Molecular Typing of Strains of *Staphylococcus aureus* Isolated from Bone and Joint Lesions in Lame Broilers by Random Amplification of Polymorphic DNA

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ABSTRACT A study was conducted to investigate the relationship between the presence of localized bacterial infection and lameness in broiler fowl (*Gallus gallus domesticus*). Isolation of bacteria from the proximal femur, proximal tibia, and tibiotarsus from broilers with lameness revealed a probable association between lameness and the presence of *Staphylococcus aureus*. Other potential pathogens, including *Escherichia coli* and DNase-negative staphylococci, were also isolated from sound and lame birds, and their association with pathologies causing lameness was less well defined. After trials with a set of twenty 10-base oligonucleotide primers, a pair of primers giving optimal performance was selected. The *S. aureus* isolates were typed by random amplification of polymorphic DNA (RAPD) by using the pair of 10-mer primers, and groupings were defined by banding patterns after agarose gel electrophoresis. The putative RAPD groupings may provide a basis for epidemiological studies of *S. aureus* in broiler production systems.

(*Key words*: broiler, lameness, leg weakness, random amplification of polymorphic DNA, bacterial chondronecrosis)

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INTRODUCTION

Bacterial chondronecrosis (BCN), previously known as femoral head necrosis and tibial osteomyelitis, causes lameness and accounts for the loss of a significant proportion of the estimated 19.5 million of birds that are culled for lameness (Pattison, 1992; Yogaratnam, 1995) in a total UK annual broiler production of 750 million birds. Broilers become lame with BCN lesions late in their growth (Butterworth, 1999; McNamee and Smyth, 2000), most commonly after 30 d of age, and thus are lost after significant economic input.

This study was performed to identify the principal bacterial pathogens associated with BCN. Lame and unlame (control) birds were sampled to differentiate nonpathogenic and commensal bacteria from those found only in birds with pathologies causing lameness. Of the potential pathogens, the *Staphylococcus aureus* isolates were typed to increase the epidemiological information available. Phage typing (Parker, 1972) of a number of isolates using a human *S. aureus* phage set was performed by the Central Public Health Laboratory[2] as an initial trial and was found to be capable of typing only 50% of the isolates submitted. The potential limitations of using phage typing for epidemiological studies of *S. aureus* is noted in recent studies from Ireland (McCullagh and McNamee, 1998).

Possible other methods for typing the *S. aureus* isolates included multilocus enzyme electrophoresis (Pujol et al., 1997; Bart et al., 1998), pulsed-field gel electrophoresis (PFGE) (Bannerman et al., 1995; Rodgers et al., 1999), bacterial restriction endonuclease digest analysis (Hampson et al., 1986), and random amplification of polymorphic DNA (RAPD) (Bart et al., 1998; Wang et al., 1993). In this study, RAPD was used for molecular typing because it is considered to offer the possibility of sensitivity at least as acute as that of multilocus enzyme electrophoresis (Wang et al., 1993) and specificity better than that of phage typing, and because this laboratory was familiar with the techniques required for RAPD bacterial grouping.

MATERIALS AND METHODS

Bacterial Isolation

Birds were assessed for lameness on the farm by using a gait scoring system (Kestin et al., 1992) and were assigned a score according to the severity of lameness (0 =

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Abbreviation Key: BCN = bacterial chondronecrosis; PCR = polymerase chain reaction; PFGE = pulsed-field gel electrophoresis; RAPD = random amplification of polymorphic DNA.

not lame to 5 = profoundly lame). The birds were humanely killed by intravenous barbiturate administration and were dissected aseptically (McNamee et al., 2000). Material aseptically extracted from the proximal femur, the proximal tibia, and the tibiotarsus was crushed in sterile bone forceps, and swabs were taken from the crushed samples. The swabs were plated onto 5% defibrinated horse blood agar and MacConkey agar, and the bacteria isolated were categorized using culture and biochemical tests including Gram stain, catalase, oxidase, indole, and DNase activity. API® 20E[3] biochemical test strips were used for bacteria not readily differentiable by the methods noted. The coagulase test has traditionally been used to identify and differentiate pathogenic and nonpathogenic staphylococci, but in this study, DNase was chosen for its greater specificity for veterinary staphylococci (Quinn et al., 1994).

