DIFFERENCES IN THE EXPRESSION OF GENES IN LAME AND NORMAL BROILER CHICKENS IDENTIFIED BY SUBTRACTION HYBRIDISATION

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Abstract *Animal Welfare 2003, **12**: 661-667*

In the UK, broiler chickens are normally slaughtered at about six weeks of age when they weigh approximately 2.2 kg; this contrasts with the growth of an 'unimproved' traditional strain of bird such as a White Sussex, which would weigh about 800 g at the same age. Lameness, characterised by abnormal gait, posture and impaired walking ability, can be prevalent in these rapidly growing birds and has been highlighted as a major welfare concern. It is during the later stages of rearing, when the bird is becoming heavy and may be achieving weight gains of over 50 g per day, that lameness begins to have an economic and welfare impact on the flock and to compromise the behaviour of large numbers of birds. A study was carried out to identify potential differences in the expression of genes between groups of lame and normal broiler chickens using subtraction hybridisation. The first group comprised lame birds with measurable gait abnormalities, and the second group comprised sound (not lame) birds. Both populations came from within the same flock. After extraction of mRNA and creation of cDNA, subtractive hybridisation was performed to eliminate genetic sequences common to both populations. The resultant DNA was separated and presented for sequence data analysis and comparison with a large sequence database. Some examples of the subtracted sequences detected are given, and the potential significance of these sequence differences at the individual and group level is discussed.

Keywords: *animal welfare, broiler, genetic, genome, lameness, subtraction hybridisation*

Introduction

In the UK, broiler chickens are normally slaughtered at about six weeks of age, when they weigh approximately 2.2 kg. In contrast, an 'unimproved' traditional strain of bird such as a White Sussex would weigh about 800 g at the same age. The rapid growth of broilers has been achieved through careful genetic selection and development of nutritional programmes. Lameness, characterised by abnormal gait, posture and impaired walking ability, can be prevalent in these rapidly growing birds (Sorensen 1992; Sanotra & Berg 2003) and has been highlighted as a major welfare concern (FAWC 1992; FAWC 1998; European Commission 2000), which is amplified by the large numbers of birds reared: 800 million annually in the UK, and approximately 15 billion worldwide. The principal causes of broiler lameness can be divided into two main categories: those of infectious origin (Butterworth 1999; Butterworth *et al* 2001), which cause small numbers of birds to become profoundly lame; and those which are caused by skeletal abnormalities (Williams *et al* 2000), which cause

larger numbers of birds to become moderately disabled. Recent studies have indicated a strong genetic influence on the incidence of lameness in broilers (Sorensen 1989; Rauw 1998; Kestin *et al* 1999).

A study of 37 000 broilers carried out by the British Poultry Meat Federation (now the British Poultry Council) suggested that the incidence of lameness, as defined by gait score category 3 and above, was 2.1%. Recent Scandinavian studies have substantiated a lameness (gait scores 3 and 4) prevalence of up to 15% in Danish and Swedish flocks (Sanotra *et al* 2001; Sanotra & Berg 2003). However, it is recognised that the relative incidence of pathologies that can cause lameness may have altered over recent years, as the primary breeder companies (ROSS plc and COBB plc, who command 90% of the broiler chick market in the UK) have identified problem areas and focused selection to control, for example, tibial dyschondroplasia. Several studies indicate that localised bacterial infections, notably bacterial chondronecrosis (synonyms: femoral head necrosis [FHN] and tibial head necrosis [THN]), and osteomyelitis are a common cause — perhaps the most common cause — of severe lameness in the broiler bird between 25 and 45 days of age (Riddell & Springer 1984; Pattison 1992; Thorp 1996; McNamee & Smyth 2000). Skeletal abnormalities, caused by the combined effects of conformation, rapid growth, rickets and heavy weight-bearing, account for nearly all the other cases of lameness (Hurwitz 1992; Kestin *et al* 1994; Thorp & Goddard 1994; Su *et al* 2000). It is during this period (25 to 45 days), during the later stages of rearing and when the bird is becoming heavy and may be achieving weight gains of 52 g per day (Farrant 1998), that lameness begins to have an economic and welfare impact on the flock (Weeks *et al* 1994) and to compromise the behaviour of large numbers of birds (Weeks & Kestin 1997; Vestergaard & Sanotra 1999). Because lameness is both an economic and a welfare concern, a study was carried out to identify potential differences in the expression of genes between lame and normal broiler chicken using subtraction hybridisation.

Introduction to subtraction hybridisation and its analysis

Subtractive hybridisation enables the comparison of two populations 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 [sound] birds), and the cDNA containing the differentially expressed transcripts as ‘tester’ (in this study, RNA derived from lame birds). The driver and tester cDNAs are hybridised and the hybridised sequences are removed. The remaining unhybridised cDNAs represent genes expressed in the tester (lame birds) but absent from the driver (sound birds), 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 offers the potential to determine whether birds that develop lameness differ in their expression of genes, be they structural genes or viral insertions.

