MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

Research and Development

Final Project Report

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CSG 15

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Project title	Identify the principle microbial agents responsible for, and factors associated with, infective causes of leg weakness.
MAFF project code	**AW0213**
Contractor organisation and location	Clinical Veterinary Science, University of Bristol Veterinary School Langford, N Somerset BS40 5DU
Total MAFF project costs	£ 379,046
Project start date	01/04/98
Project end date	21/12/01

Executive summary (maximum 2 sides A4)

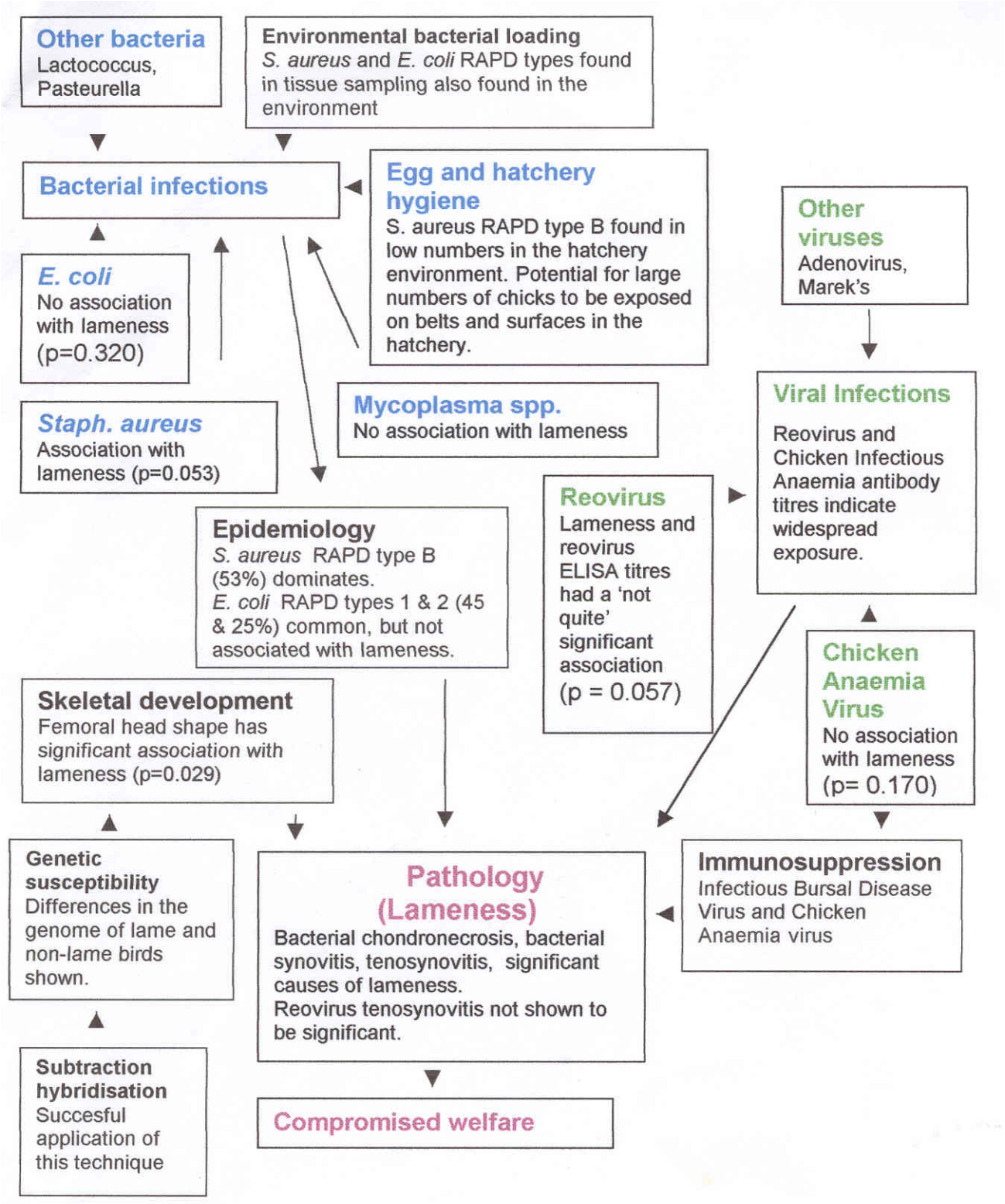
Broiler chickens (*Gallus gallus domesticus*) suffer a number of pathological conditions resulting in lameness. The incidence of Staphylococcal and *E. coli* osteomyelitis in lame and control birds sampled on commercial farms from a broad geographical sample were identified, and typing of the strains of *S. aureus* and *E. coli* performed by Random Amplification of Polymorphic DNA (RAPD). The gait score, a reproducible measure of severity of lameness in these birds, was correlated with the bacteriological findings, and with the incidence of specific *S. aureus* and *E. coli* isotypes. A correlation between the incidence of Type B *S. aureus* and high gait score was demonstrated. The incidence of isolation of the pathogens *S. aureus* and *E. coli* within the environments experienced by the bird during the growth cycle were also examined.

In a study involving detection of Mycoplasma spp. in joint associated tissues by Polymerase Chain Reaction (PCR), no association between Mycoplasma spp. and lameness was demonstrated.

Post mortem blood collection and dissection of birds which had been gait scored on farm, allowed the association between challenge with Reovirus and Chicken Anaemia Virus, as measured by antibody titre and a range of skeletal parameters, to be statistically analysed. An association which was 'not quite' significant ($p = .057$) between reovirus titre and gait score was demonstrated, with a strong correlation between tibial length and reovirus titre ($p = .006$).

The role of genomic variation in susceptibility to lameness was explored by the use of Subtractive Hybridization, with comparison of RNA derived from lame and control birds, and discussion of gene sequences produced by this subtraction study suggests that this technique has potential for differentiating genetic succeptibility to conditions which may result in lameness.

Summary flowchart of findings.



Scientific report (maximum 20 sides A4)

1 Molecular Typing of strains of Staphylococcus aureus

Bacterial chondronecrosis - BCN, previously known as femoral head necrosis (FHN) and tibial osteomyelitis, causes lameness and accounts for the loss of a significant proportion of the estimated 2.6% (approximately 20 million) of birds which are culled for lameness (Pattison, 1992; Yogaratnam, 1995) in an estimated total UK annual broiler production of 800 million birds. Broilers become lame with BCN lesions late in their growth (Butterworth, 1999; McNamee and Smyth, 2000), most commonly after 30 days of age, and thus are lost after significant economic input.
This study was performed to identify the principal bacterial pathogens associated with BCN. Both lame and non lame (control) birds were sampled to differentiate non-pathogenic 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, Colindale, UK 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* are noted in recent studies from Ireland (McCullagh and McNamee, 1998). Possible other methods for typing the *S. aureus* isolates included multilocus enzyme electrophoresis (MLEE)(Bart *et al*.,1998; Pujol et al., 1997), pulsed field gel electrophoresis (PFGE) (Bannerman *et al*., 1995, Rodgers *et al*., 1999), bacterial restriction endonuclease digest analysis (BRENDA)(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 was considered to offer the possibility of sensitivity at least as acute as that of MLEE (Wang *et al.,* 1993), specificity better than that of phage typing, and because this laboratory was familiar with the techniques required for RAPD bacterial grouping.

Visits were made to a number of farm, hatchery and processing plant sites. Birds were assessed for lameness on farm using a gait scoring system (Kestin et al. 1992) and ascribed a score according to the severity of lameness (0=non lame, to 5=profoundly lame). The birds were humanely killed by intravenous barbiturate administration, and dissected aseptically following a methodology incorporating techniques used by other workers in similar studies (McNamee & Smyth, 2000). The culture of bacteria derived from the harvesting stages described is common to all the major bacterial groups identified. The swabs were plated onto 5% defibrinated horse blood agar and MacConkey agar, the horse blood plates being cultured at 37°C in 6% CO_2 and the MacConkey plates at 37°C in air. Individual colonies of bacteria cultured at 24 hours were replated and cultured for a further 24 hours and categorised using culture and biochemical tests including Gram stain, Catalase, Oxidase, Indole and DNAse activity. API® 20E (BioMerieux[a]) 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 non pathogenic staphylococci, but in this study DNAse was chosen for its greater specificity for veterinary staphylococci (Quinn *et al*. 1994).

Femoral and tibial post mortem tissue samples were fixed in 10% buffered neutral formalin. The fixed tissues were then decalcified in an excess volume of 10% formic acid in plastic tubs placed on a slow moving mixer table in a fume cupboard. Decalcification took approximately 12 to 14 days. Microtome sections were cut at 4 µm intervals using a Leitz 1400 microtome. These sections were dried, de-waxed and stained using Gram/Twort and Haematoxylin & Eosin.

1.1 Molecular grouping

For the *Staphylococcus aureus* isolates, DNA was extracted by the following method. Single colony, purity plated isolates of *S. aureus* were cultured in broth for 24 hours and 700µl of the broth suspension was placed in a 2ml collection tube. The suspension was pelleted by centrifugation, and the pellet was resuspended in 180µl Tris HCL, pH 8.0, buffer containing 20mM Tris, 2mM EDTA and 1.2% Triton. To this buffered suspension was added 2µl (100µg/ml) of Lysostaphin® - (Sigma) a lytic agent derived from *Staphylococcus staphylolyticus,* and the mixture was incubated at 37°C for between 1 and 2 hours until the turbidity had reduced by approximately 50%.
DNA was extracted from the lysed cells using the DNeasy Tissue Kit (Qiagen®) according to established protocols.

1.2 Random amplification of polymorphic DNA (RAPD)

The DNA was amplified by random amplification of polymorphic DNA (RAPD) with 10-base oligonucleotide primers, using a methodology similar to that described by Maurer (Maurer et al., 1998). RAPD is a variation of the 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 within a close distance of 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. The optimum product yield was achieved by testing combinations of pairs of primers from a resource of twenty primers (ABgene , AB 0320 primer set).

