Severe Combined Immunodeficiency in Frisian Water Dogs caused by a *RAG1* mutation

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Abstract

Mortality of pups at 8-12 weeks of age was frequently observed in Frisian Water Dogs. Blood parameters and clinical signs of newborns from three litters were monitored. Three pups from two litters displayed strongly reduced levels of immunoglobulins and lymphocytes. These dogs were euthanized after first display of disease. Concurrent clinical and pathological features were consistent with a diagnosis of Severe Combined Immunodeficiency (SCID). Defective V(D)J recombination is one of the casuses of SCID in humans and animals. Eight genes involved in V(D)J recombination were investigated by segregation analysis of closely located microsatellite markers and by DNA sequence analysis. A nonsense mutation in the gene coding for V(D)J recombination factor RAG1 was identified in DNA from the cases at a position similar to that of nonsense mutations found in human SCID. It was concluded that SCID due to a mutation of *RAG1* led to the high mortality.

Keywords: SCID, canine, V(D)J recombination, Wetterhoun

Introduction

During the last two decades unexplained pup mortality was observed in approximately 10% of all litters of Frisian Water Dogs (FWD, FCI breed No.221, Wetterhoun in Dutch language). The observed clinical signs, like diarrhea, development of epileptic seizures and vestibular ataxia as well as pathological features like multifocal chronic encephalitis, multifocal pyogramulomatous lesions in different organs and depletion of lymphoid tissues suggested immunodeficiency as a cause of death. Inherited immunodeficiencies have been observed in man, horse, mouse, cat and dog and defects in a variety of genes were shown to be correlated with specific phenotypes and clinical symptoms, disease severity or mortality. Severe combined immunodeficiency (SCID) refers to a group of genetic disorders, generally characterized by T- and B-lymphocyte dysfunction resulting in a non-functional adaptive immune system and death during infancy[1]. The dysfunction may result from a defective *V(D)J* recombination machinery responsible for the rearrangement of the genes coding for the highly polymorphic antigen-recognition regions in T-cell receptors and immunoglobulins. Mutations in *PRKDC*, *RAG1*, *RAG2*, *DCLRE1C* and *LIG4* leading to such a defect have been described[2,3,4,5]. Only autosomal recessive and X-linked inherited forms of the disease are known. In dogs, thus far a *PRKDC* gene mutation responsible for an autosomal form of SCID in Jack Russell terriers and a mutation of *IL2RG* leading to X-linked SCID in Basset Hounds and Cardigan Welsh Corgi have been reported[6,7,8].

The present report describes the immunodeficient phenotype of affected FWD pups and the molecular genetic analysis showing its association with a specific mutation in the *RAG1* gene.

Results and Discussion

Clinical signs

Unexplained mortality of FWD pups at ages of 8-12 weeks occurred in 24 of 212 litters born between 1991-2008. The affected pups suffered from diarrhea at ages 7-8 weeks that responded well to antibiotic treatment. Neurologic signs, such as epileptiform seizures, blindness and vestibular ataxia, developed subsequently and led to spontaneous death or euthanasia. Recently three newborn litters were monitored more closely. At the age of 6 weeks all 18 pups were healthy. However, the differential white blood cell counts by bloodsmear (n=2) or automated cell counting (n=1), showed decreased numbers of lymphocytes in three pups from two litters. Percentages observed were less than 1% for the bloodsmears and 8.8% for the automated count, whereas the normal range in FWD pups was established as 15,9-50,8% (Figure 1). Immunoelectrophoresis of sera showed that the same three pups did not have detectable levels of IgM in contrast to the other pups. Since IgM is not represented in the antibody pool obtained from the mother around birth, its presence indicates production by the healthy newborns, while its absence indicates an inborn deficiency. The levels of IgG1, IgG2 and IgA were not detectable or very low in all the pups, these were probably remnants of maternal immunoglobulins. The three mothers had normal levels of IgG1, IgG2, IgA and IgM.

