Epidemiol. Infect. (2003), **131**, 931–937. © 2003 Cambridge University Press
DOI: 10.1017/S0950268803008938 Printed in the United Kingdom

Diversity and stability of the *Staphylococcus intermedius* flora in three bitches and their puppies

L. E. M. SAIJONMAA-KOULUMIES[1]*, V. MYLLYS[2] AND D. H. LLOYD[1]

[1] *Dermatology Unit, Royal Veterinary College, U.K.*
[2] *National Veterinary Food and Research Institute, Finland*

(*Accepted 27 March 2003*)

SUMMARY

The study investigated the transfer and the stability of the *S. intermedius* flora in three bitches and their puppies. A total of 240 cutaneous and mucosal isolates of *S. intermedius* was collected from three healthy Cavalier King Charles spaniels and their puppies during the immediate prepartum period and after whelping, over a total of 15 weeks. The isolates were genotyped with random amplified polymeric DNA-polymerase chain reaction analysis (RAPD-PCR) using two primers. Seventeen different genotypes of *S. intermedius* were identified. One or two of the genotypes were dominant in each of the bitches and their puppies. The rest were isolated only once or twice from the bitches or their puppies. The study indicates that *S. intermedius* flora within each studied bitch mainly consisted of one or two dominating and persistent clones, which were transferred from the dam to her puppies immediately after birth.

INTRODUCTION

Staphylococcus intermedius, the principal pathogen of canine skin [1], lives as a commensal in most dogs [2] and is carried most commonly at the mucosae. From mucosae, it is seeded to the hair and skin during grooming and other activities, providing a source of infection in the development of canine pyoderma [3–5], a major disease of dogs. Data from a recent study investigated colonization of puppies during the neonatal period and demonstrated that puppies, living in domestic environments, are colonized by *S. intermedius* almost immediately after birth [6]. Although it is assumed that this flora is transferred from their dams, there were no studies, which would confirm this assumption. In the present study, the dynamics of transfer and the stability of *S. intermedius* in the bitches and their puppies were investigated. The isolates, collected around the immediate pre- and post-whelping period were genotyped using random amplified polymorphic DNA-polymerase chain reaction analysis (RAPD-PCR). Methods based on genotyping have been used to discriminate *S. intermedius* in the past [7–12]. However, the aim of these earlier studies was to differentiate strains differing in virulence, by comparing isolates obtained from infected lesions and from healthy dogs, and not to study the dynamics of transfer between dogs. Our aim was to study the clonal relationship of the organisms at different sites of the body and between the bitches and their offspring. This knowledge might assist in the development of methods for the long-term establishment of *S. intermedius* isolates of low virulence in dogs. This approach has been employed in humans [13–18] and in pigs [19].

* Author for correspondence: Satamakatu 5B13, 00160 Helsinki, Finland.

MATERIALS AND METHODS

Bacterial isolates

A total of 240 cutaneous and mucosal isolates of *S. intermedius* was investigated. These had been collected from three Cavalier King Charles spaniels