The pair of primers giving the optimal number of bands after gel electrophoresis were;

a) (ABI– 05) 5'-TGCGCCCTTC b) (ABI-07) 5'-GGTGACGCAG

A reaction mixture containing 1μl of template (DNA solution derived from the extraction above), 100ηg primer a), 100ηg primer b) and 7μl of H_2O was mixed with 10μl Qiagen® Taq PCR mastermix and loaded into a Hybaid® Touchdown PCR block and subjected to denaturing at 94°C for 105 s, followed by 40 cycles of 94°C for 60 s, 37°C for 60 secs, T_A 72°C for 180 sec, and a final annealing period of 120 s at 72°C.

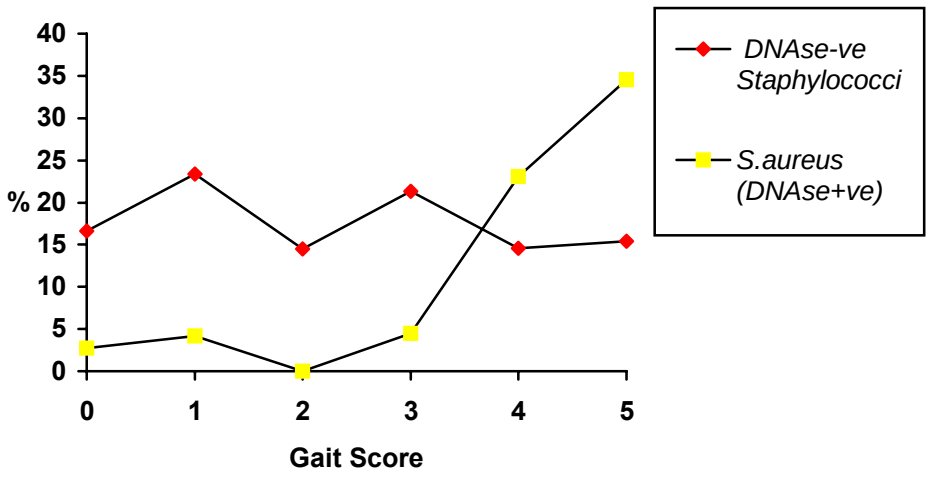
1.3 Gel Electrophoresis

To allow estimation of the molecular weights of DNA bands, a molecular weight ladder was run alongside sample and control channels whenever gel electrophoresis was carried out. In this study, Generuler®1kb DNA ladder, (MBI Fermentas) was used, providing reference bands at known molecular weights. For each PCR product, about half of the product of the amplification (10μl) was loaded into 1% agarose gels and electrophoresis carried out at 100v for 90 minutes. The remaining PCR product solution (10μl) was stored at -20°C as a reference. The bands of different molecular weight product were detected by binding of ethidium bromide to DNA in the gel, and visualisation of the fluorescence of this bound ethidium bromide in 254nm light. The images were captured using a UVP® CCD camera, and the molecular weight bands grouped on banding similarities when compared to a DNA molecular weight marker ladder (GeneRuler®). The degree of similarity between the banding pattern for different isolates was analysed by computer using UVP® Gel Works ID software. It was readily possible to differentiate the most common banding patterns into groupings by eye.

1.4 Results

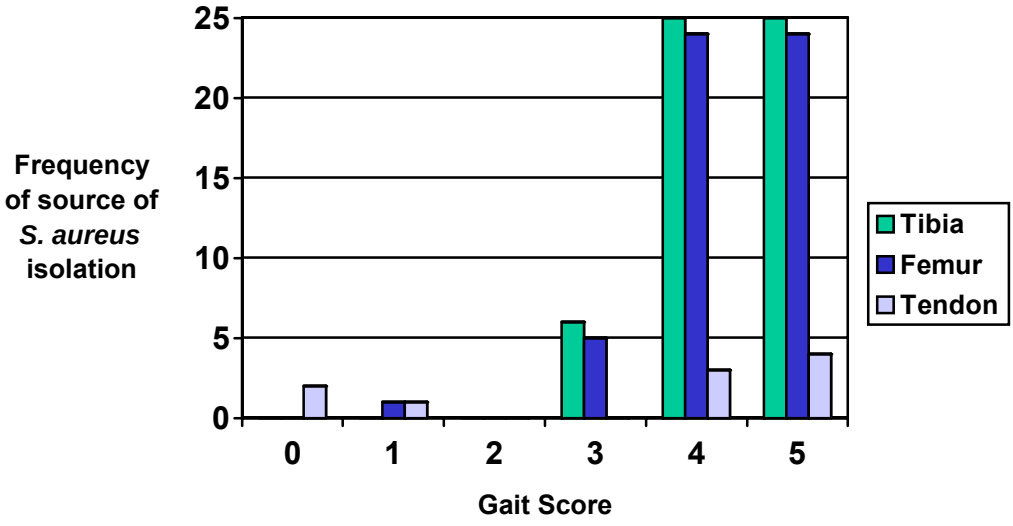
From a sample of 688 birds which were gait scored and sampled aseptically immediately *post mortem* on farm, or derived from stockman 'leg culls', culture of bacteria derived from bone of the proximal femur, proximal tibia and tibiotarsus in both control and lame birds showed DNAse negative staphylococci and *S. aureus* to be present in a number of birds (Figure 1). It is apparent that, whilst the isolation of DNAse negative Staphylococci is not uncommon, the identification of *S. aureus* is comparatively uncommon in low gait score birds, and more common in lame (higher gait score) birds.

Figure 1 Percentage of samples from birds in which isolates of DNAse negative staphylococci and *Staphylococcus aureus* (DNAse positive) were recovered. (Gait score 0 = non lame, to Gait score, 5 = worst lameness)



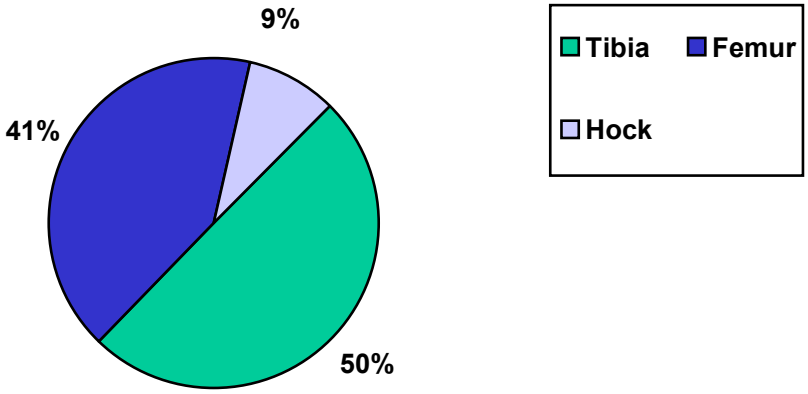
Samples were taken from the following anatomical locations by aseptic dissection: Femoral head / neck, proximal tibia and samples from the hock joint or tendon sheath distal to the hock joint (Figure 2).

Figure 2 Frequency of the source of *S. aureus* isolates for gait score groups 0 to 5.



These data (Figure 3) highlight the locality of localised *S. aureus* infection, and indicate the relative frequency of pathologies linked with *S. aureus* infection, for example, bacterial chondronecrosis of the tibia, bacterial chondronecrosis of the femur (Tibial osteomyelitis, femoral head necrosis), or hock synovitis / tenosynovitis.

Figure 3 Anatomical source of 116 *S. aureus* isolates from pooled samples for all sample locations.



1.5 Background *S. aureus* isolation results.

Additional bacteriological sampling was undertaken to gain a picture of the 'background' frequency of isolation for *S. aureus*.

Table 1 Number of samples and recovery rate of *S. aureus* for 'background' sample locations D, F, J, O & P.

ID	Date	Birds sampled	No. Sam-ples	*S. aureus* (% of sample)
D	1/7/98	25	50	2 (4)
F	5/898	72	135	8 (6)
J	30/9/98	50	98	4 (4)
O	23/1/99	18	40	5 (12)
P	11/2/99	30	54	3 (6)
	Totals	**195**	**377**	
			Mean	**6.4 %**