Flowcytometric analysis of the white blood cells showed that in FCS-SCC plots of the healthy pup all cell types, including lymphocytes could be discerned although less discretely than in those of the adult dog. In analysis of the blood of the affected FWD pup lymphocytes were undetectable, the labeled monoclonal antibodies against CD21, CD4 and CD8 did not identify positive cells (Data not shown).

After deterioration of the clinical condition and the clinical signs such as epileptic seizures and vestibular ataxia the pups were euthanized. Two pups were examined post mortem. The cortex of the lymph nodes and the thymus were hypoplastic and Hassall bodies were absent. The white pulp in the spleen showed aplasia. In addition, both pups had multifocal subacute to chronic histiocytic myocarditis and hepatitis. Assessment of the immunological parameters and the necropsy findings led to the conclusion that the FDW pups were suffering from SCID.

Genetic analysis

In order to address the mode of inheritance, the pedigree of the two litters with affected pups was combined with information from the Dutch Association for Staby- and Wetterhounen concerning suspected cases (Figure 2). Based on the pedigree information it was suggested that the mode of inheritance is autosomal recessive. This notion was strengthened by the segregation analysis which indicated that pups in sibships at risk had a probability of 0.23 of being affected. With purebred dogs recessive inheritance means almost invariably that a single DNA mutation segregates in the breed and that all cases will be homozygous for a single causative mutation[12]. Recently, it was established that SCID in Jack Russell terriers is caused by a nonsense mutation in *PRKDC* coding for the catalytic subunit of the DNA-activated protein kinase[1]. It is not uncommon that a single pathogenic DNA mutation has spread to different breeds[13]. Therefore the DNA of the affected FWD pups was analyzed for the *PRKDC* mutation, but it appeared not to be present.

Subsequently the possible involvement of the eight genes coding for factors involved in V(D)J recombination was examined. The three affected dogs, six siblings and the two mothers were genotyped for microsatellite markers located closely to *RAG1*and

RAG2, *PRKDC*, *DCLRE1C*, *LIG4*, *XRCC4*, *XRCC5*, and *XRCC6* (Supplementary Table 1). One or more of the affected pups were heterozygous for markers indicative for *PRKDC*, *DCLRE1C*, *LIG4*, *XRCC4*, *XRCC5* and *XRCC6* hence these genes were excluded from further investigations. Eight markers for the *RAG1-RAG2* gene pair were tested, but none of these was informative in the FWD litters with SCID cases. Therefore we decided to analyze the coding DNA sequences of these genes. In *RAG1* a point-mutation c.2893G>T was found, changing a GAG codon for glutamate into a TAG stop codon (p.Glu965X, Figure 3). We predict that the mutation leads to a truncated protein which lacks the C-terminal 79 amino acid residues. Stop codon mutations at similar amino acid positions 938 and 959 of RAG1 are associated with SCID in humans[3,15]. The three affected dogs were homozygous for the mutation, the two mothers were heterozygous and the siblings were either heterozygous or homozygously clear.

Apart from the litters with affected dogs described above, 138 Frisian Water Dogs were tested for the mutation and 41 carriers and no homozygous dogs were found. The group of carriers included all five available parents of litters with unexplained mortality.

The glutamate at position 965 of RAG1 corresponds to the glutamate at position 962 in a variety of other species. The residue is embedded in a domain that is present without change in all vertebrates in which the gene has been analyzed. It has been shown that the glutamate residue is one of a few acidic residues essential for the catalytic activity of RAG1 involved in the process of V(D)J recombination[16]. Thus it was concluded that the nonsense mutation causes SCID in the FWD breed responsible for the pup mortality. Since using simple PCR methodology will now enable identification of carriers, the breed can be released from the deficiency and the

underlying gene by selective breeding. Approximately 50% of T and B cell-negative SCID cases in man is the result of more than 30 different RAG1 mutations. In humans, the disease affects at least 1;100 000 births[17]. The disease as it occurs spontaneously in the FWD breed provides a natural large animal model to asses (gene-)therapies in SCID caused by *RAG1* mutations.