Materials and methods

Tissue collection

Birds were humanely euthanased by barbiturate overdose. Samples from the deep tissues of the hock joint (tibiotarsus) containing a mixture of bone, cartilage and muscle were derived by aseptic dissection and fine division of the tissues, and then subjected to extraction of RNA. The pooled samples were taken from the same farm, and on the same day, for the following reasons:

a) For subtraction to be most effective, it is necessary for the birds in both tester and driver groups to be derived from genetically similar (ideally identical) groups. It is possible for this ideal to be approached in commercial broiler chickens because their genotype has been very closely regulated by generations of selective breeding for uniform characteristics such as breast muscle conformation and growth rate.

b) The same environmental exposure to, for example, endogenous viruses, environmental bacteria, diet-derived proteins and vaccine-derived sequences was considered essential for the tester and the driver samples. If both groups have identical 'environmental' exposure, then common insertions in both tester and driver DNA will be subtracted and hence eliminated as a differentially expressed cause of pathology causing lameness.

Extraction and purification of RNA from bird tissue samples

Total RNA was extracted from lysed tissue samples using the Promega SV Total RNA Isolation System® #Z3100, according to the manufacturers' protocol. An outline of the process, which provides differential expression of ligated cDNA sequences but elimination of ubiquitous sequences, can be found in Figure 1.

Differential expression

In the case of this study, where the two populations were derived from sound and lame birds, a 'differentially expressed sequence' means, either, a sequence found in driver (sound) but not in tester (lame), or, a sequence found in tester (lame) but not in driver (sound).

However, smaller quantities of non-differentially expressed sequence may be amplified in a linear fashion, or may exist in the small initial quantities provided by the original sample. It is thus possible for small amounts of non-differentially expressed material to survive the hybridisations and the polymerase chain reaction (PCR) (Figure 1); however, these sequences should form a very small proportion of the final population of cDNA sequences resulting at the end of the subtraction hybridisations and PCR, particularly if the quantitative differences between the tester and driver cDNA populations are good.

The differentially expressed sequences synthesised by the PCR described above were then transferred to competent *E. coli* (TOPO TA® cloning vector) to create a cDNA library, and when required, plasmid DNA was separated from the entire genomic DNA of the *E. coli* (using Qiagen, QIAprep® Miniprep). The purified plasmid DNA, which contained the sequences of interest to this study, was then submitted for cycle sequencing using a Perkin Elmer Biosystems 377 DNA sequencer at the Department of Biochemistry at the University of Dundee. This machine performed repeated AmpliTaq cycles of denaturation, annealing and extension to produce amplified products labelled with a dye, which were then analysed to produce a chromatograph of the signal output for each base position in a sequence.

Results

The chromatogram files provided by the automated sequencer were converted to a sequence of bases (eg ATTTAGGCCTATNTGTAACC) which included the non-designated base position (N) for sequence positions that the automated sequencer had been unable to designate without ambiguity. The sequence data, presented as a single long string of bases A, T, G, and C, was submitted to the basic local alignment search tool (BLAST) site at the web site of the National Institutes of Health, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The sequences were compared using a large database containing reported sequences from the literature, those which have been voluntarily submitted by laboratories worldwide, and those which appear in patent applications worldwide. Selected results from the BLAST database searches are produced in Table 1.

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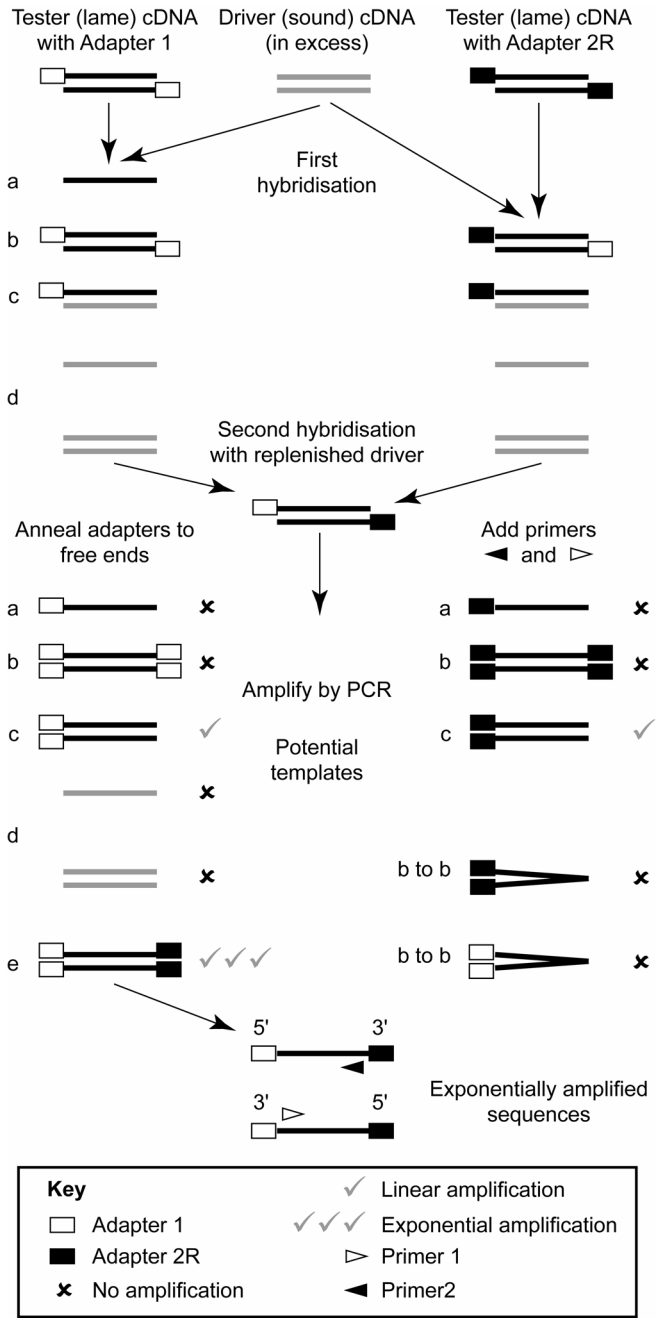