Molecular Grouping

Bacterial Lysis and Extraction of DNA. For the *S. aureus* isolates derived from 357 birds, DNA was extracted by the following method: single-colony, purity-plated isolates of *S. aureus* were cultured in broth for 24 h, and 700 μL of the broth suspension was placed in a 2-mL collection tube. The suspension was pelleted by centrifugation, and the pellet was resuspended in 180 μL buffer containing 20 m*M* Tris-HCl, pH 8.0; 2 m*M* EDTA; and 1.2% Triton X-100.[4] To this buffered suspension was added 2 μL (100 μg/mL) of Lysostaphin®,[5], a lytic agent derived from *Staphylococcus staphylolyticus*, and the mixture was incubated at 37 C for between 1 and 2 h until the turbidity had reduced by approximately 50%. DNA was extracted from the lysed cells with the DNeasy Tissue Kit[6] according to the manufacturer's recommendations.

RAPD

The DNA was amplified by RAPD with 10-base oligonucleotide primers, using a methodology similar to that described by Maurer et al. (1998). RAPD is a variation of polymerase chain reaction (PCR) in which there is no absolute requirement for previous sequence information to design the primers used for amplification (McClelland et al., 1994; McPherson and Moller, 2000). Short sequences of random oligonucleotides (10 bases in this case) will, by chance, be complementary to sequences within the genome. If two complementary sequences are present on opposite strands of a genomic region, and are close to each other, the DNA between them can become amplified by PCR. The amplified fragments produced are likely to be of variable lengths and can be resolved by gel electrophoresis. It is possible to increase the number of products, and hence the number of electrophoretic bands, by using two unrelated random primers of the same length in each PCR reaction (Welsh and McClelland, 1991); however, if more than 10 bands are produced on an agarose gel, then it becomes difficult to resolve each band.

By testing individual 10-mer primers, and combinations of pairs of these primers from a resource of 20 primers (AB 0320 primer set[7]) to optimize the RAPD product yield, the pair of primers giving the optimal number of bands after gel electrophoresis was as follows: Primer A, 5'-TGCGCCCTTC; and Primer B, 5'-GGTGACGCAG.

A reaction mixture containing 1 μL of template (DNA solution derived from the extraction above), 100 ng of Primer A, 100 ng of Primer B, and 7 μL of H_2O were mixed with 10 μL Taq PCR mastermix and loaded into a Hybaid®[8] Touchdown PCR block. Samples were denatured at 94 C for 105 s, followed by 40 cycles of 94 C for 60 s, 37 C for 60 s, 72 C for 180 s, and a final extension period of 120 s at 72 C.

Half of the product of the amplification (10 μL) was loaded into 1% agarose gels, and electrophoresis carried out at 100 V for 90 min. The bands of different molecular weights were detected by staining the gel for 1 h with ethidium bromide and by visualization with 254 nm light. The images were captured with a CCD camera,[9] and the molecular weight bands were grouped on banding similarities when compared to a DNA molecular weight marker ladder.[10] The degree of similarity between the banding pattern for different isolates was analyzed by computer using UVP® Gel Works ID software. It was readily possible to differentiate the most common banding patterns into groups by eye. For each *S. aureus* isolate, and for a reference isolate, repeat RAPD typing was performed on at least two occasions to determine the reproducibility of RAPD typing based on molecular weight banding patterns.

RESULTS

Bacteriological Results

Of the 357 birds examined, cultures of bacteria derived from bone of the proximal femur, proximal tibia, and tibiotarsus in control and lame birds showed *E. coli* and DNase-negative staphylococci to be present. Table 1 shows the distribution of RAPD groupings for the bacterial isolates derived from gait scored birds.

