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Genetic variation at the tumour virus B locus in commercial and laboratory chicken populations assessed by a medium-throughput or a high-throughput assay

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The tumour virus B (*TVB*) locus encodes cellular receptors mediating infection by three subgroups of avian leukosis virus (B, D, and E). Three major alleles, *TVB**S1, *TVB**S3, and *TVB**R, have been described. *TVB**S1 encodes a cellular receptor mediating infection of subgroups B, D, and E. *TVB**S3 encodes the receptor for two subgroups, B and D, and *TVB**R encodes a dysfunctional receptor that does not permit infection by any of the subgroups, B, D, or E. Genetic diversity at the *TVB* locus of chickens was investigated in both layer and broiler commercial pure lines and laboratory lines. Genotyping assays were developed for both medium-throughput and high-throughput analysis. Of the 36 broiler lines sampled, 14 were fixed for the susceptible allele *TVB**S1. Across all broiler lines, 83% of chickens were typed as *TVB**S1/*S1, 3% as *TVB**R/*R, and 14% as *TVB**S1/*R. In the egg-layer lines, five of the 16 tested were fixed for *TVB**S1/*S1. About 44% of egg-layers were typed as *TVB**S1/*S1, 15% as *TVB**R/*R, with the rest segregating for two or three of the alleles. In the laboratory chickens, 60% were fixed for *TVB**S1/*S1, 6% for *TVB**S3/*S3, 14% for *TVB**R/*R, and the rest were heterozygotes (*TVB**S1/*S3 or *TVB**S1/*R). All commercial pure lines examined in this study carry the *TVB**S1 allele that sustains the susceptibility to avian leukosis viruses B, D, and E. More importantly, the *TVB**R allele was identified in multiple populations, thus upholding the opportunities for genetic improvement through selection.

Introduction

Avian leukosis virus (ALV) is a naturally occurring avian retrovirus and is classified into six subgroups (A to E, and J) on the basis of interactions between virus-specific cell receptors and viral envelope glycoproteins (Coffin, 1992; Payne *et al.*, 1992). ALV-A and ALV-B cause lymphoid tumours primarily in egg-layer type chickens. ALV-D and ALV-C are rarely isolated from the field. ALV-E is rarely oncogenic but is commonly isolated from both egg-layer type and broiler chickens (Crittenden *et al.*, 1984; Ignjatovic, 1988; Gavora *et al.*, 1991). ALV-E alone constitutes the endogenous virus group and transmits vertically from hen to chick (Fadly & Payne, 2003). ALV-J causes myeloid tumours primarily in broilers (Chai & Bates, 2006). In addition to inducing tumours, ALV also reduces productivity, causes immunodepression, and other production, reproduction and health problems in affected flocks (Crittenden *et al.*, 1984; Gavora *et al.*, 1991; Bacon *et al.*, 2000; Fadly & Payne, 2003).

The tumour virus B (*TVB*) locus is one of four autosomal loci encoding cellular receptors for a group of avian retroviruses that induce tumours in host birds (Payne & Pani, 1971; Crittenden & Motta, 1975; Bacon *et al.*, 2004; Chai & Bates, 2006). *TVB* encodes the receptors for subgroup B, D, and E avian leukosis viruses (ALV-B, ALV-D, and ALV-E) (Adkins *et al.*, 2000), *TVA* encodes the receptors for subgroup ALV-A (Bates *et al.*, 1993; Gilbert *et al.*, 1994), *TVC* encodes the receptors for subgroup ALV-C (Elleder *et al.*, 2004) and, comparably, *TVJ* may be defined as the locus that encodes receptors for subgroup ALV-J. *TVJ* has been recently cloned and its product was identified as the chicken Na^+/H^+ exchanger type 1 protein (chNHE1) serving as the receptor for ALV-J (Chai & Bates, 2006). Comprehensive information on the receptor structures, functions, and interactions between the receptors and ALV was described by Barnard & Young (2003).

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TVB is the most complex of the four loci because it transcribes multiple alleles encoding multiple receptors that mediate infection by three of the six major subgroups of ALV (ALV-B, ALV-D, and ALV-E) (Payne & Biggs, 1966; Smith *et al.*, 1998; Klucking *et al.*, 2002; Zhang *et al.*, 2005). There are three alleles commonly identified at the *TVB* locus. *TVB**S1 encodes receptors mediating infection by ALV-B, ALV-D, and ALV-E. This is the most susceptible *TVB* allele. *TVB**S3 encodes receptors that permit ALV-B and ALV-D infection but block ALV-E from entering an infection cycle (Adkins *et al.*, 2001). *TVB**R encodes a dysfunctional receptor that is incapable of mediating any ALV-B, ALV-D, or ALV-E infection (Klucking *et al.*, 2002). This is the resistant allele for these three subgroups of ALV. *TVB**S1 is completely dominant to *TVB**S3 and *TVB**R, and *TVB**S3 is completely dominant to *TVB**R.

Because ALV can transmit vertically from dam to offspring, an embryo may or may not harbour ALV depending on its *TVB* genotype. In a situation where embryos come from ALV-infected susceptible breeder hens, tissue culture prepared from such embryos will most probably harbour these retroviruses, which could serve as a source of ALV contamination of poultry and other live virus vaccines produced with such an ingredient (Crittenden & Smith, 1984; Crittenden *et al.*, 1987; Fadly & Payne, 2003; Fadly *et al.*, 2004). Although the vaccine manufacturing industry closely follows established strict guidelines to prevent ALV contamination from occurring, accidental contaminations of live virus vaccines of poultry with ALV have been reported recently (Fadly *et al.*, 2006; Zavala & Cheng, 2006).

In addition to eradication management measures (Fadly & Payne, 2003), a long-term and more effective control of the economic loss and contaminations induced by ALV may lie in reducing genetic susceptibility of commercial egg-layer and broiler populations through selection for genetic resistance to ALV in the breeder flocks. This paper describes a medium-throughput and a high-throughput assay for *TVB* genotyping, and reports on the *TVB* gene and genotypic frequencies in commercial pure lines of egg-layer and broiler populations and laboratory chicken flocks.

Materials and Methods

Samples of chicken populations. DNA was obtained from 3,035 chickens representing 36 broiler lines and 16 egg-layer lines from multiple major commercial companies, and 22 laboratory chicken lines from seven universities and research institutes. From the commercial pure-lines, sample sizes ranged from 23 to 88 chickens per line. For the laboratory flocks, sample sizes ranged from three birds for highly inbred experimental lines up to 114 birds per line for other lines.