Figure 1 **Stages involved in the hybridisation subtraction.**

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Table 1 Selected results from the BLAST database search.

Example	Alignment	Identification	Identity (%)
1	Query: cgaattccagcacactggcggccgttactagtggatccgagctcggtaccaagctt Sbjct: cgaattccagcacactggcggccgttactagtggatccgagctcggtaccaagctt	*Gallus gallus* chTLR2 type1 mRNA for Toll-like receptor 2 type1, complete cds	56/56 (100%)
2	Query: gaggtggtggagtcaccgagcctggtggtgttca Sbjct: gaggtggtggagtcaccatccctggaggtgttca	*Gallus gallus* ornithine transcarbamylase (OTC) gene, exon 1	17/17 (100%)
3	Query: gagaggtggtggagtca Sbjct: gagaggtggtggagtca	*G. gallus* gene encoding non-histone chromosomal protein HMG-14b	17/17 (100%)
4	Query: cattaatgaatcggccaacgcccggggagaggcggtttgcgtattgggcgctcttcc Sbjct: cattaatgaatcggccaacgcgcggggagaggcggtttgcgtattgggcgctcttcc	*Staphylococcus aureus* plasmid J3356::pOX7;3 complete sequence	56/57 (98%)
5	Query: ccattggtgaagggcgaatggttggactggatgatcctgtgggtctttttcaaccttagc Sbjct: ccattggtgaagggcgaatggttggactgggtgatcctgtgggtcttttccaaccttagc	*Gallus gallus* class II cytokine receptor gene cluster, complete sequence	75/77 (97%)

Discussion and animal welfare implications

The use of subtractive hybridisation of two populations of RNA derived from grouped lame and sound birds attempts 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.

c) A large amount of *Gallus gallus domesticus* (domesticated chicken) sequence was found. The source material was, of course, RNA derived from *Gallus* tissue; 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 alignment with sequences seen in the BLAST database is likely to represent the expected small differences in structural genes between individuals.

d) Sequences for cytokine receptors are present. Cytokines are extracellular signalling proteins secreted by cells and acting on target cells. 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) Sequences coding for γ-interferon were detected. γ-Interferon is a cytokine recognised to play a role in bone turnover and remodelling, and the presence of the gene coding for production of γ-interferon in subtracted material may represent an altered state between driver and tester samples.

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f) Sequences coding for chicken cartilage matrix protein gene, exon 2, were present. The presence of a gene coding for a cartilage matrix protein in subtracted material indicates that an altered state of this gene may exist between lame and non-lame birds.

h) *Staphylococcus aureus* plasmid sequences were detected. 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. The detection of *S. aureus* sequences by subtractive hybridisation may support the proposition that *S. aureus* is a significant disease-causing agent in broiler lameness.

Subtractive hybridisation identified the presence of genes (or portions of these genes) coding for inflammatory mediators, cytokines and interferon. Additionally, the presence of *S. aureus* sequences in subtracted material may represent a secondary confirmation of the significance of this organism in broiler lameness. These findings support the hypothesis that there may be measurable differences at the level of the genome between birds which become lame, or which are susceptible to lameness pathologies, and sound birds. Recent legislation (Council Directive 98/58/EC, Article 21, *The Welfare of Farmed Animals (England) Regulations 2000*, Regulation 29) 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". Recent studies (Kestin *et al* 1999) have demonstrated that, at the present time, Ross birds have a measurably lower incidence of lameness than do Cobb birds. However, susceptibility to lameness is only one of a number of factors that are used when selecting the genotype of birds which will be used for breeding — breast muscle gain, growth rate, hatchability, survivability, cost, availability, and long-standing contractual arrangements with the breeder companies all play a role in the decision to use one or other genotype of bird. Tools such as subtractive hybridisation and analysis of quantitative trait loci raise the possibility of the selection of breeding animals on the basis of genetic traits predicted to be of importance by man, and detected at the laboratory bench. The ethical implications of the selection of breeding individuals for farmed species by laboratory-based methods has been, and is likely continue to be, contentious, as many believe that "there can be no manipulation more profound than that of another being's genetic structure" (Stevenson 1998).

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