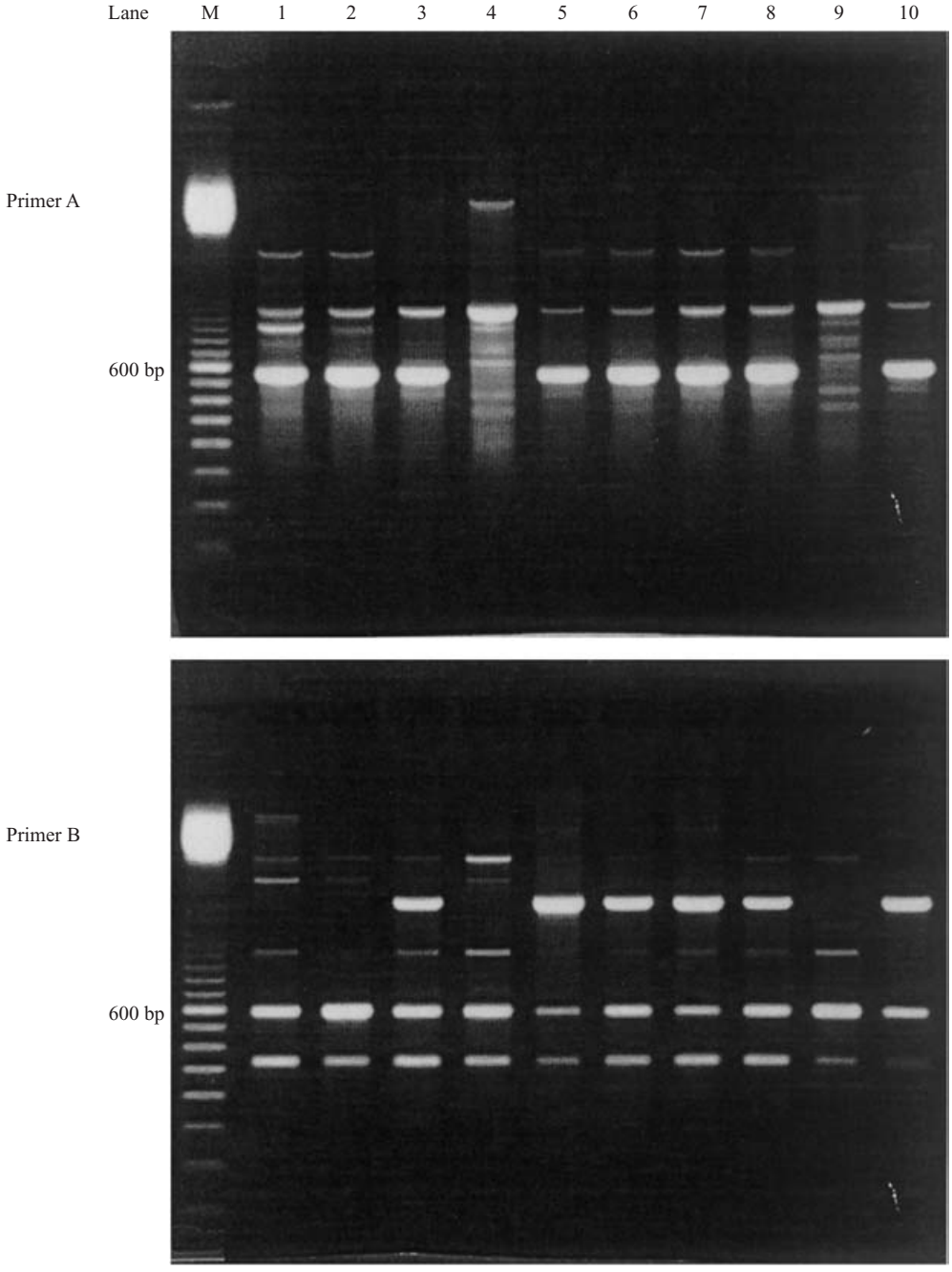

Fig. 1. RAPD-PCR typing of 10 isolates of *S. intermedius* from bitch two (D20) with two primers, A and B. Each primer gave two different fingerprints, but when combined, three types were produced. Lanes 1 and 2 represent type b, lanes 4 and 9 represent type e and lanes 3, 5–8 and 10, type c. M, molecular weight marker; bp, basepair.

and their puppies, during the immediate pre-partum period and after whelping, over a total of 15 weeks using quantitative swab procedures [20]; sampling, isolation techniques and population dynamics have been reported by Saijonmaa-Koulumies and Lloyd [6]. Briefly, up to five colonies of *S. intermedius* from each mucosal and cutaneous site (nose, mouth, anus, vulva, prepuce, ear and abdomen) were selected at random for the present study. The occasions of sampling included 6 and 3 days before the whelping, 1 day after whelping, and 1, 3, 7 and 14 weeks after whelping. Included in the study were 40 isolates from bitch one (D10) and her puppy (D11), 110 isolates from bitch two (D20) and her three puppies (D21–23) and 90 isolates from bitch three (D30) and her puppy (D31).

https://doi.org/10.1017/S0950268803008938 Published online by Cambridge University Press

The isolates were first stored at 4 °C on nutrient agar slopes for about 1 month and subsequently transferred to storage beads and kept at −70 °C until use in the study.

Genetic fingerprinting of *S. intermedius* by random amplified polymorphic DNA-polymerase chain reaction analysis (RAPD-PCR)

Chromosomal DNA for RAPD-PCR was extracted with guanidium thiocyanate according to the method described by Pitcher et al. [21].