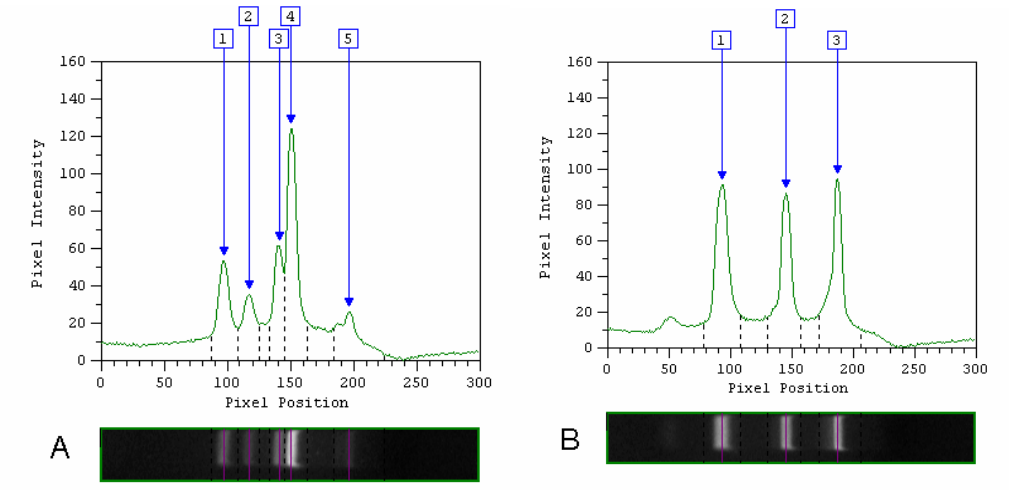
1.6 Phage typing results

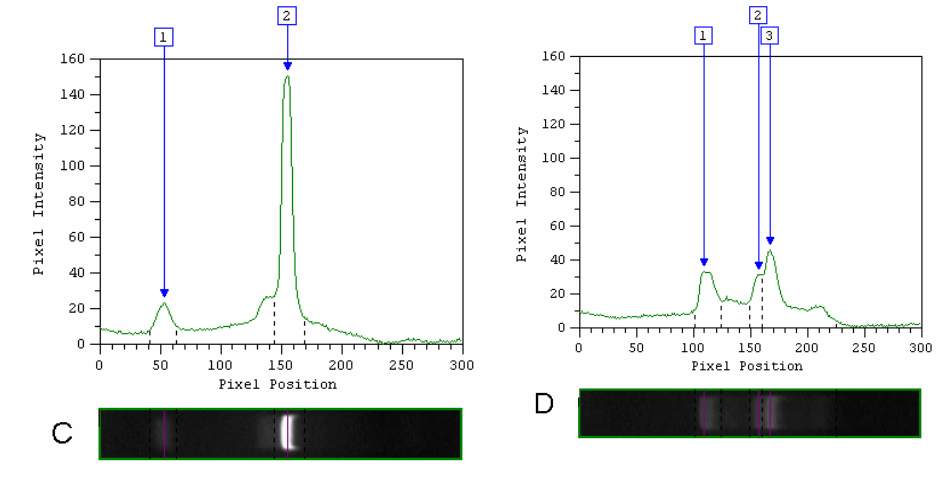
Six isolates of *S. aureus* (5 derived from sampling on farm, and one a reference strain) were typed using a human *S. aureus* phage library, by Dr Mark Ganner at the Central Public Health Laboratory, Colindale, London.
Phage typing of this small trial group of six samples indicated that the specificity was moderate, phage typing being able to differentiate 3 out of 5 bird derived samples. However, the sensitivity was poor, with only 1 isolate (T735) giving a strong phage reaction. Phage typing is widely used to type human derived strains of Staphylococci, but, the evidence of this small trial confirms than human *S. aureus* phage types are unlikely to provide a good reference for animal derived Staphylococci.

1.7 Molecular grouping results

The *S. aureus* isolates derived from the bacterial culture described were used as the source material for 'typing' by Random Amplification of Polymorphic DNA (RAPD). Extraction of DNA and amplification by RAPD produced banding patterns after electrophoresis in agarose gel which were detected in 254nm light. The banding patterns were used to separate *S. aureus* isolates into groupings based on visual separation of reproducible patterns of banding and by confirmation of the degree of similarity of banding patterns by creation of dendograms of 'relatedness' using Gelworks® image analysis software which automatically detects bands on digitised images of agarose gel's and, by comparing the pixel intensity profiles for each PCR product, ascribes a measure of 'relatedness' between PCR product banding patterns.

Figure 4 Gelworks® Band detection and pixel intensity profile for Type 'A' to 'D' *S. aureus*.

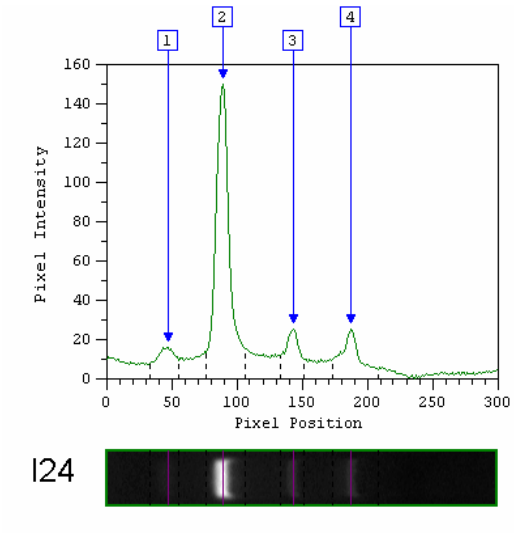




1.8 Comparison with Irish *S. aureus* reference strains.

Isolates derived from Irish flocks were kindly provided by McNamee and co-workers (DANI, Stormont) and subjected to RAPD to correlate pixel intensity profiles and RAPD grouping based on banding pattern with those produced by Pulsed Field Gel Electrophoresis (PGFE). Similarly, isolates derived from this UK based study were subjected to typing by PGFE in the Dept of Agriculture Northern Ireland laboratories.

Figure 5 Gelworks® Band detection and pixel intensity profile for Irish reference type 24 *S. aureus.*

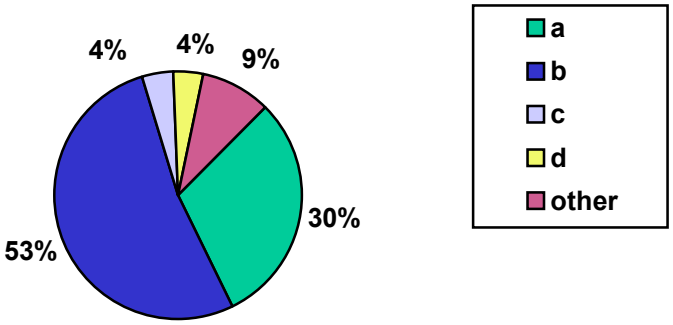


'Blind' isolates were sent to McNamee and co-workers in the Department of Agriculture, Northern Ireland (DANI), Stormont and the sensitivity of pulsed field gel electrophoresis (PFGE) was compared with the sensitivity of random amplification of polymorphic DNA (RAPD) performed in this study. A high level of agreement between the Irish PGFE typing method, and the RAPD method desribed, was found.

1.9 Statistical analysis

A breakdown of the percentage findings of groups A to D, and of 'other' (non grouped) isolates is given in Figure 6.

Figure 6 Percentage grouping of isolates from 357 broilers of *S. aureus* grouped by Random Amplification of Polymorphic DNA.



A Pearson Chi-Square test using the Xact method (StatXact 3,Cytec Software) was used to examine the association of gait score and the RAPD typing data for the *S. aureus* isolates. When $X^2 = 20.80$, with 12 degrees of freedom, **p = 0.053.** This indicates an association between high gait score (lameness) and the occurrence of RAPD types A and B, which is 'not quite' significant at the 95% confidence level, but within the 90% confidence interval.

1.10 Discussion of *S. aureus* findings.

S. aureus was isolated from aseptically derived tissue samples in up to 35% of lame birds. The Chi-Square association between the isolation of *S. aureus* and lameness (**p = 0.053**) indicates a corellation between high gait score (lameness) and the occurrence of RAPD types A and B, which is just outside the 95% confidence interval, but within the 90% confidence interval. The variability in the recovery rate for *S. aureus* from farm to farm suggests that *S. aureus* can produce 'outbreaks' of BCN / osteomyelitis causing lameness. This is supported by the findings of recent Irish studies (McNamee *et al.,* 2000; McNamee *et al*., 1998) and the field experience of poultry veterinarians who, on occasion, may choose to use antibiotics in the early growth period to reduce the occurrence rate of BCN. From the locations at which non-gait scored birds were sampled, it was possible to gain a picture of the 'background' frequency of isolation for *S. aureus,* with positive isolation in 6.4% of 195 birds. These birds represent a geographical cross section being from sites across the UK, and hence, it may be possible to infer that the rate of 'background' *S.aureus* isolation may be used as an indicator of the number of birds reaching slaughter weight which are, sub clinically infected with *S. aureus* (Not a welfare concern, but a potential human zoonotic hazard through food poisoning (Wieneke *et al.,* 1993)), or are clinically infected, likely to be lame, and harvested anyway. The typing of *S.aureus* isolates by RAPD indicated the predominance of a restricted number of types in association with pathologies causing lameness, Group B isolates (53%) being the dominant related isolate group. Group B isolates show a high degree of relatedness to Irish type 24 isolates, and the strong association with clinical disease supports the supposition (McCullagh & McNamee, 1998) that this restricted group of types are more pathogenic for broilers than other, non related isolates. This study supports findings from Irish broiler flocks (McNamee *et al.,*1998) (McCullagh and McNamee, 1998) in which *S. aureus* isolates were grouped by Pulsed Field Gel Electrophoresis - PFGE (Bannerman *et al*., 1995). The group B isolates described in this study 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. In this study, RAPD proved to be reproducible for the avian *S. aureus* strains seen, and comparison with Irish PGFE 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 which 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 & Gillaspy, 2000) may be possible if the incidence of typed strains of *S. aureus* associated with broiler lameness (BCN) are known. Early detection of pathogenic strains of *S.aureus* in breeder flocks and hatcheries and culling of lame birds to reduce dissemination of *S. aureus* may have an impact on the incidence of BCN in broilers.

2 *E. coli*

For the *E. coli* isolates derived from 375 birds, DNA was extracted by the following method. Single colony, purity plated isolates of *E. coli* were cultured in broth for 24 hours and aliquots stored at -20°C in Protect® bead storage tubes. When further culture of the isolates was required, a single bead from the stored isolates was removed and used as the source for plating onto 5% defibrinated horse blood agar and MacConkey agar. The inoculated plate was incubated at 37°C for 24 hours, and a single monotypic colony selected to grow in broth culture. After 24 hours incubation of the broth culture at 37°C, 700µl of the broth suspension was placed in a 2ml (Eppendorf) collection tube. The bacterial suspension was pelleted by centrifugation, and the pellet was resuspended in 200µl PBS by vortexing, and further diluted with 180µl buffer ATL. To this buffered suspension was added 25µl (140mAU) of the lytic enzyme Quiagen® protease, (this enzyme having reduced DNase and RNase activity when compared to protease K). This suspension was vortexed, and the mixture incubated at 55°C for between 1 and 2 hours until the fluid had almost cleared. The activity of the Quiagen® protease was then stopped by placing the vial in a heater block at 70°C. DNA was then extracted from the solution using the DNeasy Tissue Kit (Qiagen®) using established protocols.