Molecular Grouping Results

Extraction of DNA and amplification by RAPD produced banding patterns after electrophoresis and staining in agarose gels (Figure 1). The banding patterns were used to separate *S. aureus* isolates into four main groups: A, B, C, and D (Figure 2). Single isolates that did not appear to be related to other isolates by RAPD were combined as 'other' (10% of the total population).

[3]API-BioMeriuex SA, 69280 Marcy-l'Etoile, France.
[4]Merck Ltd., Merck House, Poole, Dorset, BH15 1TD, UK.
[5]Sigma Diagnostics, Poole, Dorset, BH12 4QH, UK.
[6]Qiagen Ltd., Boundary Court, Crawley, W. Sussex, RH10 2AX, UK.
[7]Abgene, Epsom, Surrey, KT19 9AP, UK.
[8]Hybaid, Action Court, Ashford, Middx, TW15 1XB, UK.
[9]UVP Inc., Upland, CA 91786.
[10]Generule, MBI Fermentas, Vilnius 2028, Lithuania.

TABLE 1. The percentage frequency of isolation of *Escherichia coli*, DNase-negative staphylococci, and DNase-positive staphylococci from gait scored birds and the random amplification of polymorphic DNA (RAPD) groupings for *Staphylococcus aureus* isolates

	Gait score						Total (% in bracket)
	0	1	2	3	4	5	
No. birds sampled in each gait score group	23	25	40	70	125	69	352
Bacteriological samples from each gait score group	72	47	62	131	226	142	680
Isolates of *E. coli*	4	0	2	12	10	6	34 (5)
Isolates of DNase-negative staphylococci	12	11	9	28	33	22	115 (17)
Isolates of DNase-positive *S. aureus*	2	2	0	6	52	49	111 (16)
Number of isolates in RAPD Type							
A	0	1	0	0	14	17	33 (30)
B	1	0	0	2	31	25	59 (53)
C	0	0	0	0	2	2	4 (3.5)
D	0	0	0	2	2	1	4 (3.5)
Other	1	1	0	2	3	4	11 (10)

Isolates derived from Irish flocks were generously provided by McNamee and co-workers (2000) and were subjected to RAPD to correlate RAPD grouping patterns with those produced by PFGE. Similarly, isolates derived from this UK-based study were subjected to typing by PFGE in the Department of Agriculture Northern Ireland laboratories)Rodgers et al., 1999). Eight isolates were analyzed by both laboratories. Agreement was achieved in seven of the eight isolates in terms of their relationships to each other and to reference isolates of UK Type B and Irish Type I24. This comparative study indicated that it was possible to group the dominant type (RAPD Type B and PFGE Type I24) reliably by either method. The Group B isolates described in this paper showed a high degree of relatedness to the dominant Irish strain, Type 24, and other isolates could be grouped readily by both methods, although the degree of specificity varied between the two methods.

Pearson's chi-squared test using the Xact method[11] was applied to the gait score and the RAPD typing data for the *S. aureus* isolates. An asymptotic $P = 0.053$ indicated a correlation between high gait score (lameness) and the occurrence of RAPD Types A and B.

DISCUSSION

In this study, the frequency of *S. aureus* isolation from lame birds (gait scores 4 and 5) was approximately 17% of the sample, which compares very closely with the recent finding of 17.3% in Irish flocks (McNamee and Smyth, 2000), but less closely matches the 38% noted by Pattison (1992). *S. aureus* BCN/osteomyelitis is one of a number of potential causes of lameness including hock lesions (Menzies et al., 1998) and skeletal effects such as valgus-varus (Singh-Sanotra, 1999).

The finding that BCN/osteomyelitis is associated with only a proportion of lame birds is consistent with the multifactorial nature of broiler lameness that is likely to be a result of the combined effects of a number of influences including weight, skeletal integrity, nutrition, and disease.