TVB pyrosequencing assay. Pyrosequencing technology is good for short-read DNA sequencing and mutation/single nucleotide polymorphism (SNP) analyses. It sequences target DNA by syntheses. For SNP analysis, it typically requires three primers for a single SNP. When two SNP targets are physically close to each other, they could be analysed simultaneously with the same set of primers in a single reaction. The sequences of forward and reverse (TVB303) primers used to generate the *TVB* amplicons were previously reported (Zhang *et al.*, 2005). The only difference for this study is that the TVB303 reverse primer was biotinylated at the 5′ end and was high-performance liquid chromatography purified. The third primer is a 16-base forward sequencing primer (5′-GGC AAA TGA CTC CAT C-3′) that is positioned immediately upstream of the first (C/T) SNP at position 172 and 12 bases away from the second (A/T) SNP, as shown in Figure 1. A 20 µl polymerase chain reaction (PCR) was generated for each sample for the subsequent SNP analysis by pyrosequencing.

The pyrosequencing analysis protocol is similar to reported procedures (Zackrisson & Lindblom, 2003; Garsa *et al.*, 2005). Briefly, the single-stranded DNA template was prepared in a 96-well PCR plate with 20 µl PCR applicon, 60 µl binding reaction solution consisting of 3 µl streptavidin Sepharose beads (Amersham Biosciences AB, Uppsala, Sweden), 40 µl binding buffer (10 mM Tris–HCl, pH 7.6, 2 M NaCl, 1 mM etnylenediamine tetraacetic acid, 0.1% Tween 20), and 17 µl ddH_2O each. After shaking for 30 min at 1350 rpm in room temperature, the immobilized PCR products were washed, denatured, and then transferred from the 96-well PCR plate into a 96-well pyrosequencing plate containing an annealing reaction mixture (39.5 µl annealing buffer and 0.5 µl of 40 µM sequencing primer per sample well) using a Pyrosequencing Vacuum Prep Tool (Biotage). The single-stranded DNA sample plate was then analysed on a PSQ™ 96MA Pyrosequencing system (Biotage). The *TVB* simplex assay that was set up for the analysis included the Sequence to Analyse, T/CAGTGCCTCCCAA/TGTAAG AAA. The SNP data were analysed with a PSQ™ 96MA SNP Software v 2.1 (Biotage, Charlottesville, Virginia, USA), which projected a histogram illustrating each of the six observed *TVB* genotypes (Figure 2). This pyrosequencing *TVB* assay is considered a medium-throughput assay.

TVB Illumina's GoldenGate™ Assay. An Illumina GoldenGate™ *TVB* genotyping assay was developed for typing the two *TVB* SNPs at position 172 and 184 (Figure 1) during typing for an additional 3,070 SNPs in the chicken genome (data not shown) at Illumina Inc. (San Diego, California, USA). The GoldenGate™ SNP assays use multiplexing to accommodate for large numbers of SNP typing and are proven to be of high throughput and highly repeatable (Shen *et al.*, 2005). To develop the GoldenGate™ assay for *TVB* SNP typing, a sequence about 120 bases before and 120 bases after each of the two *TVB* SNP at 172 and 184, respectively, were submitted to Illumina. Both base letters of a target SNP within the sequence were given in a squared bracket and separated by a single forward slash (as shown below in boldface). Each, non-targeted SNP within the sequence was indicated with a corresponding IUPAC code. The sequences submitted to Illumina for the *TVB* assay development were: TCTTGCTGCAAA GACGTCTCCACGTCTCGGCAGCACCGTGTCTCCAATGTGGT GCATGAACTCATACTTCTTTCCATTCCACCCCTTCTTGCAGG CACCTATGAGGCAAATGACTCCATC**[C/T]**AGTGCCTCCCAWG TAAGAAAGACGAGTACACCGAGTATCCAAATGACTTTCCCA AGTGCCTGGGCTGCCGGACGTGTAGGGAAGGTATGAGCTG GTGTGAGGGAGGGTTGCTATCAAAG, and GACGTCTCCACG TCTCGGCAGCACCGTGTCTCCAATGTGGTGCATGAACTCAT

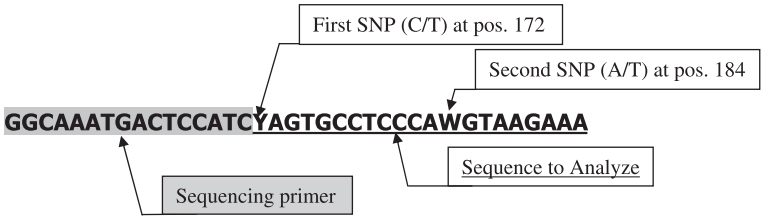

Figure 1. *Partial TVB sequence showing the sequencing primer (highlighted), the two SNPs analysed for TVB genotypes, and the Sequence to Analyse (underlined), which was entered in the Simplex Entry in setting up the TVB pyrosequencing protocol.*

