolates showed phenotypic characteristics consistent with *S. aureus*. Resistance to staphylococcal β-lactamase-stable penicillins was not detected in the four analysed isolates. One of the bovine isolates from the herd under investigation had an illegible banding pattern, and it was excluded from the analysis. The 15 control isolates derived from 12 different farms. PFGE of *Sma*I restriction fragments produced banding patterns consisting of 15–20 bands that ranged in size from 31 to 450 kb pairs. The 25 bovine isolates exhibited 16 distinct banding patterns, indicating an adequate discriminatory power of the method (Fig. 1). Three control isolates originating from different farms had indistinguishable banding patterns. The 10 isolates from man and cows from the herd under investigation produced three different banding patterns. The human isolate had a banding pattern indistinguishable from that of three of the bovine isolates from his herd. One of these originated from a cow sampled by one of the authors one week after the isolation of the human strain, ruling out possible cross-contamination of the milk sample. The other six isolates from cows on that farm were closely related to the human isolate (up to 3-band difference), and to each other. In contrast, all pair-wise matchings of the strains from the control group and the 10 isolates from the investigated farm resulted in at least a 7-band difference (unrelated). The nasal strain was also unrelated to the human strain.

Figure 2 depicts a dendrogram with the positioning and topological relationships between the different genetic lineages found in the study, except the nasal strain.

DISCUSSION

The indistinguishable but unique banding pattern of the human and three bovine isolates from the same farm, combined with the epidemiological temporal–spatial link between the disease in the farmer and in his cows (same farm, same period), provided evidence of a common, clonal aetiology in one episode of disease in the man, and in his cows. The special circumstances and the astute supposition formulated by the farmer and his veterinarian constituted a strong prior, and therefore it is conceivable that the same clone also caused other episodes of disease in the farmer. However, it must be emphasized that only one isolate from an abscess in the man was available for comparison.

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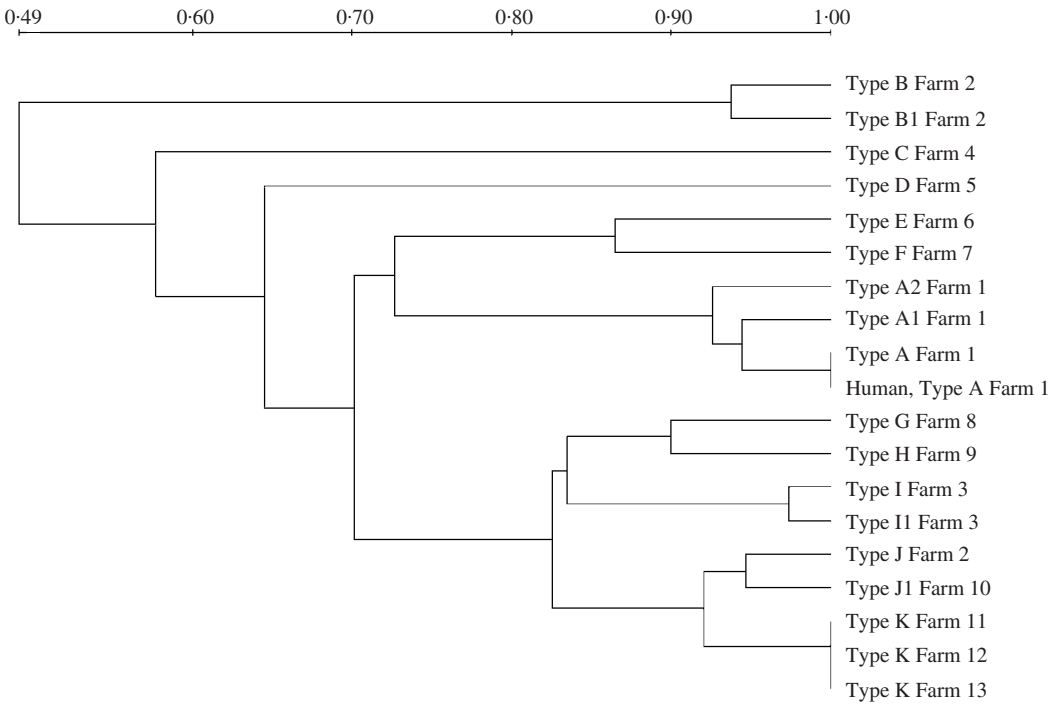

Fig. 2. Dendrogram constructed with the UPGMA method using the Dice coefficient matrix of the 25 isolates used in this study. The scale on the top indicates the unweighted arithmetic means. Subtypes were defined and represented according to Tenover et al. [18]. The human isolate and the different farms are defined.

The farmer was found to be a nasal carrier of *S. aureus* on two different occasions during the observation period. On one occasion, the nasal isolate was typed, the result being unrelated to the strain causing the skin infection. However, we could not rule out nasal carriage with the clone causing his skin disease and the mastitis in cows, since only one colony from the nasal swab culture was picked for the analysis.

Veterinary epidemiologists have traditionally distinguished between environmental and contagious bacterial causes of bovine mastitis, and *S. aureus* was considered one of the archetypes of the contagious group of agents. This is not merely a semantic distinction, having operational implications related to the different prophylactic approaches. However, whereas there are reports describing within-farm monophyletic infections with *S. aureus*, others have described multiple-strain infections [5, 22–26], questioning whether contagiosity is, or is not, a constant feature. Our findings indicate a monophyletic infection, since all the isolates from the investigated farm formed a distinct cluster of indistinguishable, or closely related isolates, which did not recur in the other farms and were unrelated to the other strains in the control set (Fig. 2). We attribute this distinct clustering to the presence of a clonal lineage of high infectivity evolving within the herd and the 2- to 3-band differences to possible random genetic events altering the banding patterns during the course of the outbreak [18, 27]. However, there was an infected stock-handler in close contact with the cows and we cannot assume only a cow-to-cow transmission, since a man-to-cow route was also possible.

In conclusion, this is the first report in New Zealand (and perhaps elsewhere), confirming with molecular methods the emergence and rapid in-herd spread of a clone of *S. aureus*, which caused multiple cases of mastitis in cows and a purulent cutaneous infection in a worker. Besides the obvious implications as a human health issue, this case provides further evidence of the major impact of mastitis-causing clones of *S. aureus* of high infectivity on dairy farms, and also raised a number of important questions: did we face a human-to-cow, or a cow-to-human transmission, or both? Is this an exceptional case, or does it reflect a wider, underestimated condition? In analogy to the food industry, should we advise stock-handlers to self-test for cutaneous infections in the occurrence of outbreaks of mastitis in herds? Could highly virulent 'species jumper' clones emerge in dairy farms and spread to the community?

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In spite of these uncertainties, physicians and veterinarians should be aware of these possibilities, due to the close human–bovine interaction characterizing dairy farming and the severe implications that this condition might have on the everyday life of the people involved.

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