2.1 Random amplification of *E. coli* polymorphic DNA (RAPD)

The DNA derived from *E. coli* isolates was then amplified by Random Amplification of Polymorphic DNA (RAPD) with selected primers (Advanced Biotechnologies Ltd) using a methodology similar to that described by Maurer (Maurer *et al*., 1998). The primers were chosen from a set of 5 possible primers (below) by testing combinations, and single primers, to optimise the RAPD PCR product yield. The primer giving the optimal product yield was;

5'-AAACGGTTGGGTGAG - 3'

A reaction mixture containing 2µl of template (DNA solution derived from the extraction above), 100ηg primer e), and 7µl of H_2O, were mixed with 10µl Qiagen® Taq PCR mastermix and loaded into a Hybaid® Touchdown PCR block and subjected to denaturing at 94°C for 145 s, followed by 35 cycles of 94°C for 60 s, 40°C for 60 secs, T_A 72°C for 180 sec, and a final annealing period of 120 s at 72°C.

2.2 Gel Electrophoresis

Half of the product of the amplification (10µl) was loaded into comb wells in 1% agarose gel, and electrophoresis carried out at 100v for 90 minutes. The remaining extracted DNA solution (10µl) was stored at -20°C as a reference.
After electrophoresis, the bands of different molecular weight product were detected by staining the gel with ethidium bromide and visualised in 254nm light. The images were captured using a UVP® CCD camera, and the molecular weight bands grouped on banding similarities when compared to a DNA molecular weight marker ladder (GeneRuler™). The degree of similarity between the banding pattern for different isolates was analysed by computer using UVP® Gel Works ID software. It was readily possible to differentiate the most common banding patterns into groupings by eye.

2.3 Bacteriological Results

764 samples were derived from 375 birds collected on farm and dissected aseptically. Culture of bacteria derived from bone of the proximal femur, proximal tibia and tibiotarsus in both control and lame birds showed *E.coli* to be present some of samples from birds from all lameness categories. The relationship between Gait Score and the percentage occurrence of *E. coli* isolation is indicated below, Table 2.

Table 2 Number of isolations of *E. coli* from samples in each gait score Group 0 to 5.

Gait Score	0	1	2	3	4	5	total
No. birds sampled of known gait score.	23	26	40	72	127	69	357
No. bacteriological samples from each gait score.	72	47	62	131	226	142	680
No. of *E. coli*	1	0	7	13	16	11	48
***E. coli* isolates as a % of sample group.**	1.3	0	11.3	9.9	7.1	7.7	-

The relationship between the isolation of *E. coli* and Gait Score is illustrated in more detail in Figure 7. It is apparent that the isolation of *E. coli* is comparatively uncommon in sound birds (score 0 and 1), and somewhat more common in birds of gait score 2 and above.

Figure 7 Percentage of samples in which *E. coli* were isolated from all sampling locations (Gait score 0 = non lame, to Gait score, 5 = worst lameness)

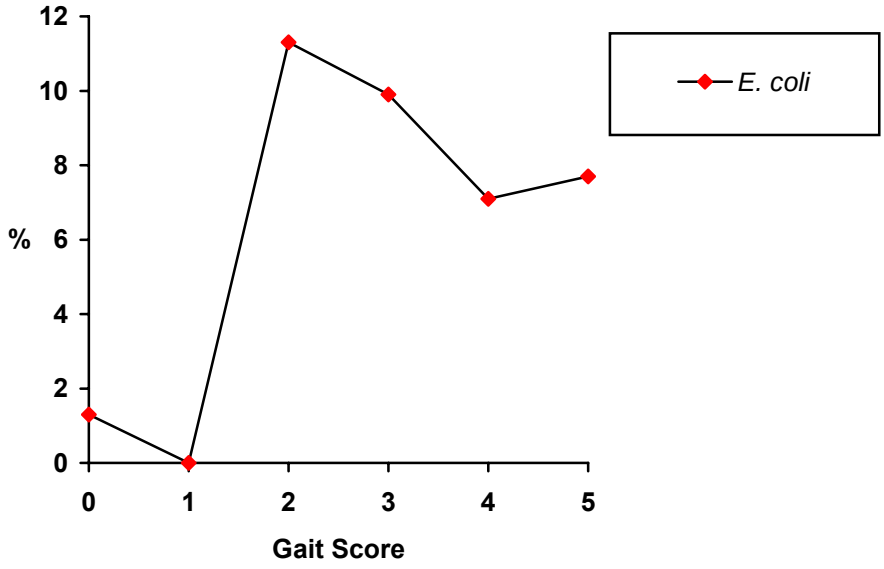


Figure 8 Frequency of *E. coli* isolation for producer A to N (Colour Key, Gait Score 0-5)

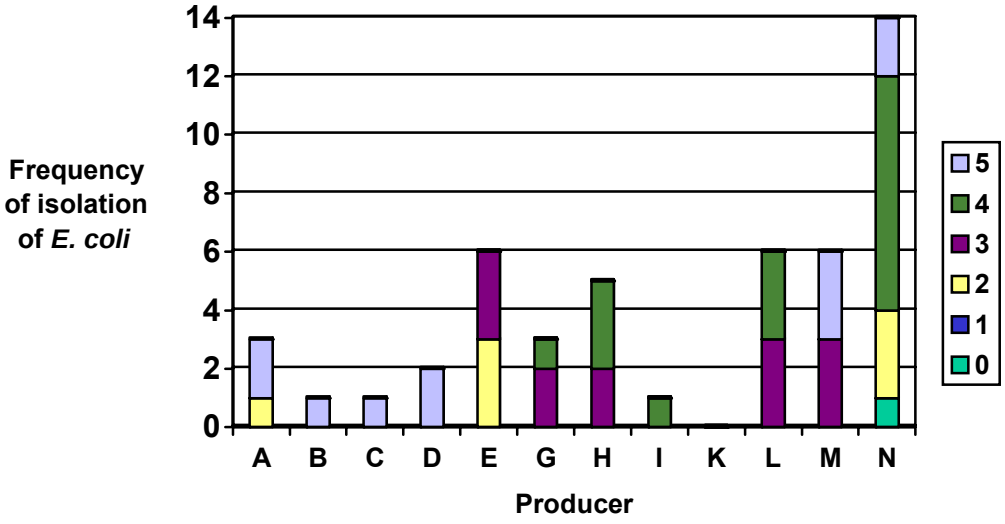
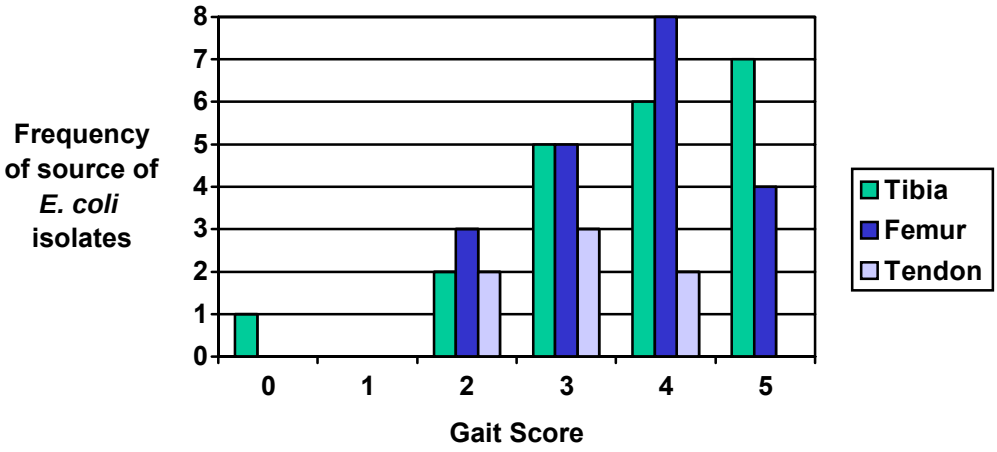
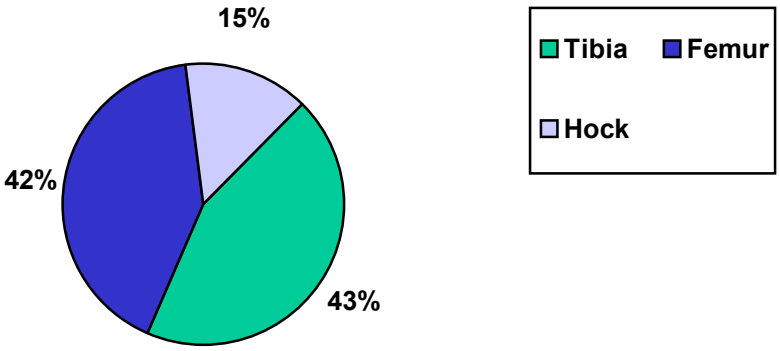


Figure 9 Frequency of the source of *E. coli* isolates for Gait Score groups 0 to 5.



Expressing the pooled anatomical source data for all farms, the pie chart in Figure 10 illustrates the percentage origin of the *E. coli* isolates from all birds sampled on farm.

Figure 10 Anatomical source of 48 *E. coli* isolates from pooled samples for all sample locations.



2.4 Background *E. coli* isolation

At a number of geographically widespread locations, birds were bacteriologically sampled to gain a picture of the 'background' frequency of isolation for *E. coli*. The results of this sampling are indicated in Table 3, below.