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Conflict of interest

None of the authors has a competing financial interest in relation to the work described.

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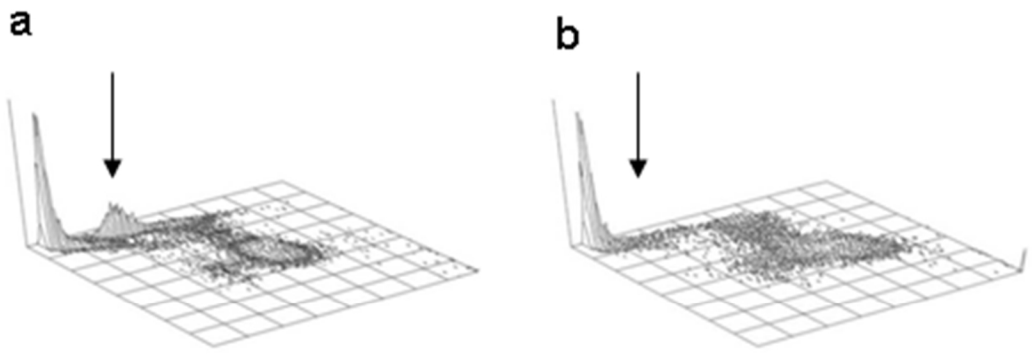

Figure 1. Hematology of newborn Frisian Water Dogs. Differential leucocyte analysis by ADVIA of a healthy pup from litter C (a) and a littermate confirmed to be affected by Severe Combined Immunodeficiency (b). The arrow indicates the position of lymphocytes.

Methods: Blood cell counts of the pups of litter A and their mother were performed by microscopical inspection of EDTA blood sample smears. EDTA blood samples from the pups of litters B and C and their mothers were also used to count the blood cells, to measure the packed cell volume and to analyze the leucocytes with an ADVIA 120 (Siemens).

The levels of immunoglobulin A, immunoglobulin M, immunoglobulin G1 and immunoglobulin G2 in the blood of the pups of litters A - C and their mothers were determined by immune electrophoresis. The Immune electrophoresis was performed as described before9. For flowcytometric analysis samples of EDTA blood were diluted 1:2 with RPMI-1640 culture medium containing heparin. The diluted samples were loaded on a ficoll gradient (Histopac) to isolate the peripheral blood mononuclear cells (PBMC), containing the lymphocytes . The gradients were centrifuged at 800G for 20 minutes at 20 °C. The PBMC were collected and labeled with monoclonal antibodies specific for CD21 and CD8 both conjugated with phycoerithrin, and an antibody specific for CD4 that was labeled with Alexa637 according to routine procedures. Labeled cell suspensions were analysed using a FACSCalibur flowcytometer.