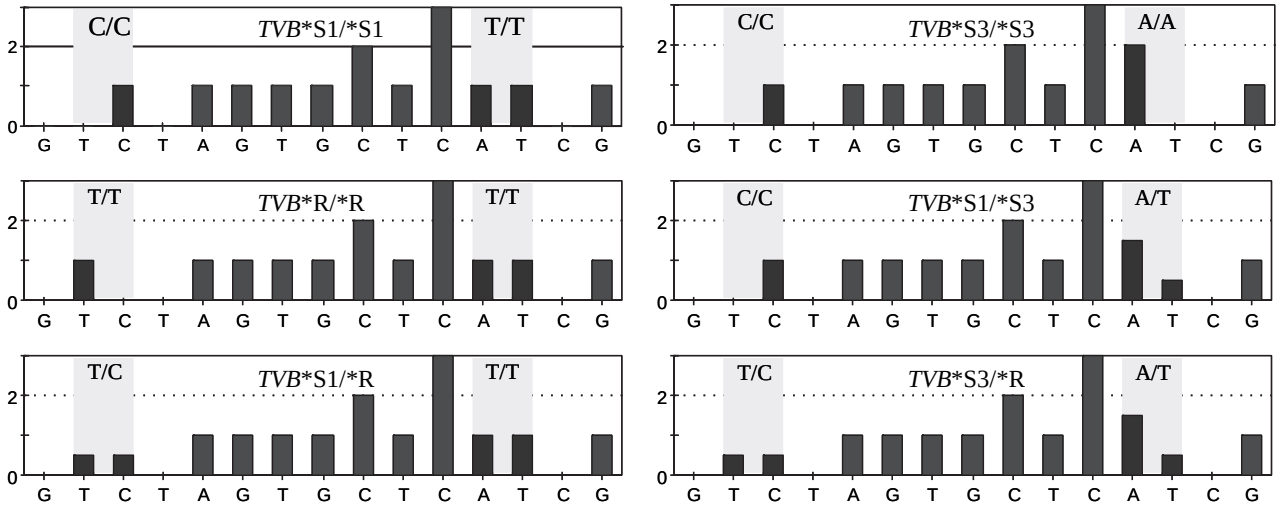

Figure 2. *Theoretical outcomes from a simplex entry of a pyrosequencing assay for each of the observed TVB genotypes: TVB*S1/*S1, TVB*S3/*S3, TVB*R/*R, TVB*S1/*S3, TVB*S1/*R and TVB*S3/*R. The two capital letters separated by a forward slash (/) on the top left of each histogram correspond to the bar(s) highlighted below the letters showing the base substitutions at position 172. The other two capital letters also separated by a forward slash on the top right of each histogram correspond to the bar(s) highlighted below the letters showing the base substitutions at position 184.*

ACTTCTTTCCATTCCACCCCTTCTTGCAGGCACCTATGAGGC
AAATGACTCCATCYAGTGCCTCCCA**[A/T]**GTAAGAAAGACGA
GTACACCGAGTATCCAAATGACTTTCCCAAGTGCCTGGGCT
GCCGGACGTGTAGGGAAGGTATGAGCTGGTGTGAGGGAG
GGTTGCTATCAAAGCTTCTGCATAAG.

Either DNA or blood samples were collected from chickens by the participating companies and institutes, and all DNA samples were quantitated at the Avian Disease and Oncology Laboratory following a PicoGreen method (Molecular Probes catalogue number R-21495; http://www.molecularprobes.com) to a final concentration of 50 ng/μl before being submitted to Illumina Inc. for SNP analysis. This developed Illumina assay is a high-throughput assay, which typed the *TVB* SNP at positions 172 and 184 with a 97.5% and a 99.6% call rate (http://www.cmmt.ubc.ca/research/core_facilities/SNP/), respectively. The 50% Gen-Call score (Oliphant *et al.*, 2002) for the 172-position SNP was 0.88, and for the 184-position SNP was 0.90.

Statistical analyses. Base substitutions in the two SNPs at positions 172 and 184 were individually converted into specific genotypes for all chickens following previously established criteria described by Zhang *et al.* (2005). The gene frequencies were estimated as $\tilde{p}_u = n_u/(2n)$, where n_u is the number of copies of allele u and n is the total number of chickens from which the gene frequency was estimated. Unbiased maximum likelihood estimates of genotype frequency were estimated as $\tilde{p}_{uv} = n_{uv}/n$, where n_{uv} is the number of chickens possessing an allele u and an allele v for $u = v$ as well as $u \neq v$. Allele diversity (Div) and observed heterozygosity (Het) for each population were calculated as $Div = 1 - \Sigma_{u=1}^{3}\tilde{p}_u^2$ and $Het = 1 - \Sigma_{u=1}^{3}\tilde{p}_{uu}$, respectively, where $\tilde{p}_u^2$ is the squared estimate of gene frequency for allele u and $\tilde{p}_{uu}$ is the estimate of homozygous genotype frequency of allele u for $u = 1$, 2, and 3. The computations were accomplished with SAS Proc Allele procedures in the SAS/Genetics package using 10 000 permutations in approximating an exact P value for the Hardy–Weinberg equilibrium test (Tables 1–4) and 1,000 bootstrap samples in acquiring the confidence intervals for the allele frequencies (SAS Institute Inc., 2004).

Results

Current status of genetic variation at the *TVB* locus in commercial pure-line broiler and egg-layer populations as well as laboratory flocks. Population types, number of lines and sample sizes are presented in Table 1. The most common allele in most populations is *TVB**S1. Commercial pure-line broiler populations have the highest average frequency (0.9) for *TVB**S1 compared with the commercial pure-line egg-layer populations (0.63) and the laboratory flocks (0.71). The differences among the average *TVB**S1 allele frequencies were significant ($P < 0.05$). The resistant allele, *TVB**R, was found in commercial pure-line broiler populations, pure-line egg-layer populations, and laboratory flocks with an average frequency of 0.10, 0.33, and 0.22, respectively. The *TVB**S3 allele was found in only three of the 36 broiler lines tested, and was at a very low frequency in those three lines. This allele was detected in five of the 16 commercial pure-lines of egg-layers and six of the 22 laboratory lines tested with an average frequency of 0.04 and 0.07, respectively.

Considering genotype frequencies, 97% of commercial broilers were either homozygous *TVB**S1/*S1 or heterozygous *TVB**S1/*R. Only 3% of chickens from the commercial pure-line broiler populations were typed as homozygous *TVB**R/*R. In contrast, 82% of the commercial pure-line egg-layer populations were typed either as *TVB**S1/*S1, or *TVB**S1/*S3, or *TVB**S1/*R, whereas 3% were typed as *TVB**S3/*R, and are resistant to ALV-E but susceptible to ALV-B and ALV-D. The remaining 15% of commercial pure-line egg-layers were typed as *TVB**R/*R. Eighty per cent of chickens of the laboratory flocks were typed either as *TVB**S1/*S1, or *TVB**S1/*S3, or *TVB**S1/*R. Six per cent were typed as homozygous *TVB**S3/*S3 and 14% were typed as *TVB**R/*R. Together, 89% of all the chickens had at least one copy of the *TVB**S1 allele and are genetically susceptible to ALV-B, ALV-D, and ALV-E; 1% were typed as *TVB**S3/*S3 and are susceptible to ALV-B and ALV-D; the remaining 9% are genetically resistant to all the three subgroups. The average allelic diversities for commercial broiler populations, commercial egg-layer populations, and the experimental flocks were 18.4%, 49.0%, and 44.8% (data not tabulated) and the estimates of observed heterozygosity were 14%, 41%, and 20%, respectively (Table 1).