Random amplified polymorphic DNA-polymerase chain reaction analysis (RAPD-PCR) was performed according to Williams et al. [22].

Purified DNA was quantitated spectrophotometrically and diluted with molecular biology water (Sigma) to a concentration of 5 ng/μl. Amplifications were performed in a DNA thermal cycler (DNA Thermal Cycler 480, Perkin Elmer) for 45 cycles of 30 s at 94 °C, of 30 s at 36 °C and 1 min at 72 °C with a 3 min initial denaturation at 94 °C and 5 min final extension at 72 °C. The reaction was conducted in a 25 μl volume containing 25 ng purified DNA and 1 U of AmpliTaq DNA-polymerase (Perkin Elmer) in a buffer provided by the manufacturer containing 3 mM $MgCl_2$, 10 mM Tris-Cl (pH 8·3), 50 mM KCl, 200 mM each of dATP, dCTP, dGTP and dTTP, 0·5 mM primer and overlaid with one drop of sterile paraffin oil (Sigma). Amplification products were analysed by electrophoresis in 1·5% agarose gels stained with ethidium bromide.

Twenty-four primers were pre-screened with five strains of *S. intermedius*, randomly selected from the study material to identify those giving the most discriminatory and reproducible results. The candidate primers were: (1) the four most discriminatory primers with *S. aureus*; OPJ5 and OPJ6 (Operon), and two primers based on the enterobacteria 1 repetitive intergenic consensus sequence (ERIC), 3237 and 4874 [23], and (2) 20 commercially available 10-base oligonucleotide primers, 60-1 to 60-10 and 70-1 to 70-10 (Genosys Europe). The screening process involved two stages. Two initial assays allowed four primers yielding either no products or unreadable banding (primers 60-5, 60-6, 60-7 and 60-10) to be eliminated. The remaining 20 primers were re-evaluated in two further assays and two primers, 70-5 (5′GAGATCCGCG3′) and 4874 (5′ATGTAAGCTCCTGGGGATTCAC3′), giving original and distinguishable patterns, were selected. Reproducibility of results with the two primers was confirmed in two assays with 30 isolates of *S. intermedius*, randomly selected from the study material. The pictures of the gels were analysed visually. Differences in the number and location of bands indicated a novel type. Variations in the intensity of the bands were disregarded. An alphabetical code combining both primers was given for each genotype (Fig. 1). Single unique isolates were called Si.

The pictures of the gels were also digitized with a scanner and analysed with Gel Compare software (Applied Maths).

Table 1. *Number of genetic fingerprints with RAPD-PCR using two primers (A and B) of 240 isolates of* S. intermedius *from three bitches and their puppies (D1, D2 and D3)*

Dogs	Primer A	Primer B	Primers (A + B)
D1	2	1	2
D2	6	9	13
D3	4	4	5
All	8	12	17

RESULTS

RAPD-PCR

With the two primers, 70-5 (A) and 4874 (B), the 240 isolates of *S. intermedius* were separated into 17 different genotypes. Primers A and B divided the isolates into 8 and 12 fingerprints respectively (Table 1).

Among the 40 isolates of *S. intermedius* from the first bitch (D10, $n=15$) and her puppy (D11, $n=25$), 39 isolates were of the same genotype with both primers (type a). One of the isolates in the puppy had a different fingerprint with primer A (Table 2, Fig. 2). Among the 110 isolates of *S. intermedius* from the second bitch (D20, $n=63$) and her three puppies (D21, D22, D23, $n=47$) 13 different genotypes were discovered. Two of these were dominant (types c and b) in the bitch (76·2 and 7·9% respectively) and the puppies (42·6 and 36·2% respectively) and were isolated throughout the study both in the bitch and the puppies (Table 2, Fig. 2). Among the remaining 11 genotypes, 9 were isolated only either from the bitch or puppies and 8 were isolated only on one sampling occasion. With the primer A, 6 different genotypes and primer B, 9 genotypes were identified.

https://doi.org/10.1017/S0950268803008938 Published online by Cambridge University Press

Table 2. *Distribution of fingerprints of 240 isolates of* S. intermedius *at different sites and occasions of sampling among three bitches* (*D10, D20, D30*) *and their puppies* (*D11, D21–23, D31*)

