Characterisation of *Staphylococcus intermedius* isolates from canine pyoderma and from healthy carriers by SDS-PAGE of exoproteins, immunoblotting and restriction endonuclease digest analysis

R. P. ALLAKER†, N. GARRETT, LOUISE KENT*, W. C. NOBLE* and D. H. LLOYD

Dermatology Unit, Royal Veterinary College, Hawkshead Lane, North Mymms, Hertfordshire AL9 7TA, * Department of Microbial Diseases, St John's Institute of Dermatology, St Thomas's Hospital, London SE1 7EH and † Department of Oral Microbiology, The London Hospital Medical College, Turner Street, London E1 2AD

Summary. Ten *Staphylococcus intermedius* isolates from cases of canine pyoderma and 10 from healthy carriers were examined by SDS-PAGE of exoproteins, immunoblotting and restriction endonuclease digest analysis. Similarities between banding patterns of the isolates were calculated as Dice coefficients for all three methods. For SDS-PAGE and immunoblotting, no significant differentiation was found between the pyoderma and "healthy" groups. Analysis of DNA digested with *Bgl*II indicated that *S. intermedius* is genetically heterogeneous; Dice coefficients for the pyoderma group were distinct from those for the healthy group ($p < 0.001$), and cluster analysis confirmed that the pyoderma isolates (9) formed a group separate from the majority (6 of 9) of the normal isolates.

Introduction

Many factors have been suggested as contributing to the pathogenesis of canine pyoderma, including bacterial infection, impaired immunity, endocrine abnormalities, seborrhoea and allergic conditions.¹ *Staphylococcus intermedius* is the predominant coagulase-positive staphylococcus isolated from normal and infected canine skin.² Production of potential virulence factors, including haemolysins, enterotoxins, epidermolytic toxins, proteases and protein A, have been compared in *S. intermedius* isolates from healthy dogs and dogs with pyoderma.^{3–5} However, no clear differences in the toxin profiles between these groups of isolates have been demonstrated.

In this study, *S. intermedius* isolates from healthy dogs and from canine pyoderma were examined by three methods: SDS-PAGE analysis of concentrated extracellular proteins (CEP); immunoblotting of CEP with hyperimmune rabbit serum; and DNA restriction fragment length polymorphism (RFLP) analysis. The banding patterns produced allowed comparison of phenotypic (SDS-PAGE of CEP; immunoblot) and genotypic (RFLP) characteristics of the two groups of isolates.

Materials and methods

Bacterial isolates

Skin isolates of *S. intermedius* were obtained from 20 distinct sources;³ 10 from clinically normal dogs and 10 from dogs classified as cases of superficial pyoderma according to Muller *et al.*¹ Identification to species level was by the methods of Hajek.⁶ Isolates were maintained on Protect beads (Lab M) at -20°C until required.

Preparation of samples

Two culture methods were used. Exoproteins for SDS-PAGE and immunoblotting were obtained by culturing *S. intermedius* on the exterior of dialysis bags (*c.* 12 cm $\times$ 5 cm) containing 150 ml of broth enclosed in glass flasks as described by Ikigai.⁷ Broth culture medium contained Tryptone (Oxoid, L42) 1.7% w/v, yeast extract (Oxoid, L21) 1% w/v, NaCl 0.5% w/v and K_2HPO_4 0.25% w/v. Cultures were incubated at 37°C in air for 24 h on a shaking platform (100 rpm), the broth was centrifuged and the supernate was membrane filtered (Millipore; 0.45 μm) to remove residual cells. Supernate protein concentrations were measured by the BioRad Protein Assay and the supernate was diluted to a standard protein concentration (3.63 SE 0.39 mg/ml) for subsequent analyses. For RFLP analysis, isolates were grown on Blood Agar Base (Oxoid, CM271), and colonies were

Table. Similarity matrices for pyoderma (DP) and healthy (DN) isolates in (A) SDS-PAGE exoprotein, (B) immunoblot and (C) *Bgl*II RFLP analyses