Variability of the *TVB* gene and genotype frequencies of commercial pure-line boiler populations. The estimated gene and genotypic frequencies of the commercial

Table 1. *Current status of genetic variation at the tumour viral B locus in commercial pure-line and laboratory chicken populations*

Population	Lines (n)	Chickens (n)	Allele frequency[a]			Genotype frequency						Heterozygosity (%)
			S1	S3	R	S1/S1	S3/S3	R/R	S1/S3	S1/R	S3/R	
Broilers	36	1534	0.90^{D}		0.10^{A}	0.83		0.03		0.14		14
Layers	16	839	0.63^{A}	0.04^{A}	0.33^{C}	0.44		0.15	0.05	0.33	0.03	41
Experimental chickens	22	662	0.71^{B}	0.07^{B}	0.22^{B}	0.60	0.06	0.14	0.04	0.16		20
Overall	74	3035	0.78^{C}	0.03^{A}	0.19^{B}	0.67	0.01	0.09	0.02	0.20	0.01	23

[a]Gene frequencies not sharing a common superscript capital letter within a column are statistically significantly different ($P < 0.05$).

broiler lines varied greatly, and *TVB**S1 was the most common allele in all commercial broiler lines except one (C28; Table 2). About 39% (C1 to C14) of commercial broiler lines examined were completely fixed for *TVB**S1/*S1. In the other 61% of the broiler lines (C15 to C36), the *TVB**S1 frequency varied from 0.35 to 0.99 while the frequency for *TVB**R was between 0.01 and 0.65. The *TVB**S3 allele was only detected in 8% of the lines, all with very low frequencies (0.01 to 0.03). The *TVB**S1/*S1 and *TVB**S1/*R genotype frequencies ranged from 0.10 to 0.97 and from 0.03 to 0.50, respectively. The genotypic frequency for *TVB**R/*R varied from 0 to 0.40. In the lines segregating for different alleles, all but one of the lines had gene and genotype frequencies at Hardy–Weinberg equilibrium, and the observed heterozygosity varied between 3% and 50% (Table 2).

Variability of the *TVB* gene and genotype frequencies of commercial pure-line egg-layer populations. Thirty-one per cent of the commercial lines were fixed for the *TVB**S1 allele (Table 3). Among the segregating commercial egg-layer lines, the estimated gene frequency for *TVB**S1 varied from 0.15 to 0.93 while the estimate for *TVB**R ranged from 0.07 to 0.85. About 30% of the lines were segregating for a low frequency (0.01 to 0.14) of the *TVB**S3 allele. The estimated genotype frequencies for *TVB**S1/*S1, *TVB**S1/*S3, and *TVB**S1/*R, varied from 0 to 1, from 0 to 0.16, and from 0 to 0.65, respectively. The estimated frequencies for *TVB**S3/*R ranged from 0 to 0.16, and *TVB**R/*R varied from 0 to 0.70. About one-third (36%) of the commercial egg-layer lines segregating for different alleles had frequencies that departed from Hardy–Weinberg equilibrium ($P < 0.01$ or $P < 0.05$). Observed heterozygosity for the commercial egg-layer lines ranged from 9% to 86% among the segregating lines (Table 3).

Variability of the *TVB* gene and genotype frequencies among laboratory chicken flocks. About 36% of the lines of the experimental populations were fixed for the *TVB**S1 allele. One out of the 22 lines sampled was fixed for *TVB**S3, and two were fixed for the resistant allele *TVB**R. The remaining 50% of laboratory lines were segregating for more than one *TVB* allele, and the gene frequencies ranged from 0.20 to 0.89 for *TVB**S1, from 0 to 0.24 for *TVB**S3, and from 0.11 to 0.74 for *TVB**R. The estimated genotypic frequencies of the segregating lines for the homozygous genotypes *TVB**S1/*S1, *TVB**S3/*S3, and *TVB**R/*R ranged from 0 to 0.78, from 0 to 0.13, and from 0 to 0.63, respectively. The ranges of genotypic frequencies were from 0 to 0.40 for the heterozygous genotype *TVB**S1/*S3, and from 0.10 to 0.62 for *TVB**S1/*R. Chickens with the heterozygous genotype *TVB**S3/*R were not found in the laboratory lines. More than 50% of the lines segregating at the *TVB* locus departed from Hardy–Weinberg equilibrium. The observed heterozygosity in the sampled experimental lines ranged from 17% to 75% (Table 4).

Discussion

A basic PCR-restriction fragment length polymorphism assay has been developed for genotyping the *TVB* locus in chickens (Zhang *et al.*, 2005). However, high-throughput assays are needed to enable large numbers of samples to be analysed quickly. Therefore, two different *TVB*-typing assays were developed. One is considered a medium-throughput assay using a pyrosequencer (Biotage), and the other as a high-throughput assay using the Illumina GoldenGate™ SNP-typing technology (Illumina, Inc.). Compared with the PCR-restriction fragment length polymorphism assay, the pyrosequencing *TVB* assay only involves one PCR applicon and does not require use of any restriction endonuclease. In addition, one can expect to identify any novel base substitutions within the sequence to analyse domain that may occur in tested samples. In fact, we have identified a novel SNP in the *TVB* coding sequence using this newly developed assay in an inbred line of chickens, which predicts an amino acid residue change in the *TVB* encoded cellular receptor and therefore differentiates the *TVB* allele in this line from all the three known *TVB* alleles, *TVB**S1, *TVB**S3, and *TVB**R (data not shown).

The high-throughput Illumina *TVB* assay was developed when two *TVB* SNPs were typed along with 3070 additional SNPs in a genome-wide SNP project with the commercial pure-line broiler, egg-layer, and laboratory populations. To SNP-type only for the *TVB* locus, one may prepare all DNA samples as required (Fan *et al.*, 2003) and simply submit the samples to Illumina for genotyping when the Fast-track Genotyping Service on the BeadXpress platform is available. The required GoldenGate Chemistry reagents developed by Illumina for the *TVB* gene are SNP_Names TVBS3-chr22-1253083 and TVBR-chr22-1253071, and they were used in the genome-wide SNP project along with an additional 3070 SNPs in the Fast-track Genotyping Service on an Illumina BeadArray Reader. The BeadArray Reader performs genotyping simultaneously for 384 to 1536 SNPs in a single reaction, whereas the BeadXpress reader is appropriate for genotyping between 1 and 96 SNPs (personal communication, Illumina Tech Support staff scientists). Alternatively, one may request the required reagents from Illumina Inc. and perform the genotyping in a laboratory where one has access to an Illumina BeadXpress Reader. In either case, the *TVB* Illumina assay allows one to genotype a large number of samples in the shortest period of time.