	Day						Day				
	−6*	−3*	1	7	21	49	1	7	21	49	98
	D10						D11				
Nose				a†			a, Si				
Mouth			a					a			
Anus						a					
Vulva						a					
Prepuce											
Ear							a	a			
Abdomen				a			a	a			a
	D20						D21–23				
Nose		c, e	c				c, Si, Si				
Mouth	c	c, Si	b, c			e	b, c, Si			d, g, h	
Anus	c	c	c				b, c			b, c	
Vulva	c	b, c	c, e			c					
Prepuce							c				
Ear							b				
Abdomen		b, g	b, e, f, g, Si			b	c, Si				
	D30						D31				
Nose			b				b		c		
Mouth			c		c	b, c	c		b	b, c	e
Anus		b, c	b, c		b, c		c				
Vulva		c			c	b, c					
Prepuce							j			b, c	
Ear							b		b		
Abdomen		Si	c		b	b, c	b, c		c	c	e

* Days −3 and −6, before whelping.
† Alphabetic codes for fingerprints; Si, single unique isolate.

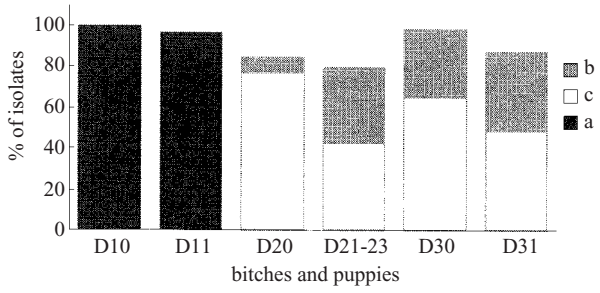

Fig. 2. Frequency of three most dominant genotypes (a, b and c) with RAPD-PCR of 240 isolates of *S. intermedius* in three bitches (D10, D20, D30) and their puppies (D11, D21–23, D31).

Among the 90 isolates of *S. intermedius*, from the third bitch (D30, $n=51$) and her puppy (D31, $n=39$), 5 different fingerprints were discovered. Two of these (c and b) were dominant among the isolates both in the bitch (64·7 and 33·3% respectively) and the puppy (51·3 and 38·5% respectively) (Table 2, Fig. 2). The remaining three types were isolated only on one sampling occasion either from the bitch or the puppy. Individually, both primers, A and B, each revealed four genotypes.

The dominant fingerprint (a) obtained from the first bitch and her puppy was different from the other two dominant genotypes (b and c), isolated from the other bitches and their litters. These latter two bitches shared the same domestic environment. When the sources of different clones were plotted, no consistent pattern relating to particular clonal types could be discerned (Table 2).

Although the intensity of the bands varied somewhat between the tests, good reproducibility of the fingerprints was obtained in this study; in over 95% of the 30 duplicate isolates of *S. intermedius*, the number and location of the bands were identical. Computerized analysis of the PCR patterns using Gelcompar software did not yield any improvement

https://doi.org/10.1017/S0950268803008938 Published online by Cambridge University Press

compared to visual inspection of the gels. On the contrary, many of the patterns that were clearly distinct when visually inspected, clustered together with different patterns when computerized, and patterns that were identical when visually inspected sometimes fell into different clusters.

DISCUSSION

The findings in this study indicate that the *S. intermedius* flora within each studied bitch mainly consisted of one or two dominant and persistent clones, which were transferred from the mother to her puppies immediately after birth.

Similar findings have been reported from pigs. Allaker et al. [24] demonstrated that the skin of the piglet is colonized within 12 h of parturition by bacteria, including *S. hyicus*, which present the spectrum found on the adults. Also, Wegener and Skov-Jensen [25] showed that among the vaginal strains of *S. hyicus* in gilts, one or two phage types were dominant and persistent in each herd and were isolated from the skin of the piglets three weeks after farrowing.

Allaker et al. [26] also studied the colonization of neonatal puppies by *S. intermedius*. Although they isolated reasonably high counts of *S. intermedius* from the vaginal vestibule in the bitches, when compared to other sites, they concluded that, unlike in gilts, the flora at the vaginal vestibule was not the major source of the cutaneous *S. intermedius* flora in puppies. They also concluded that increases in the populations of *S. intermedius* in the period following whelping at the oral and abdominal sites on both the bitches and puppies indicated transfer of staphylococci between mother and offspring and that contact during feeding would contribute to these rises. No typing of individual isolates was carried out in their study.