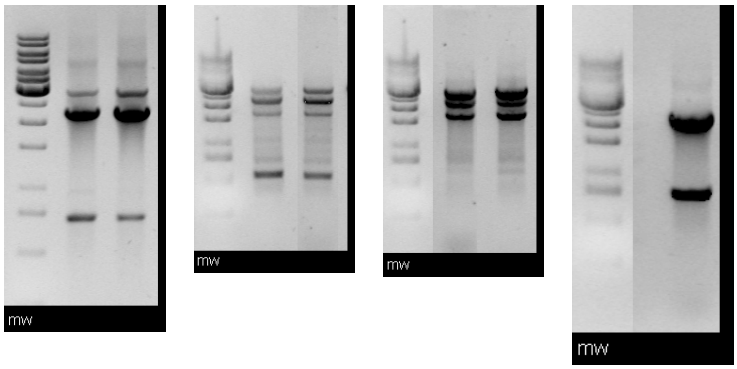
Table 3 Number of samples and recovery rate for *E. coli* isolations for 'background' sample locations D, F, J, O & P.

ID	Date	Birds sampled	No. Sam-ples	E. coli (% of sample)
D	1/7/98	25	50	4 (8)
F	5/898	72	135	4 (3)
J	30/9/98	50	98	5 (5)
O	23/1/99	18	40	13 (32)
P	11/2/99	30	54	2 (4)
	Totals	**195**	**377**	
			Mean	**10.4%**

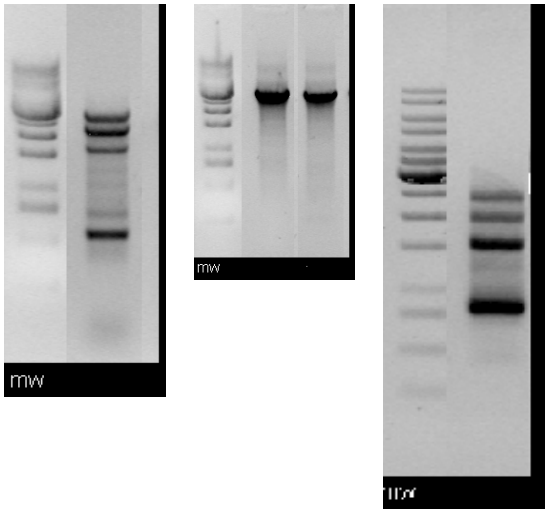
2.5 Molecular grouping results

The techniques for visualisation of PCR product in ultraviolet light after gel electrophoresis are as described for *S. aureus*. Extraction of DNA and amplification by RAPD produced banding patterns after electrophoresis in agarose gel which were detected in 254nm light. The banding patterns were used to separate *E. coli* isolates into 7 main groupings. Reference gel banding profiles for groups 1 to 7 (Figure 11) were produced to help the visual grouping of bands on gels visualised under ultraviolet light.

Figure 11 Reference 1½% agarose gel profiles for isotypes of *E. coli* derived by aseptic sampling.



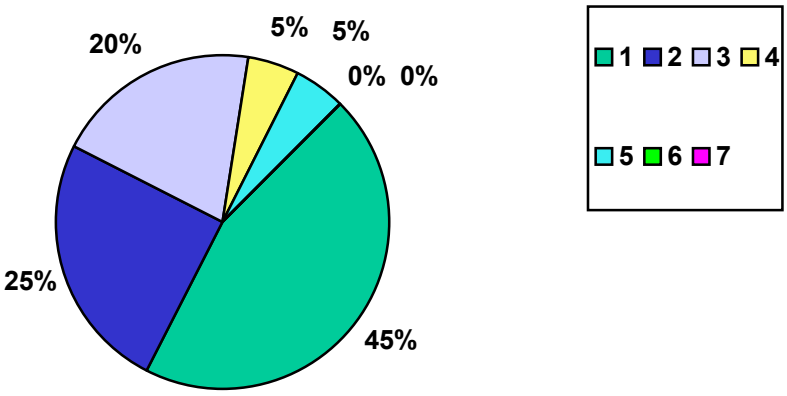
1	2	3	4



5	6	7

The relative frequency of RAPD groups 1 to 7 for the *E. coli* isolates derived by aseptic dissection in gait scored birds is illustrated as a pie chart in Figure 12.

Figure 12 Relative proportions of reproducible RAPD groups for 48 *E. coli* isolates derived by aseptic dissection.



*Note that *E. coli* RAPD types 6 and 7 (Colour Key Bright Green and Pink , Figure , above) are not identified (0%) in *E. coli* isolates derived by aseptic dissection. However, these RAPD types do feature in the environmental isolates which will be discussed below. The implication of this finding is that *E. coli* RAPD types which were recovered from birds are a sub-set of *E. coli* likely to be found in the poultry environment. It is also possible that these RAPD types have simply not been detected in bird derived samples in this study.

2.6 Statistical analysis

A Pearson Chi-Square test (StatXact 3,Cytec Software) was used to examine the association between *E. coli* isolation and gait score for 375 gait scored birds. With a Pearson Chi-Square statistic CH(X) = 13.83, and 12 degrees of freedom, **p = 0.31,** indicating that there was no significsant association between high gait score (lameness) and the occurrence of the common *E. coli* RAPD types 1 and 2.

2.7 E. coli Discussion

The incidence of *E. coli* recovery by aseptic dissection at farms A to N (Figure 8) indicates the variability of *E. coli* detection, with a relative recovery rate of from 14% (N) to 0% (K). The mean recovery rate for *E. coli* of 7% indicates the comparatively uncommon recovery of this organism from tissues. The corellation between the detection of *E. coli* and lameness is **p = 0.31** indicating that there was no statistically significant link between high gait score (lameness) and the occurrence of the common *E. coli* RAPD types 1 and 2 at the 95% confidence level. This statistical finding fails to support the clinical finding that *E. coli* may be isolated in cases of BCN (McNamee, 2000; Reece, 1992; Thorp, 1996) , but may support the hypothesis that, although *E. coli* may be isolated from lame birds, it is not likely to be the primary cause of lameness. *E. coli* may be an opportunisitic pathogen, making use of the niche provided by anatomy, the breakdown in defences caused by other organisms (including *S. aureus ?*), or the localisation of *E.coli* circulating as part of a generalised coli-septicaemia. Thus, it is possible that some of the *E. coli* isolated in lame birds may represent circulating *E. coli* from early stage colisepticamia. From the locations at which birds which were sampled as control background samples, it is possible to gain a picture of the 'background' frequency of isolation for *E. coli,* with positive isolation in 10.4% of 195 birds. These birds represent a geographical cross section of UK producers and hence, it may be possible to infer that the rate of 'background' *E. coli* isolation may be used as an indicator of the number of birds reaching slaughter weight which are;

a) Sub clinically infected with *E. coli* – Not a welfare concern, but a potential human zoonotic hazard.

b) Clinically infected, perhaps with generalised *E. coli* septicaemia, but harvested anyway.

c) The typing of *E. coli* isolates by RAPD indicate the predominance of a restricted number of types which could be isolated aseptically from birds. Types 1 and 2, defined on their RAPD DNA product agarose gel banding characteristics, dominate with 45 and 25% respectively of isolates derived from aseptic sampling.

The use of molecular methods for typing bacteria has been questioned (Cookson *et al*. 1996; Tenover *et al*. 1994) 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 sub-groups is valuable, even without complete correlation with other methods such as phage typing. A reproducible method for sub-division allows tracing of sub-populations of bacteria in animals and in the environment. In this study, RAPD

proved to be reproducible for the avian *E. coli* strains. The grouping of avian *E. coli* 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 which regulate the dominance of these strains may be possible.

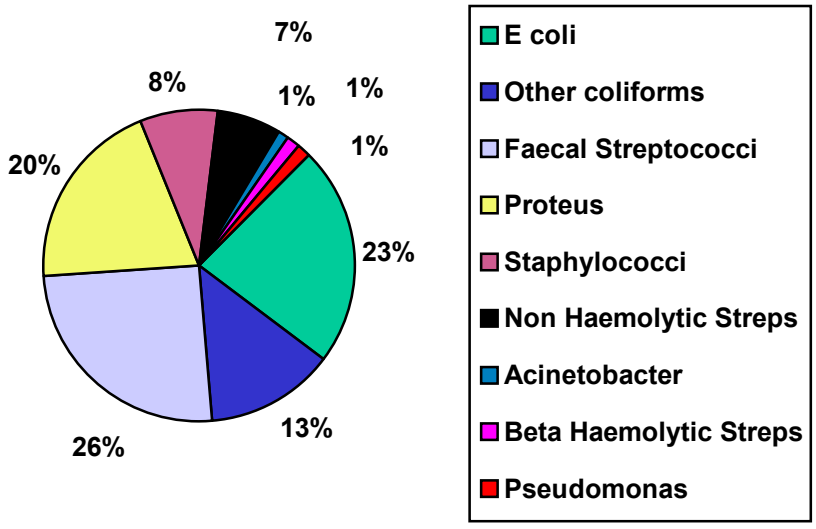
3.0 Hatchery Exposure Sampling

This aims of the environmental sampling were to identify the range of organisms to which eggs and chicks might be exposed in the hatchery, and to identify 'Critical Control Points'. For the purpose of this study the identification of an organism on a culture plate was considered as a 'positive' identification, and described as 1 (one) isolation. Thus, in the summary results described below, an 'isolation' indicates the presence of an organism at a given sampling site but does not describe the density (colony count) of the organism recovered at this site. For isolates derived from the sample sites described in the tables above, the frequency of isolation of the most common (as defined by number of isolations) bacterial pathogens is described in Table .

Table 4 Summary tabulation for isolation of the common bacterial species identified during hatchery sampling.