Table 2. TVB *gene and genotypic frequencies of commercial pure-lines of broilers*

Line	Number	Allelic frequency			Genotypic frequency				HWE test probability	Heterozygosity (%)
		S1	S3	R	S1/S1	R/R	S1/S3	S1/R		
C1[a]	40	1			1					
C2[a]	40	1			1					
C3[a]	40	1			1					
C4[a]	40	1			1					
C5[a]	36	1			1					
C6[a]	40	1			1					
C7[a]	40	1			1					
C8[a]	36	1			1					
C9[a]	39	1			1					
C10[a]	37	1			1					
C12[a]	40	1			1					
C12[b]	50	1			1					
C13[b]	49	1			1					
C14[b]	49	1			1					
C15[a]	40	0.99		0.01	0.97			0.03	>0.05	3
C16[a]	40	0.92		0.08	0.85			0.15	>0.05	15
C17[a]	40	0.97		0.03	0.95			0.05	>0.05	5
C18[a]	40	0.92		0.08	0.85			0.15	>0.05	15
C19[a]	40	0.91		0.09	0.82			0.18	>0.05	18
C20[b]	52	0.90		0.10	0.81			0.19	>0.05	19
C21[a]	40	0.96		0.04	0.92			0.08	>0.05	8
C22[a]	40	0.89		0.11	0.77			0.23	>0.05	23
C23[a]	40	0.90		0.10	0.80			0.20	>0.05	20
C24[a]	40	0.87		0.13	0.80	0.05		0.15	>0.05	15
C25[a]	40	0.62		0.38	0.45	0.20		0.35	>0.05	35
C26[a]	39	0.90		0.10	0.82	0.03		0.15	>0.05	15
C27[a]	40	0.75		0.25	0.57	0.08		0.35	>0.05	35
C28[a]	40	0.35		0.65	0.10	0.40		0.50	>0.05	50
C29[a]	33	0.80		0.20	0.64	0.03		0.33	>0.05	33
C30[a]	40	0.69		0.31	0.50	0.13		0.37	>0.05	37
C31[a]	40	0.69		0.31	0.50	0.13		0.37	>0.05	37
C32[b]	47	0.90		0.10	0.83	0.02		0.15	>0.05	15
C33[b]	50	0.92		0.08	0.88	0.04		0.08	<0.05	8
C34[b]	59	0.72	0.01	0.27	0.48	0.03	0.02	0.47	>0.05	49
C35[b]	59	0.78	0.03	0.19	0.61	0.05	0.07	0.27	>0.05	34
C36[b]	59	0.98	0.01	0.01	0.97		0.015	0.015	>0.05	30

HWE, Hardy-Weinberg equilibrium.
[a]Typed by the Illumina Beadstation System. [b]Typed on a PSQ™96MA Pyrosequencer.

About 5% of the chickens from five different experimental lines were typed by both the pyrosequencing assay and the Illumina assay, and contained individuals representing all three *TVB* alleles and the three homozygous genotypes (Table 4). The concordance between the genotypes determined by both assays was 100%, showing complete agreement of the genotype data generated either by the pyrosequencing assay or by the Illumina assay.

A total of 3035 chickens were obtained from 74 lines representing pure-line chicken populations managed by multi-major commercial broiler and egg-layer companies and academic institutes. The genetic variability of these chicken populations at the *TVB* locus was assessed for the first time with a molecular genetics assay (either the *TVB* pyrosequencing or the *TVB* Illumina SNP typing assay). Among the three types of chicken populations, the ranking of heterozygosity was egg-layers > laboratory birds > broilers. Across all three types of the chicken populations, the allelic diversity, an estimate of the expected heterozygosity, was calculated as 35.1%, but the observed average heterozygosity was 22.9%. This suggests that there may be selection pressure favouring *TVB**S1/*S1 or *TVB**R/*R homozygotes in some of the lines (Table 1).

The *TVB* gene frequency of 22 laboratory lines has been evaluated. Five lines from the ADOL provide standard controls for the development of ALV assays. As expected, both assays identified fixation of *TVB**S1 in inbred lines 6_3, 7_1 and $15I_5$, *TVB**S3 in line 0, and *TVB**R in inbred line 7_2. A summary of the details on the selection and genetic characteristics of these lines was recently published (Bacon *et al*., 2000). Seventeen additional experimental lines from seven universities or research institutes were tested. The frequencies of the *TVB* alleles in these lines reflect those observed in the commercial egg-layer strains except for a higher frequency of the *TVB**S3 allele.

The higher frequency of *TVB**R in layers than broilers was unexpected based on earlier studies that evaluated *TVB* alleles (Calnek, 1968; Crittenden, 1968). We cannot explain the difference in frequency in *TVB* alleles in broilers versus egg-laying strains, but it is of interest that numerous studies have shown that the number and frequency of ALV-E genes is generally higher in broilers than in egg-laying strains (Ignjatovic,

Table 3. TVB *gene and genotypic frequencies of commercial pure-lines of egg-layers*

Line	Number	Allelic frequency			Genotypic frequency					HWE test probability	Heterozygosity (%)
		S1	S3	R	S1/S1	R/R	S1/S3	S1/R	S3/R		
C37[a]	40	1			1						
C38[a]	40	1			1						
C39[a]	39	1			1						
C40[a]	40	1			1						
C41[a]	40	1			1						
C42[b]	27	0.93		0.07	0.85			0.15		>0.05	15
C43[a]	40	0.15		0.85		0.70		0.30		>0.05	30
C44[a]	23	0.35		0.65	0.30	0.61		0.09		<0.01	9
C45[a]	40	0.62		0.38	0.30	0.05		0.65		<0.05	65
C46[a]	39	0.36		0.64	0.05	0.33		0.62		>0.05	62
C47[b]	73	0.66		0.34	0.46	0.14		0.40		>0.05	40
C48[b]	83	0.65	0.01	0.34	0.35	0.04	0	0.60	0.01	<0.01	61
C49[b]	84	0.47	0.08	0.45	0.21	0.23	0.12	0.39	0.05	>0.05	56
C50[b]	57	0.42	0.12	0.46	0.03	0.11	0.160	0.61	0.09	<0.01	86
C51[b]	86	0.38	0.14	0.48	0.13	0.21	0.13	0.37	0.16	>0.05	66
C52[b]	88	0.64	0.07	0.29	0.39	0.09	0.13	0.37	0.02	>0.05	52

HWE, Hardy-Weinberg equilibrium.
[a]Typed by the Illumina Beadstation System. [b]Typed on a PSQ™96MA Pyrosequencer.

