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A new method for rapid detection of the mutant allele for Chediak-Higashi syndrome in Japanese Black cattle

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ABSTRACT

Chediak-Higashi syndrome (CHS) is an autosomal recessive hereditary disorder in Japanese Black cattle, caused by a mutation of the *Lyst* gene. So far, the mutation has been detected by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. However, this method is disadvantaged by its low-throughput performance. Here, we report an alternative method involving real-time PCR with TaqMan minor groove binder probes, which shortens the total assay time by more than 120 min, analyzing 10 samples in a duplicated manner. Using this method, we examined 102 Japanese Black cattle and found that 8.8% of the cattle were CHS-carriers. These data indicate that our technique is useful for routine diagnostic testing for CHS in Japanese Black cattle.

KEY WORDS: Chediak-Higashi syndrome, Japanese Black cattle, PCR-RFLP, real-time PCR with TaqMan minor groove binder probes

Chediak-Higashi syndrome (CHS) is an autosomal recessive disease caused by mutation of the *Lyst* gene [2, 4, 5]. CHS has been reported in many mammalian species including humans, cattle, mice and rats [6, 7, 9]. In Japan, Japanese Black cattle affected by CHS were frequently seen in the South Kyushu area more than a decade ago [8, 14]. Japanese Black cattle with CHS exhibit variable degrees of oculocutaneous albinism, easy bruisability and a bleeding tendency, causing an economic loss to cattle farms [8, 12, 14]. It has been thought that the clinical manifestations are related to malfunction of the protein encoded by the *Lyst* gene, a lysosomal trafficking regulator protein, leading to alterations in the size, structure and function of lysosomes [3, 12, 13].

The *Lyst* gene is located in the proximal region of bovine chromosome 28. In 1999, two research groups independently found the *Lyst* mutation in Japanese Black cattle with CHS as a G to A nucleotide substitution at position 6044, resulting in substitution of the amino acid histidine to arginine [4, 15]. Based on these observations, the PCR restriction fragment length polymorphism (PCR-RFLP) method has been established for detection of heterozygous and homozygous mutant alleles [4, 15] in Japanese Black cattle, and it is now widely used as a gold standard method for diagnosis in Japan. Although PCR-RFLP analysis is specific, sensitive and reproducible, it is disadvantaged by being a slow process, involving several steps, such as restriction enzyme digestion, electrophoresis and DNA staining, taking more than 180 min to analyze 10 samples in a single sample test. This slow processing limits the method's routine diagnostic use [1, 11]. Here, we report a new method with a high throughput performance for detection of CHS in Japanese Black cattle.

Genomic DNA was isolated using the commercially available QIAamp DNA Blood Mini Kit (Qiagen®, Tokyo, Japan) in accordance with the manufacturer's instructions. For PCR-RFLP, the DNA was PCR-amplified using the primers: forward (5'-

GAAAATTACAGCAGAAGTCCTTGG-3') and reverse (5'-TGACAAACATAAGTATTAGTAGGAGG-3'). Conditions for cycling were at 94°C for 5 min, followed by 40 cycles of 94°C for 45 sec, 56°C for 45 sec and 68°C for 45 sec. After amplification, amplicons were subjected to digestion with the *Fok-I* restriction enzyme (Takara, Tokyo, Japan) at 37°C for 1 hr. Finally, fragment length was analyzed after separation by agarose gel electrophoresis (2%) and ethidium bromide staining.

For real-time PCR, TaqMan minor groove binder (MGB) probes and primers were designed using software provided by Life Technologies (Tokyo, Japan). The mutant probe was 5'-AGCAGTTCGTCCTC-3', the wild type probe was 5'-AGCAGTTCATCCTC-3' and the 5' end of each probe was labeled with a different fluorogenic dye, FAM and VIC, respectively. Also, the MGB moiety, which stabilizes the hybridization of the probes with single-stranded DNA targets, and a non-fluorescent quencher were attached to the 3' end of each probe. The primers used in this study were 5'-CGGATTTGGAATTATTGACGATTA-3' for forward and 5'-CTATGTGCAAAGAAAAATAGAAGTTTGTG-3' for reverse, respectively. Cycling conditions were at 95°C for 10 min followed by 40 cycles of 92°C for 15 sec and 60°C for 1 min. All reactions were performed in duplicate with a 7900HT fast real-time PCR instrument, and the data were analyzed using software (Life Technologies).