Bacteria	Number of swab isolations	Number of sponge derived isolations	Number of fluff / egg shell derived isolations	Number of chick oral swab isolations	Number of chick skin and feet isolations
Samples	304	48	82	32	39
E. coli	96	16	8	5	3
Other coliforms	33	2	4	0	0
Faecal streptococci	104	16	7	2	4
Proteus	80	20	6	0	0
Staphylococci	16	1	2	1	1
Non Haemolytic Streptococci	26	0	2	0	0
Beta Haemolytic Streptococci	7	0	1	0	0
Pseudomonas	6	0	2	0	0
Acinetobacter	6	0	0	0	0

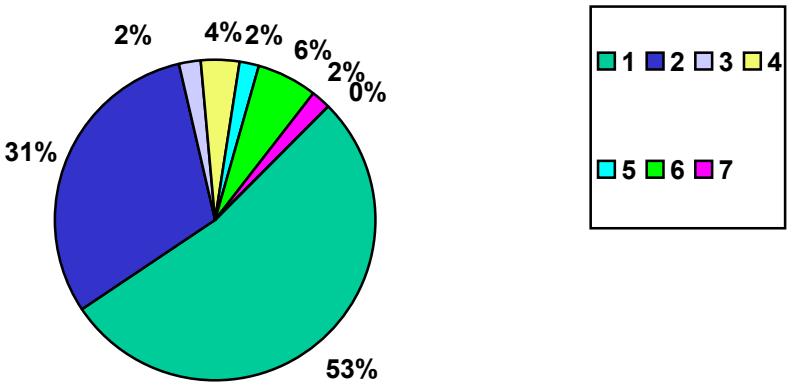
Figure 13 Relative frequency of isolation for the common bacteria identified during hatchery sampling.



RAPD typing of the *E. coli* isolates from the hatchery environment produced a distribution of types with a predominance of Type 1 and Type 2. Isolates which produced their own unique banding pattern i.e. those which only occurred once , and

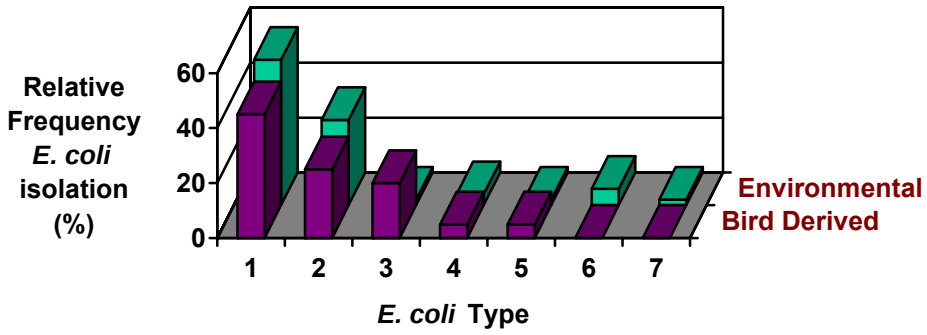
therefore could not be considered as Reference Types accounted for 22 % (23 of 112 isolates) of the sample. These 'other' types are not included on the frequency pie chart seen in Figure 14.

Figure 14 Relative distribution of reproducible RAPD groupings 1 to 7 for *E. coli* isolated from the hatchery environment. Non-typable isolates (22%) are not indicated.



The distribution of RAPD types for *E. coli* derived by sampling in the environment is compared with the distribution of *E. coli* types derived from aseptic tissue samples in birds in Figure 15.

Figure 15 Comparison of the relative distribution of *E. coli* RAPD type 1 to 7 for isolates derived by aseptic dissection of broiler birds, and from the hatchery environment. Non-typable isolates (22% of environmental isolates) are not included.



Sampling from a number of sites within the hatchery environment determined that *E. coli* was a common environmental bacterial isolate (23% of all isolations). With reference to the relationships between lameness and *E. coli* , there was no statistical association between lameness and the isolation of *E. coli* (p=0.31). This study suggests that, although *E. coli* may be commonly isolated in the hatchery environment, this pathogen does not appear to play a statistically significant role in lameness in the growing bird.

Staphylococci were comparatively uncommonly isolated in the study of the hatchery environment (8% of all isolations), but *S. aureus* formed only a small part of these (1.4% of all isolations). However, with reference to the relationship between lameness and *S. aureus* demonstrated insection 1.09 – when the X^2 statistic = 20.80, with 12 degrees of freedom, p = 0.053**.** This indicates an association between high gait score (lameness) and the occurrence of RAPD types

A and B, which is 'not quite' significant at the 95% confidence level, but within the 90% confidence interval. The finding that RAPD type 'B' isolates dominate in isolations from the hatchery and also in isolations from growing birds supports the supposition that Type B isolates may be 'avian adapted' strains, and that, although not present within hatchery environements in high density, it is likely that the hatchery is a key source of early introduction of these *S. aureus* types into the rearing environment via early chick exposure.

4.0 Mycoplasma spp.

One hundred and twenty six (126) birds were gait scored on farm, 94 being of high gait score (4 and 5), and 32 control (low gait score 0 and 1). The birds were selected from a number of farms, and on a number of occasions, to ensure a wide spread of potential exposure to Mycoplasma species in the sample group

A DNA suspension derived from these samples was subject to PCR using the following primers;

MYCOFOR - 5' AGATAATGGAAGAACACCA

Tm = 53.4°C MW = 6151

MYCOREV - 5' TTAACCTCCACTATATCTCTATAGC

Tm = 55.7°C MW = 7496

A reaction mixture containing 1μl of template (DNA solution eluted from the extraction above), 100 ηg primer MYCOFOR, 100ηg primer MYCOREV, and 7μl of H_2O were mixed with 10μl Quiagen® Taq PCR mastermix and loaded into a Hybaid® Touchdown PCR block preheated to 80°C, and subjected to denaturing at 94°C for 105 s, followed by 40 cycles of 94°C for 30 s, 45°C for 30 s, T_A 72°C for 30 s, and a final annealing period of 10 s at 72°C. Half of the product of the amplification (10μl) was mixed with 5μl fluorescent binding agent Cyberdye® , and the mixture loaded into comb wells in 1% agarose gel and electrophoresis carried out at 100v for 60 minutes. The remaining PCR product solution (10μl) was stored at -20°C as a reference. The bands of different molecular weight product were detected by visualisation in 254nm light. The images were captured using a UVP® CCD camera, and the molecular weight bands grouped on banding similarities when compared to a DNA molecular weight marker ladder (GeneRuler™). The degree of similarity between the banding pattern for different isolates, and for three known reference *isolates M. gallisepticum, M. iowae* and *M. synoviae* were assessed visually.

4.1 Results

126 tissue samples derived from 94 lame and 32 control birds were subject to extraction of DNA, PCR amplification and agarose gel lectrophoresis. Simultaneous PCR of reference *Mycoplasma synoviae*, *M. iowae* and *M. gallisepticum* strains indicated that the PCR was capable of amplifying Mycoplasma derived DNA. However, no Mycoplasma derived samples provided banding patterns similar to those for reference strains. It was concluded that Mycoplasma derived DNA was not present in the samples analysed by PCR. Confirmation of this finding was achieved by probing of the PCR product produced above using the ECL detection for bound probe. No positive response to probes designed for reference *Mycoplasma synoviae*, *M. iowae* and *M. gallisepticum* was detected and it was concluded, as a result of these two tests, that Mycoplasma DNA was not present in the tissues sampled in this study. Thus, despite the use of a sensitive PCR technique for the detection of Mycoplasma DNA in tissue, and confirmation of the specificity of the test with control samples provided by the University of Liverpool**, no Mycoplasma DNA was detected in either lame or control samples**. This may have been for the following possible reasons –

a) There was no Mycoplasma DNA present in the samples – i.e. the birds had either not been exposed to *M. synoviae, M. Gallisepticum* or *M. iowae*, or if exposed, these organisms, or their DNA, were not present in the localised tissues sampled.

b) Any Mycoplasma DNA was degraded by handling and storage. This seems unlikely in view of the repeated detection of control samples of *M. synoviae, M. Gallisepticum M. iowae.*

The non-detection of Mycoplasma DNA in these birds may reflect the pronounced efforts which the breeder companies have made in recent years to eliminate Mycoplasma in breeder flocks through vaccination and flock serological testing. Of the Mycoplasma, *M. gallisepticum* appears to be the current dominant organism causing outbreaks of respiratory disease and is, at present, less stongly linked with lameness pathologies than it has been historically (Jordan, 1996).

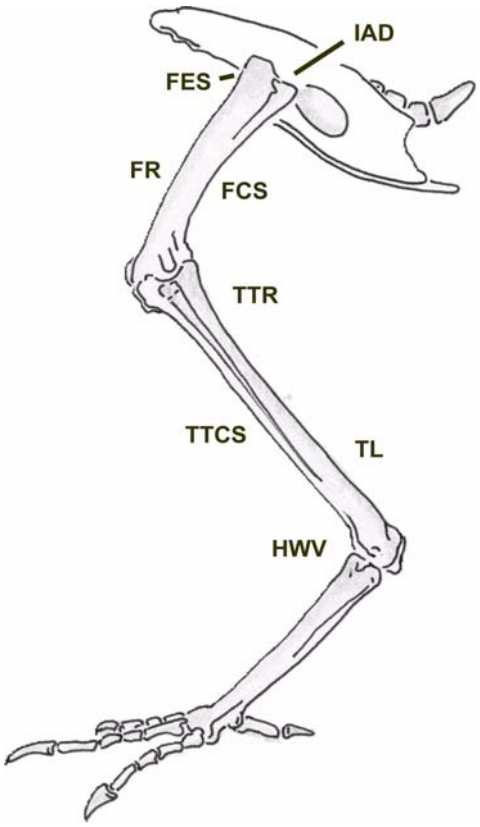
5.0 Skeletal Pathologies and their potential association with Reovirus and Chicken Infectious Anaemia (CIA)

A total of 401 Cobb genotype birds were assessed for walking ability using a gait scoring system (Kestin *et al*., 1992). The birds were gait scored *in situ* i.e. their walking ability was assessed in the environment they were familiar with. The scoring of birds *in situ* avoids artefact caused by changes in behaviour seen in broilers if they are handled or examined in an unfamiliar environment. A sub-set of the birds had their gait score assessment 'audited' using the 'water test', as a further confirmation of the degree of gait impairment. In this test, a pen (2 x 2 m) was constructed in the poultry house and the bottom portion sheeted with a plastic liner and filled to a depth of 2cm with tepid water. Small groups of the selected birds were placed in this pen, and their aversion to the water determined by the duration of time for which they would stand. The relationship between standing time and gait score has been shown in previous studies by this group (Weeks *et al*., Veterinary Record, in press) to correlate well with gait score - those birds with higher gait score (more lame) standing for shorter periods than those with low gait score. The birds were marked with their gait score category and were humanely killed using commercial slaughter equipment and blood collected at bleed out. Blood was collected into plain and anticoagulant (EDTA) tubes and centrifuged as soon as was practicable. The blood was spun at 6000 rpm for 15 minutes and serum and plasma collected and stored in 2ml aliquots at -20°C. A random selection of 82 of the bird carcases were subjected to post mortem examination for soft tissue, skeletal and other pathologies.