Isolate no.	Percentage similarity with isolate no.																		
	DP1	DP2	DP3	DP4	DP5	DP6	DP7	DP8	DP9	DP10	DN23	DN25	DN26	DN31	DN41	DN47	DN49	DN51	DN52
A	DN53	78	66	71	91	86	89	84	92	68	62	67	69	58	83	79	84	94	99
	DN52	83	76	90	87	88	87	86	86	92	64	72	71	54	89	80	87	95	90
	DN51	71	67	76	92	95	90	83	93	79	57	59	67	56	82	77	83	75	
	DN49	91	89	79	75	78	77	74	80	85	81	78	76	72	92	88	78		
	DN47	94	78	80	76	78	78	72	76	85	79	79	77	73	96	64			
	DN41	94	87	83	76	78	80	77	78	83	79	75	85	73	68				
	DN31	93	83	79	75	77	79	74	75	87	76	81	83	68					
	DN26	68	69	59	53	55	55	52	58	62	91	86	79						
	DN25	79	77	70	63	66	65	62	66	73	86	77							
	DN23	77	78	68	61	64	63	60	64	68	90								
	DP10	80	81	76	74	94	72	73	85	84									
	DP9	84	72	74	86	82	90	85	79										
	DP8	87	78	82	74	67	82	79											
	DP7	81	77	72	75	81	82												
	DP6	83	68	67	69	71													
	DP5	87	85	86	82														
	DP4	84	80	83															
	DP3	81	87																
	DP2	80																	
B	DN53	77	79	76	80	84	78	80	87	81	86	65	81	72	73	82	89	84	82
	DN52	86	95	82	85	89	84	78	82	86	87	71	87	78	95	83	81	93	94
	DN51	69	75	68	76	73	70	72	89	73	91	65	74	68	83	93	61	87	
	DN49	78	83	77	88	89	83	85	78	82	87	74	78	77	95	86	81		
	DN47	80	88	75	89	87	78	75	86	65	88	61	77	72	86	87			
	DN41	76	77	68	79	76	70	71	82	62	96	61	77	68	85				
	DN31	85	86	80	90	88	82	80	84	81	86	70	85	76					
	DN26	81	87	85	80	81	98	85	70	81	69	89	86						
	DN25	91	88	86	85	94	84	86	79	74	78	80							
	DN23	84	76	89	70	74	87	83	60	74	63								
	DP10	77	85	73	92	77	68	73	97	74									
	DP9	95	83	86	81	90	98	93	77										
	DP8	75	83	70	81	84	72	70											
	DP7	95	87	85	80	85	98												
	DP6	88	85	78	74	79													
	DP5	90	87	85	84														
	DP4	85	86	80															
	DP3	86	87																
	DP2	88																	
C	DN53	37	64	56	57	62	38	35	45	61	54	36	64	30	69	48	45		38
	DN52	42	27	36	56	61	33	40	53	40	43	42	53	24	43	33	53		
	DN49	40	52	52	54	50	55	48	50	38	33	30	70	56	50	53			
	DN47	50	36	55	56	61	25	40	53	40	35	21	63	59	35				
	DN41	48	67	52	67	79	48	32	42	32	50	33	50	36					
	DN31	42	29	48	42	36	43	34	56	63	36	33	56						
	DN26	48	43	52	46	50	73	48	30	29	42	40							
	DN25	48	35	52	31	58	56	48	30	48	33								
	DN23	62	52	67	60	71	76	56	58	72									
	DP9	69	50	33	59	64	62	64	67										
	DP8	48	52	52	62	42	45	57											
	DP7	62	25	58	44	64	62												
	DP6	53	50	64	65	55													
	DP5	62	52	67															
	DP4	55	48	41															
	DP3	57	54																
	DP2	36																	

harvested to yield *c.* 0.4 g wet weight/isolate for subsequent DNA extraction.

reached the resolving gel (30 mA) and the end of the gel (40 mA). Gels were stained with silver stain (BioRad).