Figure 1A shows the result of PCR-RFLP analysis with samples from three Japanese Black cattle that had been diagnosed as a wild-type, heterozygous carrier and homozygous mutant (CHS), by PCR-RFLP analysis, blood coagulation test and blood smear test, at the University of Miyazaki. The animals had no clinical symptoms at the time of the diagnosis. *Fok-I*-digestion of DNA from wild-type clearly produced the two digested fragments. On the other hand, when DNA derived from homozygous mutant was digested with *Fok-I*, no

digested fragments were detectable. In a sample from heterozygous carrier, *Fok-I*-digestion produced three bands.

Next, using these samples in a sextuplicated manner, we verified the method for real-time PCR with TaqMan minor groove binder probes. In a preliminary experiment, the amplification curve obtained with each sample did not show any significant non-specific allelic amplification under the cycling conditions we employed. Figure 1B shows the result of allelic discrimination cluster analysis at the end point of the PCR reaction, indicating four separate distinct clusters that represent the no-template controls and three genotypes. The VIC fluorescence for DNA from wild-type was markedly high, whereas that for FAM was rather low. On the other hand, analysis of DNA from the homozygous mutant revealed a mirror-image fluorescence pattern. For the DNA from heterozygous carrier, the intensities of both types of fluorescence were moderately increased by the PCR-reaction. These fluorescence patterns clearly discriminated samples from wild-type, heterozygous carrier and CHS animals. Assessment of the time required for analysis showed that our new technique could be carried out in only 60 min, thus shortening the total assay time by more than 120 min, in comparison with PCR-RFLP analysis.

Next, exploiting the high-throughput performance of our real-time PCR with TaqMan minor groove binder probes, we used it to examine the genotypes of 102 Japanese Black cattle from a cattle farm whose manager had approved this study. We found that 93 (91.2%) of these cattle were wild-type, but unexpectedly, 9 (8.8%) of the cattle were heterozygous carriers. These diagnoses were confirmed by PCR-RFLP analysis. No cattle with the homozygous mutant allele were detectable. Based on these data, we investigated the pedigree records of the carriers, and found that 8 of the 9 heterozygous animals were offspring of a single known carrier sire (Sire A). Further investigation revealed that Sire A had produced at least three

heterozygous carrier dams that had not been diagnosed. For the remaining heterozygous animal, we were unable to find a potentially causative individual harboring the mutant allele in the first two generations.

In this study, we have developed a new high-throughput method for accurate detection of heterozygous and homozygous mutant alleles for CHS. In addition to the rapid processing offered by this method, it has other advantages over PCR-RFLP. It has been reported that partial digestion is one of the main problems associated with SNP genotyping by PCR-RFLP, because undigested PCR products can be confusing and hinder accurate judgement [1]. Also, as mentioned earlier, the PCR-RFLP requires several steps, which increase the risk of contamination [1, 10]. Our method simply produces allele-specific fluorescence, and detection can be performed in a single reaction tube or well, thus minimizing any false results or contamination.

After the introduction of PCR-RFLP analysis more than a decade ago [4, 15], many animals with heterozygous and homozygous mutant alleles have been excluded from breeding, and therefore, we had initially assumed that heterozygous carriers would have been largely eradicated from the cattle population. However, our findings clearly indicated that some heterozygous dams remained, and that unfortunately they had been used for breeding. This might have been due to the lack of a rapid and convenient molecular method for screening large numbers of cattle. Our present results clearly indicate that this new method for real-time PCR with TaqMan minor groove binder probes is rapid, specific, sensitive and reproducible. We anticipate that this method will be used to screen large numbers of cattle, thus helping to eradicate CHS and its carriers.

In conclusion, to our knowledge, this is the first reported study to have used the method for real-time PCR with TaqMan minor groove binder probes for genotyping of heterozygous

and homozygous CHS alleles in Japanese Black cattle. The proposed technique would be similarly applicable for rapid genotypic screening of other genetic defects in different breeds and species.