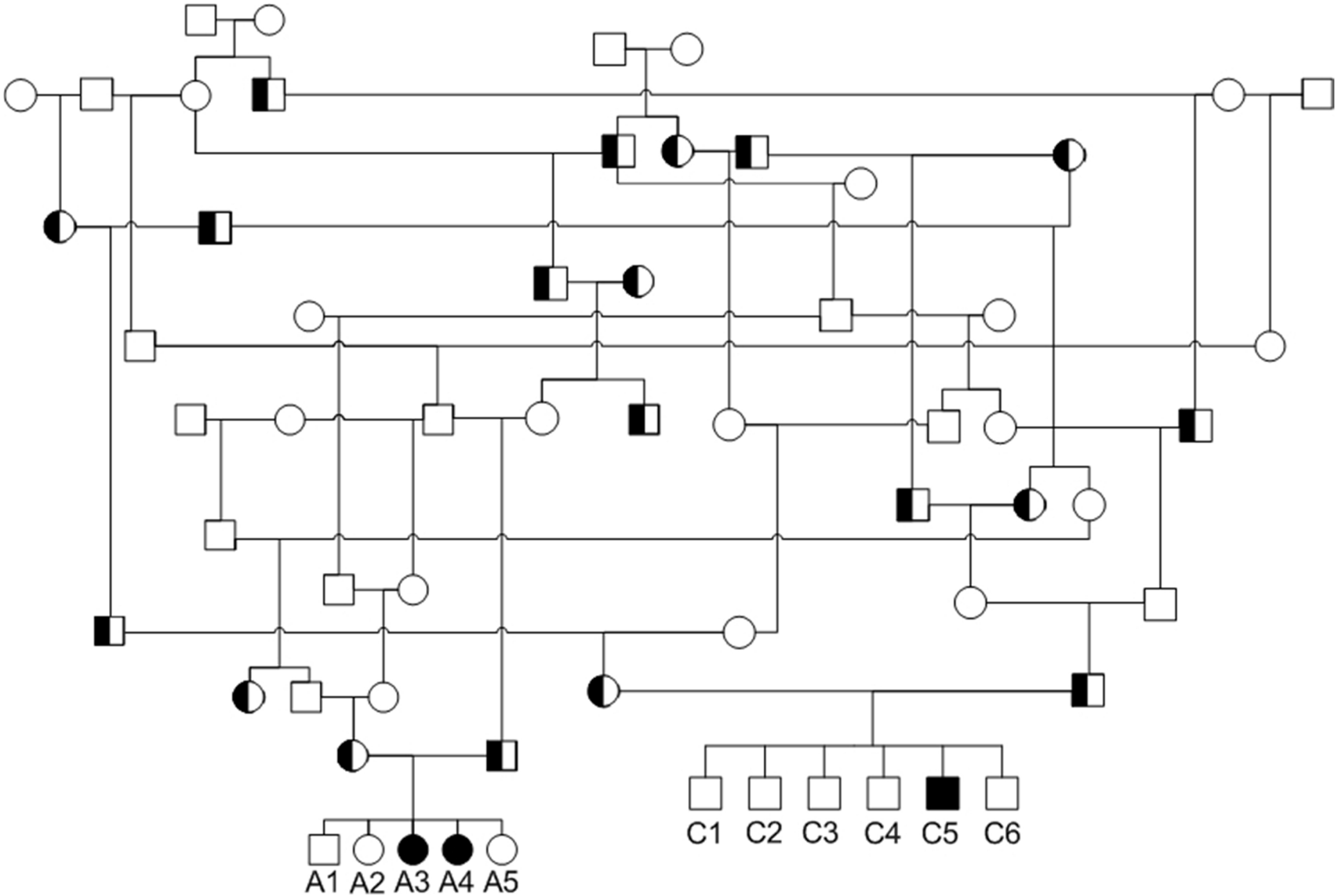

Figure 2. Pedigree of a Frisian Water Dog family segregating pup mortality. The partially filled symbols represent dogs that have bred offspring with signs of immunodeficiency. The filled symbols represent confirmed cases of Severe Combined Immunodeficiency.

Methods: The pedigree was constructed based on information provided by the Dutch Association for Staby- and Wetterhounen. The newborn dogs A1-A5 and C1-C6 were subject of the present study. The DNA of the mothers was also available. The dogs were privately owned and included with informed consent of their owners. The 7 dogs of litter B were not affected and are not depicted in the pedigree diagram. The dogs A3, A4 and C5 were euthanized and A3 and A4 were examined post mortem. A segregation analysis was performed with the singles method as described10. This analysis was based on the composition of 19 litters with 38 suspected cases and 89 healthy dogs. There were 11 litters with one suspected case. The DNA was isolated from EDTA blood samples as described.