Most puppies are born with the amniotic sac intact. This then is opened and the umbilical cord cut by the bitch with her teeth. Therefore the first contact is not the vaginal but rather the buccal flora of their mother. In the present study, however, no evidence of a site of preference for certain clones of *S. intermedius* was obtained and the most dominant fingerprints of *S. intermedius* were isolated at different sites of the body both in the bitches and their puppies.

In addition to the dominant and persistent strains of *S. intermedius*, other types were identified which could be isolated only once or twice from the bitches or the puppies. Among the reasons why these were not able to become established could be competitive adherence or other mechanisms involved in bacterial interference. It has been demonstrated, both in man and animals, that precolonization by one strain of pathogenic staphylococci prevents the establishment of the subsequent strain [13–16, 19, 27–29]. Further studies are needed to study the adherence of different fingerprints isolated in this study to canine epithelial cells and to determine whether the dominant clones of *S. intermedius*, transferred from the dams, persist through life and generations of dogs, and whether recurrence of pyoderma in certain susceptible animals could be associated with the persistence of the more virulent variants. The need for such investigations is supported by studies of bacterial interference in the treatment of recurrent furunculosis in man; relapse of furunculosis occurred if *S. aureus* 502A, the interfering strain, was lost and the original strain reacquired [17].

Several studies have confirmed the suitability of RAPD-PCR for genetic fingerprinting of staphylococci. For instance in 1993, Saulnier et al. [30] reported that RAPD-PCR-method showed good reproducibility of the profiles of methicillin resistant strains of *S. aureus*. In 1994, van Belkum et al. [31] suggested that, due to ease of performance, PCR fingerprinting may become the method of choice for establishing clonal relationships among isolates of *S. aureus*. In 1995, van Belkum et al. [32] concluded that randomly primed PCR was well suited for genetic analysis and monitoring of nosocomial spread of *S. aureus*. In the veterinary field, this method has been used to study the epidemiology of bovine *S. aureus* mastitis [23, 33]. RAPD-PCR was chosen here for genotyping isolates of *S. intermedius*, because it had not been used before to study the genetic variability of this bacterial species and thus offered a novel approach.

The choice and number of primers is critical and affects the discriminatory power of the test [23, 32]. In the present study, the primers were first investigated in a preliminary study. The two primers, which then were adopted for the definitive study, provided good reproducibility, although the intensity of the bands was variable in some cases. The number of genotypes differed with each primer. By adding more primers to the study, the discriminatory power of this technique might have been further improved. In the present study it was found that visual interpretation of the fingerprints was very time-consuming, owing to the large number of isolates. A similar conclusion was reported by Saulnier et al. [30] who confirmed

that the RAPD assay was easy to perform with a single primer but it became more cumbersome when the number of primers increased. To solve this problem in the present study, computerized analysis of the gels with Gelcompar was performed. However, the results were not satisfactory because in many cases they disagreed with those obtained from the visual judgement. It seems that the PCR data generated in the present study was not suitable for computerized analysis. It may have been too sensitive and unduly influenced by artefacts. Van Belkum et al. [32] also reported that Gelcompar analysis of their results was disappointing. They claimed that it was due to lack of contrast, excessive 'smiling' of the gels, and low-resolution photography; they concluded that Gelcompar analysis is heavily influenced by electrophoretic and photographic artefacts. If the problems with computerized analysis could be corrected, RAPD-PCR could prove to be a very useful technique to explore the epidemiology of canine pyoderma and to answer some of the questions already identified as important such as whether there are distinct follicular and mucosal populations of *S. intermedius*, as suggested by Harvey and Lloyd [34]. Also this method could help to confirm whether clinical infection by *S. intermedius* is caused by strains carried as commensals or of exogenous origin and whether reinfection occurs with the same or different strains.

In the current study visual evaluation proved functionally effective and enabled transfer of *S. intermedius* strains between bitches and their puppies to be critically evaluated for the first time.