5.1 Dissection

The following measures were recorded before dissection; Weight (kg), Gait Score, Presence of Valgus or Varus (angular measure in degrees), Intertarsal angulation (angular measure in degrees) and during dissection, measurements were made at the sites illustrated in Figure 16, below.

Figure 16 Skeletal sampling sites on the broiler appendicular skeleton.



IAD - Inter Acetabular Distance (mm)

FES - Femoral Epiphyseal Score (0-3**)**
Scored from 0, very round femoral head with a distinct neck to 3, triangular proximal femoral head with no discernable neck.

FR - Femoral Rotation (deg)

FCS - Femur Curvature Score (0-3)
From straight (0) to very curved (3).

TTR - Tibiotarsal Rotation (degrees)

TTCS - Tibiotarsal Curvature Score (0-3)

TL - Tibial Length (mm)

HWV - Hock Width Variability (mm)
A measure of the maximum difference in between the widest and narrowest regions of the hock joint (tibiotarsus and tarsometatarsus)

5.2 Statistical Summary

A summary of the skeletal measurements made, and their association with the gait score, reovirus titres, the occurrence of tenosynovitis, and bird weight for 82 birds may be seen in Table 5, below.

A 95% level of confidence has been adopted ($p <= 0.05$). For values where p is close to this value, the term 'not quite' significant has been used to draw attention to these values.

Table 5 Summary tabulation of the statistical outcomes for skeletal analyses.

Variable 1	Variable 2	Statistical test or model	p	Significant ?
Gait Score	Reovirus titre	ANOVA	0.057	Not quite significant
Gait Score	Femoro tibial joint angulation	Pearson Chi-Square	0.682	Not Significant
Weight	Inter Acetabular Distance	Linear Regression	<0.001	Significant ***
Reovirus titre	Inter Acetabular Distance	Linear Regression	0.059	Not quite significant
Gait Score	Femoral Epiphyseal Score	Pearson Chi-Square	0.029	Significant ***
Gait Score	Femoral Curvature Score (Left)	Pearson Chi-Square	0.603	Not Significant
Gait Score	Femoral Curvature Score (Right)	Pearson Chi-Square	0.069	Not Significant
Gait Score	Femoral Rotation	Linear Regression	0.867	Not Significant
Reovirus titre	Tibial Length (Left)	Linear Regression	<0.001	Significant ***
Reovirus titre	Combined Tibial Length (L & R)	ANOVA	0.014	Significant ***
Reovirus titre	Combined Tibial Length (L & R)	Pearson Chi-Square	0.006	Significant ***
Gait Score	Tibiotarsal Curvature Score	ANOVA	0.643	Not Significant
Reovirus titre	Tibiotarsal Rotation	Linear Regression	0.459	Not Significant
Reovirus titre	Presence or absence of tenosynovitis	Two sample t - test	0.065	Not Significant
Reovirus titre	Presence or absence of tenosynovitis	Pearson Chi-Square	0.165	Not Significant

5.3 Discussion of Reovirus study.

Post mortem, dissection and skeletal assessment of the birds in the study described above confirm that skeletal effects are a cause of lameness. Table 5 summarisess the associations between gait score and the skeletal parameters measured. In interpreting the following associations between skeletal parameters, lameness and reovirus titres, the influence of bird weight must not be forgotten. The influence of the weight / gait score curve may mask the (potentially subtle) effects of other influences (Kestin *et al.,* 2001). Of the relationships which are statistically significant at the 95% level of confidence, the shape of the femoral head has a stong corellation with severity of lameness, p = 0.029.
The shape of the head of the femur is recognised as an important influence on lameness in dogs as a result of a condition known as hip dysplasia, and a system for 'Hip Scoring' of large breeds of dog, regulated by the Royal College of Veterinary Surgeons through the British Small Animal Veterinary Association (BSAVA) is a recognised part of the control of pedigree breed lines by the Kennel Club. In the canine, poor hip conformation, in combination with selective breeding from a comparatively small gene pool in some breeds, and rapid growth rate as a result of good nutrition, has predisposed some breeds of dog to debilitating lameness. Once the problem was recognised, control measures based on ascribing 'value' to a low hip score have been successful in reducing the impact of hip dysplasia in dogs through voluntary selection for breeding animals with good hip conformation.
The genotypic selection of broilers for hip conformation could be complicated by the following factors;

a) Hip shape (Femoral Epiphyseal Score) is likely to have a more significant effect on gait score in heavy 'finisher' birds than in lighter breeder stock, and so carriage of genes for poor hip conformation is more readily detected in progeny rather than the breeder stock themselves.

b) Good visualisation of the hip joint morphology is achieved only at post mortem. It is unlikely that *in vivo* assessment of hip score could be achieved with the ease that it was for tibial dyschondroplasia using a portable x ray source (lixiscope)(Thorp *et al.,* 1997).

The association between Reovirus antibody titre and gait score was significant at the 90% level of confidence (p = 0.057), but 'not quite' significant at the 95% level. The interaction between previous exposure to the ubiquitous Reovirus (as indicated by antibody titre) and the development of lameness pathologies is likely to be a subtle one because all birds show antibody titres to this virus, but very few show overt disease. Reovirus has been clearly shown to be associated with tenosynovitis (Hill *et al.,* 1989a; Tang *et al.,* 1987b) and different reovirus strains appar to have different degrees of pathogenicity in relation to lameness (Hieronymus *et al.,* 1982; Tantanwi *et al.,* 1984; Takase *et al.,* 1987). In this study, the weak association of reovirus titre and lameness supports the clinical finding from field veterinarians that reovirus is not a cause of an identifiable disease progression which could be recognised as 'Reovirus lameness'. However, it is possible that subtle localised or immunosuppressive effects of reovirus may have a role in disease causing lameness in broilers. To identify these localised effects at the cellular level would require recognition of viral sequences incorporated into specific cells associated with joint and ligament, because detection of reovirus alone is not enough to link presence with pathology, since reovirus may be isolated from nearly all chicken tissue, including that derived from SPF (specified pathogen free) birds. There appears to be a close association of Reovirus and Tibial Length (p = 0.014). Further examination of this relationship suggests that this relationship is a result of the real (expected) corellation between Tibial Length and body weight. As body weight increases, body size and hence tibial length also increases. A 'weak' association exists between Body Weight and Reovirus antibody titre (p = 0.057) such that, in the range 1.5 to 2.5 kg, the Reovirus titre scales in a linear fashion with body weight. It is thus possible that the apparent association between Tibial Length and Reovirus titre is influenced by the relationship between body size and reovirus titre.

6.0 Chicken Infectious Anaemia (CIA)

Serum samples from 150 gait scored birds were tested for the presence of CIA antibodies by ELISA. A robust linear regression model of the form y = (se)x + c was used to examine the association between CIA antibody titre and gait score.

With c = -1.38, a standard error (se) = 40.73, **p = 0.17**

This showed no significant association between CIA antibody titre and gait score.

Statistical analysis of the association between CIA antibody titre and gait score showed there to be no significant association. However, an association between CIA titre and body weight was shown, birds with the highest body weights having low antibody titres, and those with the highest antibody titres being in the middle of the weight range. These findings may support the hypothesis that CIA is not a direct influence on, or cause of, lameness, but may influence growth rate, body weight, and hence gait score. These findings suggest that CIA challenge does not have any potentiating effect on pathologies causing lameness. This does not follow the findings of recent experimental work carried out by McNamee and co workers in Stormont (McNamee *et al.*, 1999) in which it was possible to potentiate the effects of artifical induction of BCN using *S. aureus* combined with challenge with CIAV. It is likely that the gait score / CIAV serology study described here, and based on birds sampled 'on farm', with lameness assessed by gait score '*in situ'* on farm cannot be usefully compared with the experimental model described by McNamee *et al.*