1986; Kuhnlein *et al.*, 1989; Aarts *et al.*, 1991; Gavora, *et al.*, 1991; Sabour *et al.*, 1992). Indeed, Benkel (1998) has stated that the average modern egg chicken has only one to three ALV-E elements, whereas meat birds typically carry six to 10. The consistent presence of ALV-E genes in all the chickens in a broiler line would potentially provide a uniform expression of ALV-E proteins. The uniform expression of ALV-E genes in a line of broilers as opposed to egg-layer strains may somehow affect the type of *TVB* receptors, and therefore the Hardy–Weinberg equilibrium. Interestingly, Hardy–Weinberg disequilibrium was identified in only one of 22 broiler lines, as opposed to four of 11 egg-layer strains. Another possibility that has led to the differences in observed Hardy–Weinberg disequilibrium may be the actual sampling processes used by the companies. Some breeders in some of the lines may have obtained samples from previously selected chickens that were not completely randomly sampled.

The classical methods for detecting ALV-E resistance are relatively complicated in contrast to the DNA methods described in this paper. Genetic cellular resistance to exogenous infection by ALV of subgroups A, B, C, and E were first evaluated using Rous sarcoma viruses (RSVs) (Crittenden, 1975). The RSVs were used to infect immunologically incompetent recipients; for example, the chorioallantoic membrane of embryos, or the cranium of 1-day-old chicks. Susceptibility (or resistance) to the respective subgroup of ALV was based on the development (versus absence) of pocks on the chorioallantoic membrane (Prince, 1958), or of a tumour in the brain (Waters & Burmester, 1961). Results indicated a single-autosomal *dominant gene controlled susceptibility* to RSV at three tumour virus loci (i.e., *TVA*, *TVC*, and *TVB*). Tissue culture of cells from individual embryos permitted identification of foci induced by RSV, and genetic resistance identified by the *in-vitro* tests corresponded with results of the *in-vivo* methods (Rubin, 1960; Calnek, 1968; Crittenden, 1968). However, these tests were not adapted by commercial companies for selection of chickens genetically resistant to ALV-A and ALV-B. This may be attributed to several factors, including the requirement of expertise and facilities to perform and evaluate the complex RSV assays, and to test for ALV antibodies. Also, ALV resistance is conferred by a recessive allele found in low frequency, and therefore the selection progress is slow. The advantage of either of the current methods described in this research is that they do not require viral assays and are not influenced by infection of chickens with ALV-B or ALV-E, or of the presence of antibodies or ALV-E genes. The only requirement is acceptable collection of blood from pedigreed breeders and subsequent analysis of the purified DNA.

The chickens used in these studies were from flocks where efforts have been made to eradicate exogenous ALV. Breeders have been selected for over 12 years in broilers and over 25 years in egg-layers for the absence of p27, the gag capsid ALV protein present in all exogenous and most endogenous ALV-E (Crittenden & Smith, 1984; Spencer, 1984; McKay & Rosales, 2000). Therefore, could selection against p27 influence ALV-B, ALV-D, ALV-E receptor gene frequencies? When chickens have complete ALV-E genes, as is frequent in broiler lines (Benkel, 1998), and in slow-feathering broiler and egg-laying strains with ALVE21, this leads to high expression of p27 unless one of two factors exist (Crittenden, 1991). First, chickens may be homozygous for *TVB**R/*R or *TVB**S3/*S3 and lack receptors or ALV-E. Indeed, one study showed that the *TVB*S* S3/*S3 genotype was very effective in decreasing the tolerance inducing influence of ALVE21 against exogenous ALV infection in chickens lacking envelope expressing genes like ALVE6 (Smith & Crittenden, 1988). Still, the present study clearly shows that few commercial breeder chickens are homozygous for *TVB**R/*R or *TVB**S3/*S3 genes, so another mechanism must be prominent in controlling low expression of p27. Alternatively, chickens may possess the envelope glycoprotein, gp85, encoded by an incomplete ALV-E gene, which inhibits viral penetration by blocking subgroup E cell membrane receptors for ALV-E on *TVB**S1 chickens (Weiss, 1969; Hanafusa *et al.*, 1970; Crittenden, 1991). Two commonly observed genes of this type are ALVE6

Table 4. TVB *gene and genotypic frequencies of 22 laboratory chicken flocks*

Line	Number	Allelic frequency			Genotypic frequency					HWE test probability	Heterozygosity (%)
		S1	S3	R	S1/S1	S3/S3	R/R	S1/S3	S1/R		
6_3^{ab}	3	1			1						
7_1^{ab}	3	1			1						
15I5[ab]	3	1			1						
0[ab]	23		1			1					
7_2^{ab}	3			1			1				
E6[a]	32	1			1						
E7[a]	109	1			1						
E8[a]	37	1			1						
E9[a]	12	1			1						
E10[a]	78	1			1						
E11[a]	15			1			1				
E12[a]	16	0.66		0.34	0.38		0.06		0.56	>0.05	56
E13[a]	114	0.89		0.11	0.78		0.01		0.21	>0.05	21
E14[a]	18	0.31		0.69	0.22		0.61		0.17	<0.05	17
E15[a]	16	0.31		0.69	0.19		0.56		0.25	>0.05	25
E16[a]	13	0.31		0.69			0.38		0.62	>0.05	62
E17[a]	10	0.25	0.20	0.55			0.50	0.40	0.10	<0.01	50
E18[a]	16	0.62	0.13	0.25	0.25			0.25	0.50	>0.05	75
E19[a]	27	0.20	0.06	0.74		0.04	0.55	0.04	0.37	<0.05	41
E20[a]	24	0.48	0.14	0.38	0.21	0.12	0.13	0.04	0.50	<0.01	54
E21[a]	63	0.46	0.24	0.30	0.16	0.13	0.11	0.22	0.38	<0.01	60
E22[a]	27	0.20	0.08	0.72	0.07	0.04	0.63	0.07	0.19	<0.01	26

HWE, Hardy-Weinberg equilibrium.
[a]Typed by the Illumina Beadstation System. [b]Typed on a PSQ™96MA Pyrosequencer.