SDS-PAGE

CEPs were analysed by the SDS-PAGE technique of Laemmli.⁸ Broth supernate was mixed in equal proportions with 1 M Tris-HCl, pH 6.8, containing SDS 3% w/v, glycerol 15% w/v, 2-mercaptoethanol 7.5% w/v and bromophenol blue tracking dye 0.0005% w/v, boiled for 5 min and centrifuged to remove insoluble debris. The treated sample was applied to a well in an acrylamide 3.9% stacking gel over an acrylamide 12.5% resolving gel. Electrophoresis was performed with a discontinuous buffer system at room temperature in a vertical electrophoresis tank (model Protean II; BioRad). Constant current was used until the leading edge of the dye

Immunoblotting

Preparation of antiserum. Dialysis culture broth supernate from isolate DP10 (1 ml) was mixed with 1 ml of Freund's incomplete adjuvant (Sigma). Dutch rabbits were inoculated twice, with a 14-day interval, and bled at 28 days.

CEP SDS-PAGE was performed as above, but with an acrylamide 10% resolving gel. Proteins were transferred from acrylamide gels to nitrocellulose membranes (BioRad) by overnight electrophoresis at room temperature in 25 mM Tris-190 mM glycine buffer, pH 8.3, containing methanol 20% v/v.⁹ Free protein-binding sites on the nitrocellulose were saturated by incubation for 1 h in blocking-washing buffer

Figure. Dendrograms obtained from cluster analysis of: (A) SDS-PAGE exoprotein; (B) immunoblot; and (C) *Bgl*II RFLP dice coefficient matrices for pyoderma (DP) and healthy (DN) isolates. Distances that clusters combine rescaled from (A) 69–99%, (B) 77–98% and (C) 40–79%.

(phosphate-buffered saline, pH 7.4, plus Tween 20 0.05% v/v) with fetal calf serum (Gibco) 5% v/v. The membrane was incubated for 1 h with rabbit antiserum

(diluted 1 in 50), washed and incubated for 1 h with goat anti-rabbit IgG biotin conjugate (Sigma; diluted 1 in 250). Membranes were then washed and incubated

with streptavidin-biotin-horseradish peroxidase complex for 30 min. Staining was with diaminobenzidine tetrahydrochloride (Sigma).

Restriction endonuclease analysis

Chromosomal DNA was extracted by the guanidium thiocyanate method,¹⁰ modified to exclude plasmid DNA by the addition of Brij 58 1% w/v, sodium deoxycholate 0.4% w/v and EDTA 2% w/v.¹¹ Extracted DNA was digested with the restriction enzymes *Hae*III, *Bgl*II or *Eco*RI, according to the manufacturer's instructions (Pharmacia and Gibco BRL). Digest fragments were separated by horizontal electrophoresis in agarose (1% w/v) gel at a constant 40 V for 20 h. A 1-kb DNA ladder (Gibco BRL) was used as a marker, with bromophenol blue as the tracking dye. Gels were stained with ethidium bromide and photographed under UV light.

Numerical analysis of banding patterns

The similarity between the banding patterns was calculated as a Dice coefficient,¹² relating the number of common bands to the total number of bands present. Banding patterns were compared within gels for the two isolate groups run on successive occasions under strictly standardised conditions. Dice coefficients were compared in the Mann-Whitney test, and cluster analysis performed (average linkage between groups) in the Statistical Package for Social Sciences (PC version).

Results

CEP-SDS-PAGE patterns for all 20 isolates showed similarities > 50% (mean 78%; table, A), and immunoblot patterns showed similarities > 60% (mean 81%; table, B). However, there were no consistent, significant pattern differences between the pyoderma and healthy groups in either method (figure A and B).

Of the endonuclease enzymes, *Eco*RI digestion gave well resolved bands for all isolates, but non-discriminatory band patterns, and *Hae*III gave too many bands for clear interpretation. *Bgl*II gave interpretable band patterns between 3.1 and 7.1 kb, with good interstrain discrimination. However, two strains (DP10 and DN51) did not give restriction patterns.

Similarities calculated from the *Bgl*II digest patterns varied between 20 and 80% (table, C). Strains of the pyoderma group (mean 55%) were significantly ($p <$

0.001) more similar to one another than strains of the healthy group (mean 44%). Indeed, the strains of the healthy group were clearly heterogeneous, so much so that mean similarities within this group were as low as the mean similarities between individual members of the healthy group and the pyoderma group. In cluster analysis, all the pyoderma isolates grouped together, along with three healthy isolates (figure, C).