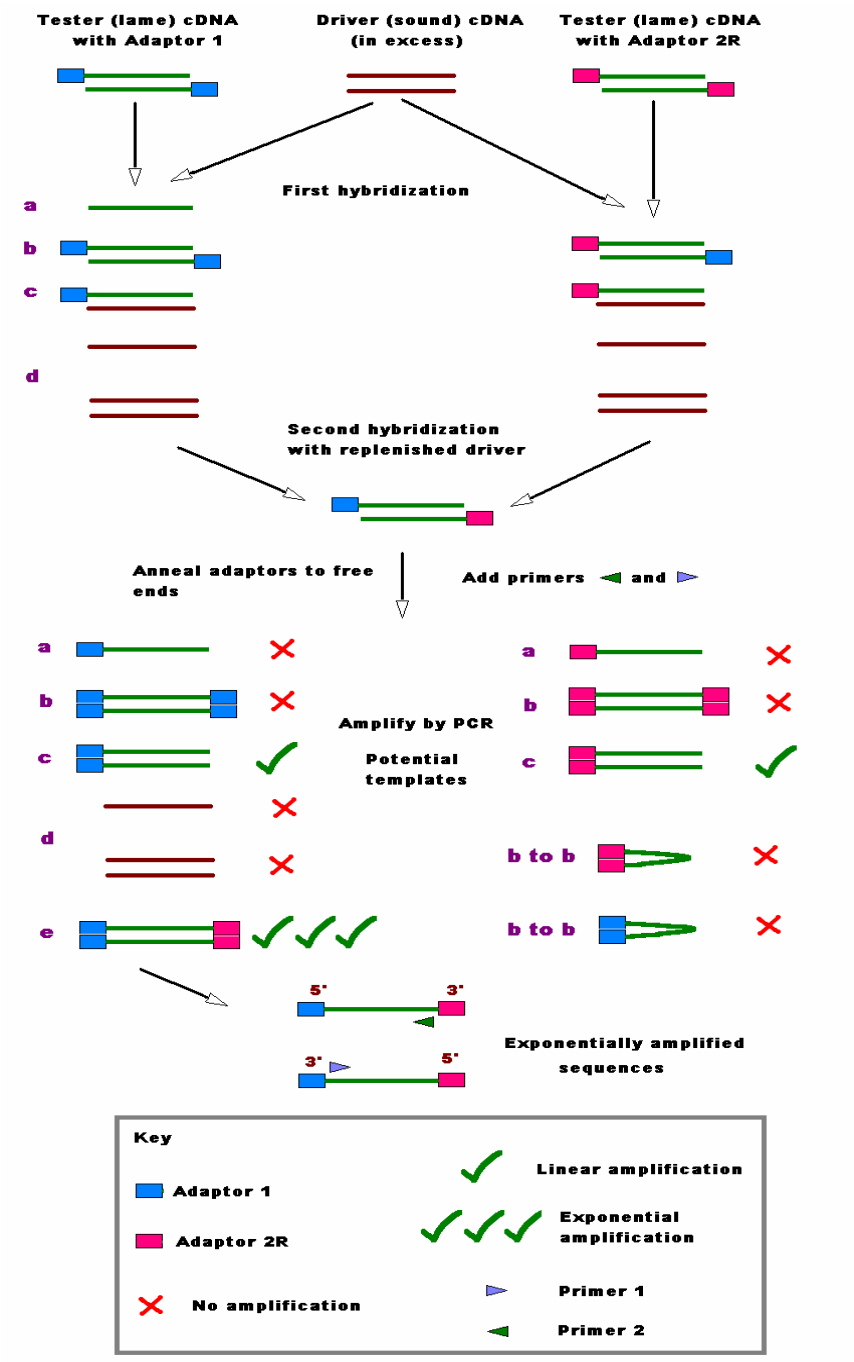
7.0 Subtractive Hybridization

Subtractive hybridization enables the comparison of two populations (Lame and Sound) of mRNA by separation of clones of genes which are expressed in one population and not in the other. The mRNA is first converted into cDNA. The reference cDNA is known as 'driver', (in this study, RNA derived from non-lame birds), and the cDNA containing the differentially expressed transcripts as 'tester', (in this study RNA derived from lame birds). The target and tester c DNAs are hybridized and the hybridized sequences are removed. The remaining unhybridized cDNAs represent genes expressed in the tester (lame birds,(L)) but absent from the driver (sound birds, (S)), or present in the driver and absent in the tester. Thus, any genes that do not occur in both tester and driver are expressed. This technique offered the potential to determine whether birds which developed lameness differed in their expression of genes, be they structural genes or viral insertions.

Samples from the deep tissues of the hock joint (tibiotarsus) containg a mixture of bone, cartilage and muscle were derived by aseptic dissection and were snap frozen in liquid Nitrogen (-196ºC) and then transferred to a freezer at -80ºC for storage. The samples were handled with sterile gloves, and RNA source material prepared in a clean area, and all practical precautionstaken to reduce the risk of contamination of the sample material with environmental and human derived RNAases. The material was broken up with an electrical macerator and the finely divided tissue subjected to

extraction of RNA. Ten samples were derived in this way, five from gait score 4 and 5 birds (lame, tester) and 5 from gait score 0 and 1 birds (sound, driver). The five tissue homogenates from each of these groups were pooled to provide one pooled tester sample and one pooled driver sample.

Figure 17 Schematic of the stages involved in the hybridization subtraction.



The of subtractive hybridisation of two populations of RNA derived from lame and sound birds was used to assess whether it was possible to identify variation at the level of the genome to explain differences in susceptibility to disease, or predisposition to skeletal lameness. The results provide the following findings;

a) Differences between populations of RNA could be detected.

b) Some common genes were present in the subtracted material, notably a number of variations of the 16s ribosomal RNA subunit, found repeatedly (for example AF076035 Haemophilus influenzae 16S ribosomal RNA. This is to be expected because the 16s subunit is a housekeeper gene common to many types of cell.

c) A large amount of *Gallus gallus domesticus* (domesticated chicken) sequence was found. This confirmed that the the source material was, RNA derived from Gallus tissue (not contaminant RNA), however, subtraction hybridisation acts to eliminate common structural gene sequences found in both driver (sound) and tester (lame) samples, and so the large number of *Gallus* sequence portions which found significant alignement with sequences seen in the BLAST database was, perhaps, unexpected.

d) Sequences for cytokine receptors are present (Gallus gallus class II cytokine receptor gene). Cytokines are extracellular signalling proteins secreted by cells and acting on target cells. Cytokines ae involved in the signalling responsible for numerous targeted cell activities such as monocyte/macrophage (heterophils in birds) responses and activation of osteoblasts and osteoclasts. It is possible that the presence of cytokine receptor genes in the subtracted material, represents an altered state of this cytokine receptor between lame and non lame birds.

e) Interferon gamma sequences are detected. Interferon gamma is a cyokine recognised to play a role in bone turnover and remodelling, and as described above, the presence of the gene coding for production of interferon γ in subtracted material may represent an altered state between driver and tester (Lame and Sound) samples.

f) Chicken cartilage matrix protein gene, exon 2 is detected. The presence of a gene coding for a cartilage matrix protein in subtracted material may (speculation) imply that an altered state of this gene exists between lame and non lame birds.

g) The presence of *Meleagris* (American Wild Turkey), *Geotrygon* (Dove), *Columba* (Pigeon) sequences in the subtracted material supports the strong convergence of genomic avian sequences, and reassures us that the subtraction is capable of carrying the sequence information through from sample, though vector, to the detection stage. However, the presence of these sequences from related species of birds does lead one to question the interpretation of the subtracted findings in these cases, where sequences shared with other species are being selectively expressed by birds from two closely related sample populations (tester & driver).

h) *Staphylococcus aureus* plasmid sequences are present. The presence of a plasmid from *S. aureus* may represent a true difference between the driver and tester groups, but could also represent contamination of the samples with this *S. aureus* plasmid from the environment. These studies indicate that the relative contribution of *S. aureus* to pathologies causing lameness (femoral head necrosis, chondronecrosis, osteomyelitis), and the isolation of *S. aureus* in approximately 17% of birds in gait score groups 4 and 5 suggests that it should be possible to detect *S. aureus* derived sequences in pooled material derived from lame birds. In this case, the samples from 5 lame birds were pooled as the tester sample – if the expectation of detecting *S. aureus* by bacterial culture is 17% (approximately 1 in 6) then it would not be far from expectation to have detected the presence of *S. aureus* sequences in a pooled sample from five birds of high gait score if it assumed that subtraction hybridisation can detect bacterial sequences if they are present (this is not clear, as the amount of RNA derived from bacteria in a sample would be much less than the RNA derived from the much greater mass of bone and cartilage tissue sampled). The detection of *S. aureus* sequences by subtractive hybridization may further support the proposition that *S. aureus* is a significant disease causing agent in broiler lameness.

i) The subtractive hybridisation study identifies the presence of *S. aureus* sequences in subtracted material, a secondary confirmation of the significance of this organism, and also demonstrates the presence of genes (or portions of these genes) coding for inflammatory mediators, cytokines and interferon. These findings support the hypothesis that there may be measurable differences at the level of the genome between birds which become lame, or are succeptible to lameness pathologies, and sound birds. Recent European legislation (Council Directive 98/58/EC , Article 21) determines that '*No animal shall be kept for farming purposes unless it can reasonably be expected, on the basis of its genotype or phenotype, that it can be kept without detrimental effect on its health or welfare*'. This Directive has been incorporated in UK legislation (The Welfare of Farmed Animals (England) Regulations 2000), Regulation 29) '*No animals shall be kept for farming purposes unless it can be reasonably expected, on the basis of their genotype or phenotype, that they can be kept without detrimental effect on their health and welfare'.* Susceptibility to lameness is only one of a number of factors which are used when selecting the genotype of birds which will be used – breast muscle gain, growth rate, hatchability, survivability, cost, availability, and long standing contractual arrangements with the breeder companies influence the decision to use one or other genotype of bird.

Technology transfer.

03/99 Broiler Lameness - State Veterinary Service training day, Langford.
07/99 Broiler Lameness - Tesco Producer Group training day, Langford.
05/00 Broiler Lameness - FAWC Whitehall
03/00 Welfare, Handling & Transport of Poultry, Trading Standards Officers, Langford.
04/01 Broiler Lameness - Lloyd Maunder Producer Group training day, Taunton.

10/01 Managing Broiler Lameness, - Widely attended Industry Day, Langford.
11/01 Broiler Lameness - Sun Valley Producer Group training day, Hereford.

Publications resulting from this study to date.

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