The typing of *S. aureus* isolates by RAPD indicated the predominance of a restricted number of RAPD types in association with pathologies causing lameness. Group B isolates (53%) were the dominant related isolate group. Group B isolates show a high degree of relatedness to Irish type 24 isolates. The strong association with clinical disease supports the hypothesis (McCullagh and McNamee, 1998) that this restricted group of types is more pathogenic for broilers than other, unrelated isolates.

The use of molecular methods for typing staphylococci has been questioned (Tenover et al., 1994; Cookson et al., 1996) in terms of reproducibility and the ability to correlate molecular groupings derived in one laboratory with those found in other laboratories, or to correlate groupings with other typing methods (Gilligan et al., 2000; Weller, 2000). However, as an epidemiological tool, the ability to break down bacterial species identified by culture and biochemical means into subgroups is valuable, even without complete correlation with other methods such as phage typing. A reproducible method for subdivision allows tracing of subpopulations of bacteria in animals and in the environment. In this study, RAPD proved to be reproducible for the avian *S. aureus* strains observed, and comparison with Irish PFGE typing indicates that it is possible to make some correlation between differing molecular typing methods performed in different laboratories.

The grouping of avian *S. aureus* isolates by RAPD may provide epidemiological data of value in the control of broiler lameness. By determining where pathogenic strains are found in production systems, control of the factors that regulate the dominance of these strains may be possible. Targeted or reduced use of antibacterials, or the creation of vaccines based on surface protein characteristics (Smeltzer and Gillaspy, 2000), may be possible

[11]StatXact 3, Cytec Software Corp., Cambridge, MA 02139.

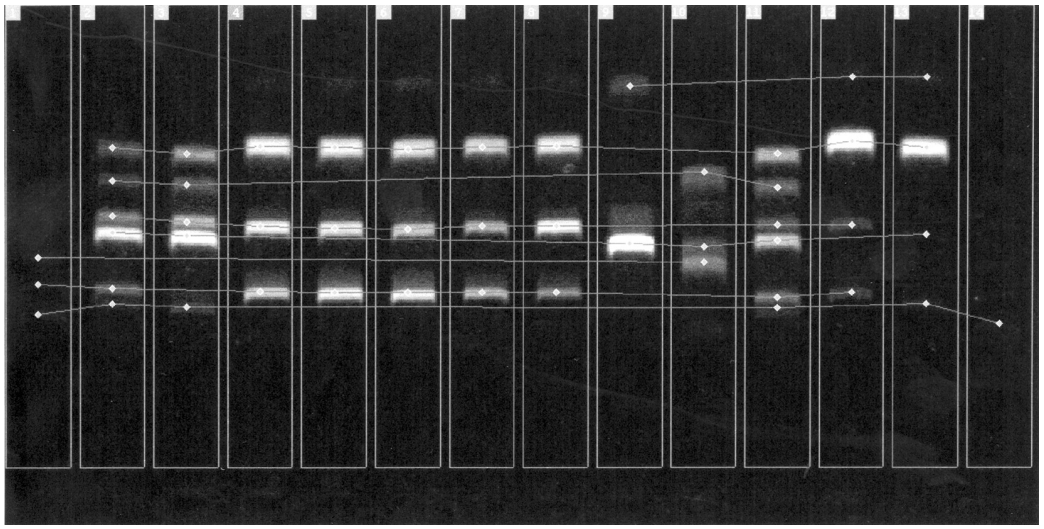

FIGURE 1. Example of random amplification of polymorphic DNA products derived from avian *Staphylococcus aureus* isolates separated by electrophoresis on 1% agarose gel.