Discussion

The complexity of the exoprotein profiles made a detailed comparison of strains difficult. Although the immunoblot technique produced much simpler patterns, subdividing the isolates according to immunoblot or SDS-PAGE differences was difficult, as no constantly variable antigens or proteins could be identified. Certain band differences between isolates may be due to the limits of detection of the method, rather than the presence or total absence of the protein.

In endonuclease digest analysis, discrimination varied depending on the restriction enzyme used. DNA fingerprints from *Eco*RI digestion were identical, but *Bgl*II gave strain-specific digestion patterns. However, not all isolates could be analysed with *Bgl*II. The chromosomal DNA from strains DP10 and DN51 remained intact after incubation with *Bgl*II, but were readily restricted with *Eco*RI. Resistance to digestion with *Bgl*II has also been reported for the chromosomal DNA of phage Group II *S. aureus* strains.¹³ This resistance may be explained by a different methylating system employed by these strains, protecting the DNA from restriction at these sites. This concept has recently inspired the conversion of common restriction enzymes to "rare cutters" by methylating the majority of restriction sites previously available to them.¹⁴

It is possible that dogs carry more than one strain of *S. intermedius*¹⁵ and perhaps those isolates possessing the required virulence determinants may be able to initiate disease, or are selected for, under the correct environmental conditions. The isolates examined in this study have been assayed for the production of a range of potential virulence factors.³ However, there was no obvious correlation between production of these factors and either the cluster patterns presented here, plasmid carriage (unpublished observations) or the pathogenic origin of the strains. It would appear that recognised staphylococcal toxins are not important virulence markers in canine infections with *S. intermedius*. A novel toxin or other unrecognised factors may be involved.

These studies were supported by a grant from the Wellcome Trust no. 19683/1.5. The authors also gratefully acknowledge M. J. Cottey, University of Hertfordshire, for assistance with the statistical analysis.

References

1. Muller GM, Kirk RW, Scott DW. Small animal dermatology, 4th edn. Philadelphia, Saunders. 1989.
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. Allaker RP, Lamport AI, Lloyd DH, Noble WC. Production of 'virulence factors' by *Staphylococcus intermedius* isolates from cases of canine pyoderma and healthy carriers. *Microb Ecol Hlth Dis* 1991; **4**: 169–173.
4. Allaker RP, Grzywacz M, Lloyd DH. Proteolytic zymograms of *Staphylococcus intermedius* isolates from cases of canine pyoderma and healthy carriers. *Microb Ecol Hlth Dis* 1992; **5**: 163–165.
5. Fehrer SL, Boyle MD, Halliwell RE. Identification of protein A from *Staphylococcus intermedius* isolated from canine skin. *Am J Vet Res* 1988; **49**: 697–701.
6. Hajek V. *Staphylococcus intermedius*, a new species isolated from animals. *Int J Syst Bacteriol* 1976; **26**: 401–408.
7. Ikigai H, Seki K, Nishihara S, Masuda S. Simplified method for preparation of concentrated exoproteins produced by *Staphylococcus aureus* grown on surface of cellophane bag containing liquid medium. *Microbiol Immunol* 1988; **32**: 225–228.
8. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
9. Burnette WN. "Western Blotting": electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 1981; **112**: 195–203.
10. Pitcher DG, Saunders NA, Owen RJ. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* 1989; **8**: 151–156.
11. Clowes RC. Molecular structure of bacterial plasmids. *Bacteriol Rev* 1972; **36**: 361–405.
12. Dice LR. Measures of the amount of ecologic association between species. *Ecology* 1945; **26**: 297–302.
13. Richardson JF, Noble WC, Marples RR. Species identification and epidemiological typing of the staphylococci. In: Board RG, Jones D, Skinner FA (eds) Identification methods in applied and environmental microbiology, Blackwell Scientific Publications for The Society for Applied Bacteriology. Oxford, Blackwell. 1992: 193–219.
14. Kur J, Koob M, Burkiewicz A, Szybalski W. A novel method for converting common restriction enzymes into rare cutters: integration host factor-mediated Achilles cleavage (IHF-AC). *Gene* 1992; **110**: 1–7.
15. Wegener HC, Pedersen K. Variations in antibiograms and plasmid profiles among multiple isolates of *Staphylococcus intermedius* from pyoderma in dogs. *Acta Vet Scand* 1992; **33**: 391–394.