and ALVE9 (Benkel, 1998). Blockage of the *TVB* receptor leads to very low or non-detectable gag in chickens with complete ALVE genes (Crittenden, 1991; Smith *et al*., 1990, 1991). Thus the low expression of p27 in chickens may be explained by the presence of dominant genes like ALVE6. Unfortunately, numerous experiments have shown that ALVE6-type of genes can also have undesirable affects for chickens as they may induce tolerance to pathogenic ALV. One very well-designed experiment explored whether chickens with resistance at the *TVB* receptor level remain resistant to pathogenic ALV in the presence of ALVE6. *TVB**S3/*S3 early-feathering female chickens produced by late-feathering and early-feathering dams with and without ALVE6 were exposed at hatch to subgroup A RPL-40 virus. Subsequently the ALVE6-positive chicks were less able to produce antibodies to ALV, and developed a higher incidence of lymphoid leukosis, than ALVE6 negative hatch-mates (Smith *et al*., 1991). Thus, the capacity of ALVE6 type genes to induce tolerance to ALV-A may defeat any benefit *TVB**S3 or *TVB**R genes provide by controlling maternal transmission or expression of complete ALV-E like ALVE21.

For selection of *TVB**R to be tenable it is desirable to know whether the *TVB* allele may adversely influence production traits of chickens. An exceptional study has evaluated the effect of selection for *TVB**R and *TVA**R genes on egg productivity, and resistance to ALV, in three productive egg layer strains of chickens (Crittenden, 1993; Hartmann *et al*., 1984). Initially pedigreed chicks were injected in one wing with subgroup B RSV, and in the other wing with subgroup A RSV, and then breeders were selected based on the progeny test. ALV-susceptible and ALV-resistant hens were evaluated for egg production, shedding of ALV (determined by p27 in the albumen) and mortality, including lymphoid leukosis. On average, the resistant lines produced more eggs and had lower mortality than the susceptible lines, and lymphoid leukosis was greatly reduced. Thus, there was no evidence that chickens homozygous for *TVB**R/*R (as well as *TVA**R/*R) had decreased productivity compared with those possessing the *TVB**S1 (and *TVA**S) gene. Another study using experimental slow-feathering White Leghorns that possess ALVE21 explored the influence of TVB receptor alleles on growth rate of chicks. Interestingly, *TVB**R/*R chicks were significantly heavier than *TVB**S1/*R hatchmates (Smith & Nelsen, 1993). However, this effect in slow-feathering chickens with genes like ALVE6 has not been explored. A number of other studies have shown that the frequency of ALV-E genes changed in strains selected for productivity or disease resistance in contrast to control strains, providing indirect evidence that ALV-E genes may influence productivity (Crittenden, 1991; Gavora *et al*., 1991).

The possibility that an antiserum could identify a TVB receptor has been explored since the 1960s. In 1970, an alloantiserum was identified that would agglutinate red blood cells of some TVB*S1/*S1 or TVB*S1/*R chickens but not TVB*R/*R chickens (Crittenden *et al*., 1970). This antiserum was termed R. In 1996, additional quantities of an antiserum with similar reactivity (termed R2) were developed and it was shown to agglutinate chicken cells if they were susceptible to ALV-E and expressed an ALV-E envelope gene (Bacon *et al*., 1996). In 2000, a flow cytometry method was developed that would detect ALV-E envelope in chicken plasma. If chickens lacked ALV-E, the R2 plasma assay could differentiate between ALV-E-susceptible and ALV-E resistant siblings (Bacon, 2000). The R antisera have been beneficial in studies of experimental lines, and for purposes of developing a chicken strain free of ALV-E and with resistance to ALV-E (Bacon *et al*., 2004). However, the limited supply of these

antisera, the complexity of the flow cytometry assay, and the variability of ALV-E genes in commercial strains make it unlikely that R antisera can be a practical method for evaluating ALV-E receptor genotypes in commercial strains.

This study has developed tests that permit rapid identification of *TVB* alleles in large numbers of commercial chickens. The level of *TVB**S1, *TVB**R, and *TVB**S3 alleles in many commercial broiler and egg-layer strains has been evaluated, and now it is possible to select chickens within strains segregating for *TVB**S3 or *TVB**R alleles. However, further evaluation of the affect of these genes on productivity traits, and on controlling response after infection with exogenous ALV-A, are desirable in selected strains before general selection for *TVB* resistance is tenable. For example, broiler strain C28 and egg-layer strains C43, C44 and C46 all have a high frequency of *TVB**R. Chickens from these strains could be evaluated for productivity to establish whether those with the *TVB**R/*R genotype have an advantage, or no adverse affects, on productivity. Additional chicks could be tested with ALV-A to establish whether *TVB**R/*R chicks are more responsive to the virus and resistant to tumours. Based on the experiments of Smith and cow-orkers reviewed above, it may also be important to identify ALV-E genes in the respective lines (Benkel, 1998), as *TVB**R/*R resistance may only be beneficial in the absence of envelope encoding incomplete ALV-E genes like ALVE6. With further evaluation some commercial strains may be identified where the *TVB**R/*R genotype is advantageous. Crosses of multiple lines are generally made to produce a commerical product. Therefore, if several strains are identified they could provide a hybrid cross with high productivity to provide some commercial eggs, embryos, and chickens lacking ALV-E and with resistance to ALV-E.

Conclusions

SNP typing of the chicken *TVB* locus in a comprehensive sample representing commercial broiler and egg-layer pure-line populations and a good number of experimental flocks revealed the variability of genetic susceptibility to ALV-B, ALV-D, and ALV–E. About 90% of chickens from 50 pure-line populations are genetically susceptible to these three subgroups of ALV. Based on the gene and genotypic frequencies for *TVB*, the relative genetic susceptibilities among the populations were ranked as commercial broilers > experimental flocks > commercial egg-layers. Medium-throughout and high-throughput assays were developed in this study, which make genotypic selection for improvement of genetic resistance to ALV-B, ALV-D, and ALV–E practical. Genetic resistance to these ALV will provide a second layer of protection to chicken populations against avian leukosis viruses.