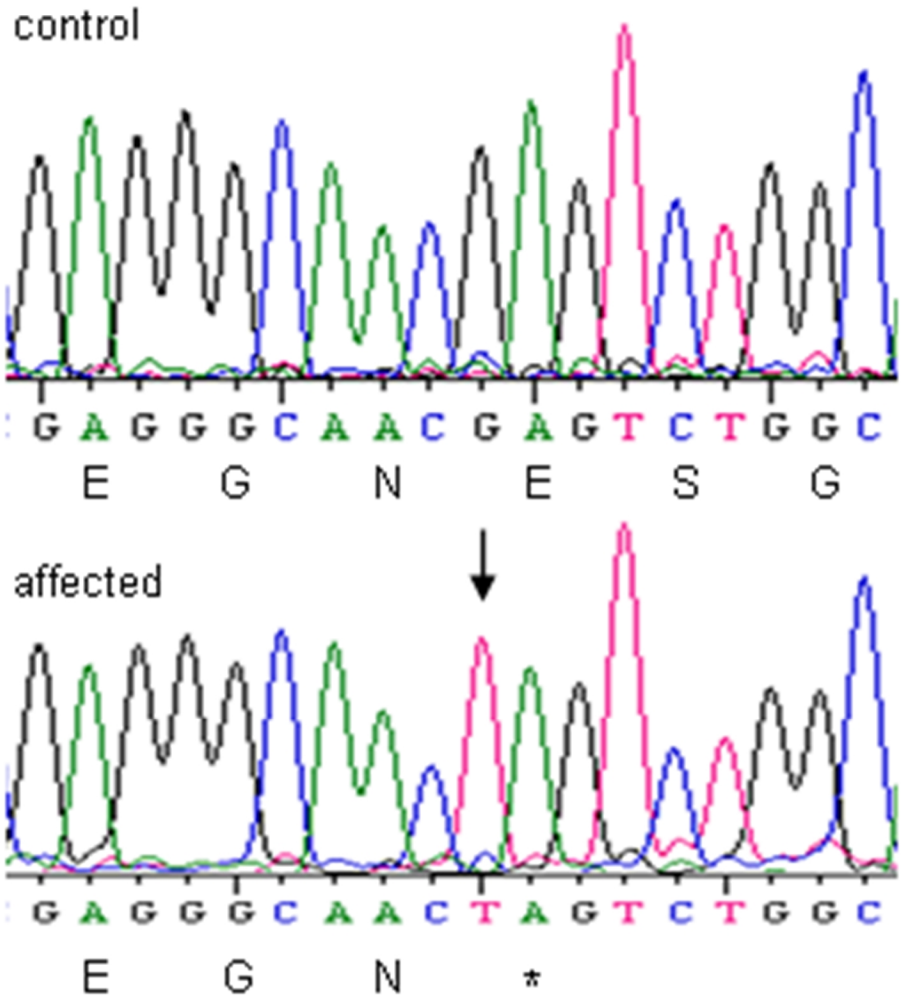

Figure 3. The mutation of RAG1 associated with Severe Combined Immunodeficiency in Frisian Water Dogs. The G>T mutation indicated by the arrow introduces a stopcodon (*) at position 965 of the protein sequence. The three affected dogs were homozygous for the mutation, all proven and suspected carriers that were available were heterozygous.
Methods: The oligonucleotides for PCR and DNA sequence analysis of the coding region of RAG1 and RAG2 and other candidate genes were selected from Dogset (http://www.vgl.ucdavis.edu/dogset/) and listed in Supplementary Table 2.
The PCR reactions for amplification of genomic DNA were performed by standard procedures. The reaction products were diluted 10-fold and 1 µl was incubated with 3 pmol of either PCR primer, 1 µl BigDye mix and 1.75 ul 5x sequencing buffer (Applied Biosystems) in a volume of 10 µl for 25 cycles of 96 °C for 30 s, 50 °C for 15 s and 60 °C for 2 minutes.
The products were purified by centrifugation on sephadex columns using a multiscreen 96-filtration plate and analyzed on an ABI Prism 3130xl Genetic Analyzer. The DNA sequences were analyzed and compared using SeqmanII software (DNAstar). The possible presence of the mutation of the PRKDC gene identified in Jack Russell terriers with immunodeficiency was investigated as described1. The primers GGCAAAAAACCCTGTTAATAAAAAA and ACCTGAATAAACCTCCTTCTG were used for amplification of the relevant PRKDC gene fragment from DNA of affected Frisian Water Dogs, followed by DNA sequence analysis as described above.