Since bacterial interference has been proposed as a method for the control of canine pyoderma [26, 35, 36] it may thus be possible to introduce antagonistic staphylococci to the puppies by establishing them first in the bitches. This might protect against the establishment of virulent *S. intermedius*.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs Merja Gustafsson at the National Veterinary and Food Research Institute, Helsinki, Finland for her technical assistance with the genetic fingerprinting and Dr Inger Kühn at the Karolinska Institute, Stockholm, Sweden for her help in analysing the data. Thanks are also due to Professor Tuula Honkanen-Buzalski at the National Veterinary and Food Research Institute, Helsinki, Finland for her help in the study design.

REFERENCES

1. Cox HU, Newman SS, Roy AF, Hoskins JD. Species of *Staphylococcus* isolated from animal infections. Cornell Vet 1984; **74**: 124–135.
2. Berg JN, Wendell DE, Vogelweid C, Fales WH. Identification of the major coagulase-positive *Staphylococcus* sp. of dogs as *Staphylococcus intermedius*. Am J Vet Res 1984; **45**: 1307–1309.
3. Devriese LA, De Pelsmaecker K. The anal region as a main carrier site of *Staphylococcus intermedius* and *Streptococcus* in dogs. Vet Rec 1987; **121**: 302–303.
4. Allaker RP, Lloyd DR, Bailey RM. Population sizes and frequency of staphylococci at mucocutaneous sites on healthy dogs. Vet Rec 1992; **130**: 303–304.
5. Saijonmaa-Koulumies LEM, Parsons E, Lloyd DH. Elimination of *Staphylococcus intermedius* in healthy dogs by topical treatment with fusidic acid. J Small Anim Pract 1998; **39**: 341–347.
6. Saijonmaa-Koulumies LE, Lloyd DH. Colonization of neonatal puppies by *Staphylococcus intermedius*. Vet Dermatol 2002; **13**: 123–130.
7. Allaker RP, Garrett N, Kent L, Noble WC, Lloyd DH. Characterisation of *Staphylococcus intermedius* isolates from cases of canine pyoderma and healthy carriers by SDS-PAGE of exoproteins, immunoblotting and restriction endonuclease analysis. J Med Microbiol 1993; **39**: 429–443.
8. Hesselbarth J, Witte W, Cuny C, Rohde R, Amtsberg G. Characterisation of *Staphylococcus intermedius* from healthy dogs and cases of superficial pyoderma by DNA restriction endonuclease patterns. Vet Microbiol 1994; **41**: 259–266.
9. Khambaty FM, Bennett RW, Shah DB. Application of pulsed-field electrophoresis to the epidemiological characterization of *Staphylococcus intermedius* implicated in a food-related outbreak. Epidemiol Infect 1994; **113**: 75–81.
10. Hesselbarth J, Schwarz S. Comparative ribotyping of *Staphylococcus intermedius* from dogs, pigeons, horses and mink. Vet Microbiol 1995; **45**: 11–17.
11. Pedersen K, Wegener HC. Antimicrobial susceptibility and rRNA gene restriction patterns among *Staphylococcus intermedius* from healthy dogs and from dogs suffering from pyoderma or otitis externa. Acta Vet Scand 1995; **36**: 335–342.
12. Shimizu A, Berkhoff HA, Kloos WE, George CG, Ballard DN. Genomic DNA fingerprinting, using pulsed-field gel electrophoresis, of *Staphylococcus intermedius* isolated from dogs. Am J Vet Res 1996; **57**: 1458–1462.
13. Shinefield HR, Ribble JC, Boris M, Eichenwald HF. Bacterial interference. Its effect on nursery acquired infection with *Staphylococcus aureus*. I. Preliminary observations on artificial colonisation of newborns. Am J Dis Child 1963; **105**: 646–654.
14. Shinefield HR, Sutherland IM, Ribble JC, Eichenwald HF. II. The Ohio epidemic. Am J Dis Child 1963; **105**: 655–662.