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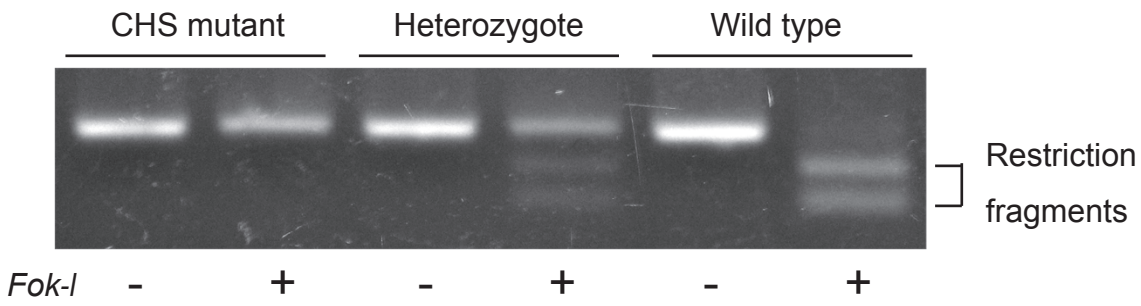
FIGURE LEGEND

Fig. 1. Genotyping of DNA samples with two different methods. A) The *Fok-I* restriction patterns of a genomic fragment produced by PCR with primers flanking the CHS mutation in CHS mutant, heterozygous and wild type cattle. (+) lanes are the *Fok-I* restriction fragments. (-) lanes are the corresponding undigested fragments.

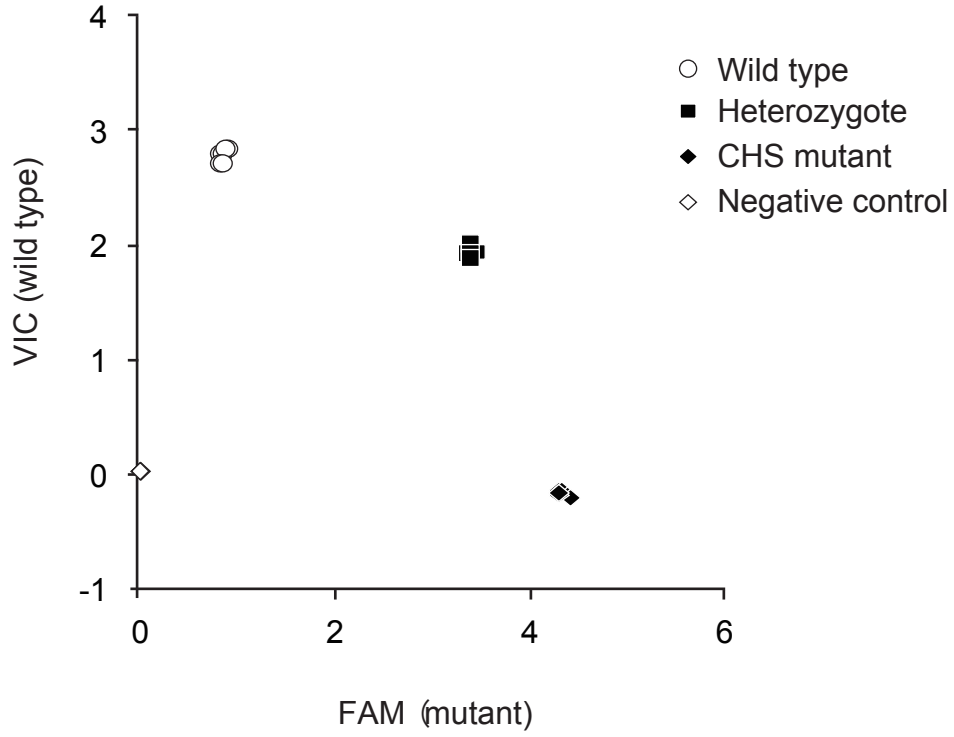
B) Allelic discrimination cluster analysis of the wild type and mutant alleles using wild type (VIC-labeled) and mutant (FAM-labeled) TaqMan probes.

Figure 1

A


CHS mutant
Heterozygote
Wild type
Restriction fragments
Fok-I - + - + - +

B


Wild type
Heterozygote
CHS mutant
Negative control
VIC (wild type)
FAM (mutant)
4
3
2
1
0
-1
0
2
4
6