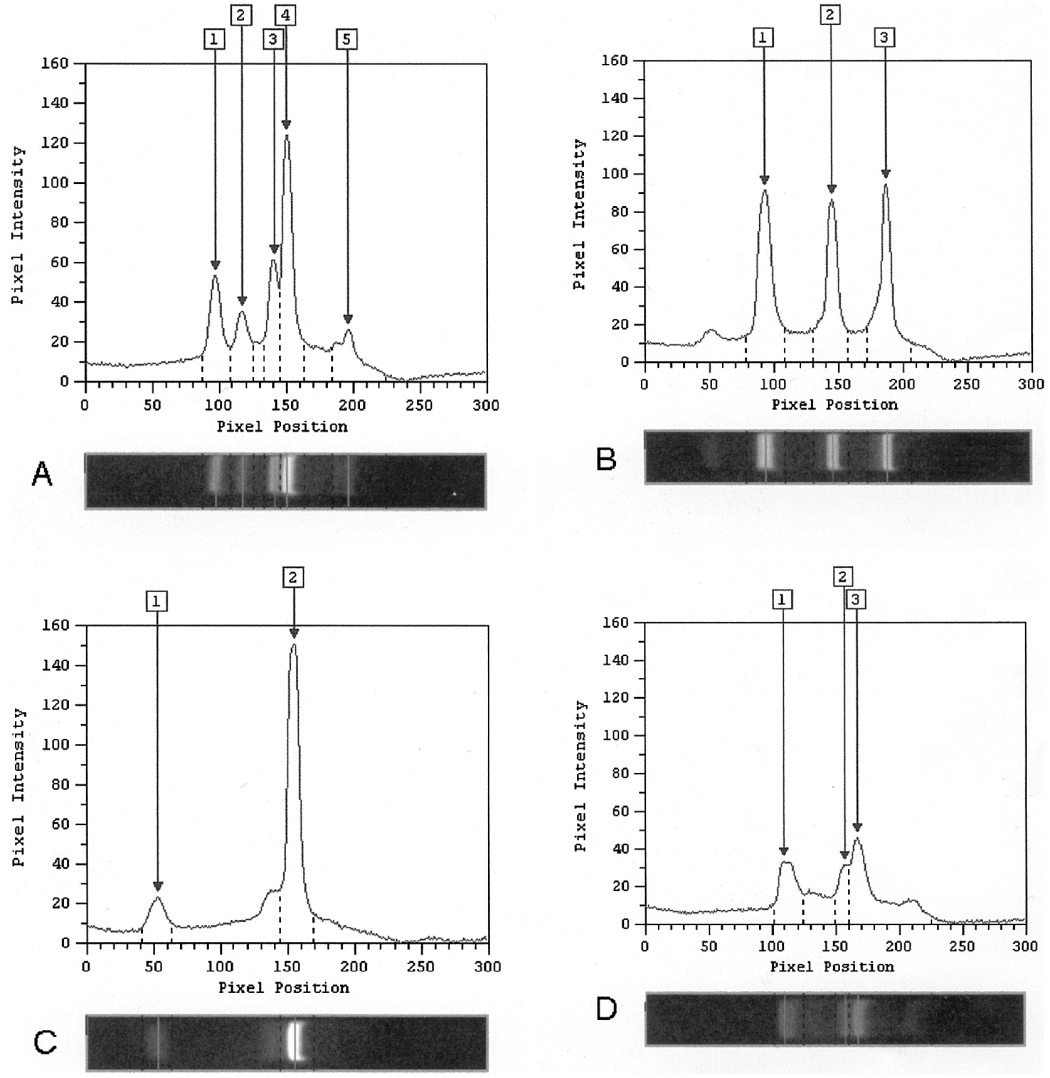

FIGURE 2. UVP® Gel Works ID pixel intensity profiles for *Staphylococcus aureus* random amplification of polymorphic DNA reference types A, B, C, and D. The molecular weights (bp) for the reference bands are as follows: A1, 500; A2, 700; A3, 1,100; A4, 1,300; A5, 2,300; B1, 450; B2, 1,300; B3, 2,300; C1, 200; C2, 1,300; D1, 650; D2, 1,350; D3, 1,400.

if the incidence of typed strains of *S. aureus* associated with broiler lameness (BCN) are known. Through early detection of pathogenic strains of *S. aureus* in breeder flocks and hatcheries and culling of lame birds to reduce dissemination of *S. aureus*, it may be possible to influence the incidence of BCN in broilers.

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