15. Shinefield HR, Boris M, Ribble JC, Cale EF, Eichenwald HF. III. The Georgia epidemic. Am J Dis Child 1963; **105**: 663–673.
16. Shinefield HR, Ribble JC, Eichenwald HF, Boris M, Sutherland JM. V. An analysis and interpretation. Am J Dis Child 1963; **105**: 683–688.
17. Strauss WG, Maibach HI, Shinefield HR. Bacterial interference treatment of recurrent furunculosis. 2. Demonstration of the relationship of strain to pathogenicity. JAMA 1969; **208**: 861–863.
18. Maibach HI, Strauss WG, Shinefield HR. Bacterial interference: relating to chronic furunculosis in man. Br J Dermatol 1969; **81**: 69–76.
19. Allaker RP, Lloyd DH, Smith IM. Prevention of exudative epidermitis in gnotobiotic piglets by bacterial interference. Vet Rec 1988; **123**: 597–598.
20. Lloyd DR, Allaker RP, Pattison AL. Carriage of *Staphylococcus intermedius* on the ventral abdomen of clinically normal dogs and those with pyoderma. Vet Dermatol 1991; **2**: 161–164.
21. Pitcher DG, Saunders NA, Owen RI. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 1989; **8**: 151–156.
22. Williams JGK, Hanafey MK, Rafalski JA, Tingey SV. Genetic analysis using random amplified polymorphic DNA markers. Methods Enzymol 1993; **218**: 705–740.
23. Myllys V, Ridell J, Björkroth J, Biese I, Pyörälä S. Persistence in bovine mastitis of *Staphylococcus aureus* clones as assessed by random amplified polymorphic DNA analysis, ribotyping and biotyping. Vet Microbiol 1997; **51**: 245–251.
24. Allaker RP, Lloyd DH, Lamport AI. Bacterial interference in the control of exudative epidermitis of pigs. In: von Tscharner C, Halliwell REW, eds. Adv Vet Dermatol, vol. 1. Baillière Tindall 1990: 327–334.
25. Wegener HC, Skov-Jensen EW. A longitudinal study of *Staphylococcus hyicus* colonization of vagina of gilts and transmission to piglets. Epidemiol Infect 1992; **109**: 433–444.
26. Allaker RP, Jensen I, Lloyd DH, Lamport AI. Colonisation of neonatal puppies by staphylococci. Br Vet J 1992; **148**: 523–528.
27. Boris M, Shinefield HR, Ribble J, Eichenwald H, Hauser G. The Louisiana epidemic. Am J Dis Child 1963; **105**: 674–682.
28. Light IJ, Sutherland JM, Schott JE. Control of a staphylococcal outbreak in a nursery. Use of bacterial interference. JAMA 1965; **193**: 699–704.
29. Light IJ, Walton RL, Sutherland JM, Shinefield HR, Brackvogel V. Use of bacterial interference to control a staphylococcal nursery outbreak. Deliberate colonisation of all infants with the 502A strain of *Staphylococcus aureus*. Am J Dis Child 1967; **113**: 291–300.
30. Saulnier P, Bourneix C, Prevost G, Andremont A. Random amplified polymorphic DNA assay is less discriminant than pulsed-field gel electrophoresis for typing strains of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 1993; **31**: 982–985.
31. Van Belkum A, Bax R, Prevost G. Comparison of four genotyping assays for epidemiological study of methicillin-resistant *Staphylococcus aureus*. Eur J Clin Microbiol Infect Dis 1994; **13**: 420–424.
32. Van Belkum A, Kluytmans J, van Leeuwen W, et al. Multicenter evaluation of arbitrarily primed PCR for typing of *Staphylococcus aureus* strains. J Clin Microbiol 1995; **33**: 1537–1547.
33. Lipman LJA, de Nijs A, Lam TJGM, et al. Genotyping by PCR of *Staphylococcus aureus* strains, isolated from mammary glands of cows. Vet Microbiol 1996; **48**: 51–55.
34. Harvey RG, Lloyd DH. The distribution of *Staphylococcus intermedius* and coagulase-negative staphylococci on the hair, skin surface, within the hair follicles and on the mucous membranes of dogs. Vet Dermatol 1994; **5**: 75–81.
35. Saijonmaa-Koulumies LEM, Lloyd DH. Carriage of bacteria antagonistic towards *Staphylococcus intermedius* on canine skin and mucosal surfaces. Vet Dermatol 1995; **6**: 187–194.
36. Saijonmaa-Koulumies LEM. Bacterial interference in the control of canine pyoderma. Ph.D. Thesis. University of London, 1999.