Supplementary Table 1. Oligonucleotides for microsatellite markers situated closely to V(D)J recombination genes.

Gene	marker	forward primer	reverse primer	alleles[a]
PRKDC	29_003B_CT	TGCTTCCTTCACTGGGCTTT	TTTCAAACTGGTTGCCTTGG	3
RAG1 and -2	18_034F_CA	ACCTGGGGAATGACAGTGA	GCTCCCTCGTTCAGGAGCTA	3
RAG1 and -2	18_034G_CA	CGAGGCCATAACATCAGCTC	GCTGCACAGTGTCTACCCCATC	2
XRCC4	03_027C_CA	TGACACACAGCATGCATGAAA	CAGATGTGAGCTGATGCCTTC	2
XRCC5	37_026A_CT	TGAAGGGAGGGGCTAATTCA	GAGGGCTTCCCTCCTTCTGT	3
XRCC5	37_026A_CA	AACAAGGAGGGACTGGAGGA	GGGACCAAACCAACCTTCAA	3
XRCC6	10_026D_CT	TGTTCTTGGGGTTAGATGAGCA	CAGCATCTAAGGTGAGCACTGA	2
XRCC6	10_027A_CA	CGTAGACTATAGGGGGCTCAA	GAAAATTGCATTAGCTGGATCA	3
DCLRE1C	02_024B_CT	GTGGCTCCCCCTG-ATGATAA	ATGAGGCTCCTCTGGCTCTG	3
LIG4	22_060A_CA	TCTTGTTTTCCGG-GGTTTGT	AAGGGGAGCTGGTTCTTTGG	4

[a] Number of alleles in investigated Frisian Water Dogs.

Methods: The microsatellite markers were used to investigate the involvement of the candidate genes by segregation analysis. The selection of candidate genes was based on known mutations in human and animal immunodeficient patients (http://www.ncbi.nlm.nih.gov/omim). The chromosomal locations of the genes were taken from the Ensemble Genome Browser (http://www.ensemble.org/index.html). The microsatellite markers and oligonucleotide sequences were selected from DOGSET (http://www.vgl.ucdavis.edu/dogset/). The distance between the markers and the candidate genes was 685 kb or less. One of the primers was tagged at the 5'-end with the DNA sequence GTTTTCCCAGTCACGAC derived from bacteriophage M13. The PCR amplification of microsatellite fragments of individual dogs was performed as described using an annealing temperature of 55 oC14. The products were mixed with LIZ500 size standard (Applied Biosystems), separated and detected with an ABI Prism 3130xl Genetic Analyzer. The data was analyzed with GeneMapper 4.0 software (Applied Biosystems).

Supplementary Table 2. Oligonucleotides for amplification and DNA sequence analysis of canine *RAG1* and *RAG2* .

Fragment	forward	reverse	Ta[a]
RAG1 A	TCGGGCAATCAGTTCATCAG	CTCCTCCAAGCCCTTCATTG	55
RAG1 B	CATGATGGGCTTCAGGTTCA	TGATGGACATGCAGGAGGAC	63
RAG1 C	CCTCATGACCGTGAACGAGA	GCTGCAGCCAGTACCACAAG	65
RAG1 D	GGTAGTCGTCCACGCAGGAG	ACGAGGAGGTCAGCTTGGAG	55
RAG1 E	GCACAAAAGTCTGGTCCGAGT	GTGGATGGCAAAACCCAAGT	63
RAG1 F	CTGTGCACTTGGCTGAGGAC	GCGTAGTGGTGCATGTTGGT	59
RAG2 G	GGAAGGTTCTGGGGCTCTTT	GGCGGATGTTATTGGCAAGT	61
RAG2 H	CCCAAAGAACCACGGAAAAA	GCTTTCCCCAGGCACTTAAT	61

[a] Annealing temperature