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Non-English Abstracts

Genetic variation at the tumour virus B locus in commercial and laboratory chicken populations assessed by a medium-throughput or a high-throughput assay

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Variation génétique au niveau du locus B des tumeurs à virus *(TVB)* chez les populations de poulets commerciaux et de laboratoire évaluée par un essai à rendement moyen ou élevé

Le locus B des tumeurs à virus (TVB) code les récepteurs cellulaires intervenant dans l'infection par les trois sous groupes de virus de la leucose aviaire (B, D et E). Trois allèles majeurs *TVB**S1, *TVB**S3, et *TVB**R ont déjà été décrits. L'allèle *TVB**S1 code un récepteur cellulaire intervenant dans l'infection des sous groupes B, D, et E. L'allèle *TVB**S3 code le récepteur pour les deux sous groupes B et D, et l'allèle *TVB**R code un récepteur dysfonctionnel qui ne permet pas l'infection par n'importe lequel des sous groupes B, D ou E. la diversité génétique au niveau du locus TVB des poulets a été étudié chez les lignées pures de poulets de chair et de pondeuses du commerce ainsi que chez les lignées de poulets de laboratoire. Des essais de génotypage ont été développés pour les analyses à débit moyen et élevé.

L'allèle de sensibilité *TVB**S1 s'est avéré fixé chez 14 des 36 lignées de poulets de chair échantillonnées. Parmi toutes les lignées de poulets de chair 83% des poulets ont été typés comme étant *TVB**S1/*S1, 3% *TVB**R/*R, et 14% *TVB**S1/*R. Chez les lignées de poules pondeuses, 5 sur les 16 testées présentaient le génotype *TVB**S1/*S1 fixé. Environ 44% des poules pondeuses ont été typées comme étant *TVB**S1/*S1, 15% *TVB**R/*R, avec le reste ségrégant pour 2 ou 3 des allèles. Chez les poulets de laboratoire 60% présentaient de façon fixée le type *TVB**S1/*S1, 6% *TVB**S3/*S3, 14% *TVB**R/*R, et le reste étaient hétérozygotes (*TVB**S1/*S3 ou *TVB**S1/*R). Toutes les lignées pures commerciales examinées dans cette étude portent l'allèle *TVB**S1 qui sous-tend la sensibilité aux virus de la leucose aviaire B, D, et E. De façon plus importante, l'allèle *TVB**R a été identifié dans de nombreuses populations, ouvrant ainsi des perspectives d'amélioration génétique au travers de la sélection.

Mittels „Medium oder High Throughput Screening" festgestellte genenetische Variation im Tumorvirus B *(TVB)*-Lokus bei kommerziellen und Labor-Hühnerpopulationen

Der Tumorvirus B (*TVB*)-Lokus kodiert zelluläre Rezeptoren, die die Infektion mit drei Subgruppen (B, D und E) des aviären Leukosevirus ermöglichen. Bislang sind drei Hauptallele, *TVB**S1, *TVB**S3 und *TVB**R beschrieben. *TVB**S1 kodiert einen zellulären Rezeptor, der die Infektion der Subgruppen B, D und E ermöglicht. *TVB**S3 kodiert den Rezeptor für die zwei Subgruppen B und D und *TVB**R kodiert für einen nicht funktionierenden Rezeptor, der eine Infektion mit keinem der Subgruppen B, D und E erlaubt. In dieser Studie wurde die genetische Diversität am TVB-Lokus des Huhns sowohl bei kommerziellen reinen Linien des Lege- und des Masttyps als auch bei Laborlinien untersucht. Genotypisierungstests wurden für das „Medium und High Throughput Screening" (HTS) entwickelt. Von den 36 untersuchten Broilerlinien wiesen 14 das empfängliche Allel *TVB**S1 auf. 83% aller Broilerlinien wurden als *TVB**S1/*S1, 3% als *TVB**R/*R und 14% als *TVB**S1/*R typisiert. Bei 5 der 16 getesteten Legetyplinien wurde das Allel *TVB**S1/*S1 festgestellt. Etwa 44% der Linien des Legetyps wurden als *TVB**S1/*S1 und 15% als *TVB**R/

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*R typisiert, während der Rest sich aufteilte auf zwei oder drei der Allele. Bei 60% der Laborlinien wurde *TVB**S1/*S1, bei 6% *TVB**S3/S3 und bei 14% *TVB**R/*R ermittelt und der Rest waren Heterozygoten (*TVB**S1/S3 oder *TVB**S1/*R). Alle in dieser Studie untersuchten reinen Linien besitzen das Allel *TVB**S1, das die Empfänglichkeit für die aviären Leukoseviren B, D und E aufrecht erhält. Wichtiger jedoch ist, dass in zahlreichen Populationen das *TVB**R-Allel identifiziert worden ist, wodurch die Chance auf eine genetische Verbesserung durch Selektion bestehen bleibt.

Variación genética en el locus del virus B tumoral (TVB) en poblaciones de pollos comerciales y de laboratorio mediante ensayos de rendimiento medio o alto

El locus del virus B tumoral (TVB) codifica para receptores celulares que median en la infección por tres subgrupos de virus de la leucosis aviar (B, D y E). Se han descrito tres alelos mayores, *TVB**S1, *TVB**S3, y *TVB**R. El *TVB**S1 codifica para un receptor celular que media en la infección de los subgrupos B, D y E. El *TVB**S3 codifica para el receptor de dos subgrupos, B y D, y el *TVB**R codifica para un receptor disfuncional que no permite la infección con ninguno de los subgrupos B, D o E. Se estudió la diversidad genética en el locus *TVB* de pollos tanto de líneas puras comerciales de puesta y broilers y de líneas de laboratorio. Los ensayos de genotipado se desarrollaron para análisis de rendimiento medio y alto. De las 36 líneas de broiler muestreadas, 14 tenían fijado el alelo susceptible *TVB**S1. De entre todas las líneas de broiler, el 83% de los pollos fueron tipados como *TVB**S1/*S1, el 3% como *TVB**R/*R, y el 14% como *TVB**S1/*R. En las líneas de puesta, 5 de las 16 tenían fijado el alelo *TVB**S1/*S1. Aproximadamente el 44% de las ponedoras se tiparon como *TVB**S1/*S1, el 15% como *TVB**R/*R, con el resto segregándose para 2 o 3 de los alelos. En los pollos de laboratorio el 60% se fijaron como *TVB**S1/*S1, el 6% como *TVB**S3/*S3, el 14% como *TVB**R/*R, y el resto fueron heterocigotos (*TVB**S1/*S3 or *TVB**S1/*R). Todas las líneas comerciales puras evaluadas en este estudio portaban el alelo *TVB**S1 que sustenta la susceptibilidad a los virus de leucosis aviar B, D y E. Más importante aún, el alelo *TVB**R se identificó en múltiples poblaciones, loscual mantiene las posibilidades de mejora genética a través de selección.