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Original article

Detection of self-biting behavior of mink by loop-mediated isothermal amplification (LAMP) and sequence-characterized amplified regions (SCAR)

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Abstract

Self-biting disease occurs in most farmed fur animals in the world. The mechanism and rapid detection method of this disease has not been reported. We applied bulked sergeant analysis (BSA) in combination with RAPD method to analyze a molecular genetic marker linked with self-biting trait in mink group. The molecular marker was converted into SCAR and loop-mediated isothermal amplification (LAMP) marker for rapid detection of this disease. A single RAPD marker A10 amplified a specific band of 1000bp in self-biting minks. The sequences of the bands exhibited 73% similarity to the Canis Brucella. SCAR and LAMP marker were designed for the specific fragment of RAPD marker A10 and validated in 30 self-biting minks and 30 healthy minks. χ^2 test showed difference ($p<0.05$) with SCAR and significant difference ($p<0.01$) with LAMP in the detection rate between the two groups, but LAMP method was more accurate than SCAR method. This indicated that LAMP can be used as a positive marker to detect self-biting disease in minks.

Key words: mink, self-biting, loop-mediated isothermal amplification, sequence-characterized amplified regions

Introduction

Stereotypical behavior is found in farmed animals but is rare in the wild. The causes are usually multifactorial, involving complex interactions between genetic, environmental, and social factors (De Bellis et al. 1999, De Bellis et al. 1999, Schore 2002, Garner et al. 2004). Stereotyped characteristics have been found to be heritable in bank voles (Clethrionomys glareolus) (Schoenecker and Heller 2000), and African striped mice *(Rhabdomys pumilio*) (Schwaibold and Pillay 2001), and the importance of genetic transmission in this regard has also been indicated in fur animals, such as mink (Hansen 1993). Lin (2007) showed that the poly-

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morphisms of the 5-hydroxytryptamine 1A receptor gene and dopamine receptor D1 and D2 genes had a direct association with self-biting behavior of mink.

Genomic analysis generally proceeds along a specific pathway of investigations in order to identify genes involved in specific traits (Andersson 2001), and determining the underlying mechanisms. Bulked sergeant analysis (BSA) combined with RAPD method has been widely used in identifying markers linked to a specific gene (Stephen et al. 2001, Wang et al. 2001, Agrama et al. 2002, Zhang et al. 2006). However, RAPD markers lack reproducibility due to mismatch annealing. Hence, some RAPD markers linked to genes of interest have been converted into sequence-characterized amplified regions (SCAR) markers (Wang et al. 2001, Agrama et al. 2002, Zhang et al. 2006, Liu et al. 2011). On the other hand, the loop-mediated isothermal amplification (LAMP) reaction has been considered a rapid method for identifying marker, which can amplify nucleic acids with high specificity, sensitivity and rapidity under isothermal conditions (Notomi et al. 2000). During the reaction, the DNA is amplified as multiple stem loops that appear as several bands of different size when visualized by agarose gel electrophoresis. In addition, the presence or absence of the target pathogen can be observed directly using stains with intercalating properties or by assessing the amount of white precipitate formed from magnesium pyrophosphate (Notomi et al. 2000). The simplicity of the technique has led to its use for detection of canine distemper virus (Cho and Park 2005), human parvovirus B19 (Yamada et al. 2006), porcine parvovirus (Chen and Cui 2009), goose parvovirus (Yang et al. 2010), and canine parvovirus (Parthiban et al. 2012), and mink enteritis virus (Wang et al. 2013).

In the present study, we used BSA in combination with RAPD to identify specific DNA sequences associated with self-biting disease, and to convert them into SCAR and LAMP markers to distinguish self-biting individuals from healthy ones.

Materials and Methods

Sample collection

Experiments were carried out at the Fur Animals Experiment Station of the Institute of Special Economic Animals and Plants of the Chinese Academy of Agricultural Sciences in Northeast China. The healthy and stereotyped minks were housed in standard roofed sheds with open sides in individual standard rearing cages. Total genomic DNAs were extracted from blood samples using a phenol-chloroform procedure. Healthy and stereotyped mink DNA pools were prepared for analysis by BSA, each containing an equivalent amount of total DNA from each group.

RAPD-PCR analysis of genomic DNA

One hundred random primers were sourced from the Shanghai Sangon Biological Engineering Technology Company for use in the RAPD-PCR analysis of the two sets of total DNA pools. PCR amplifications were performed in an Eppendorf AG thermal cycler (Gene Co., Ltd., Hamburg, Germany) in a 15 μl total volume containing 1U TaKaRa Taq DNA polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China), 2.5 μL of 10×PCR buffer (containing 500 mM KCl and 15 mM MgCl2), 0.2 mM of each dNTP, 6 μM of each primers, and 2 μl of DNA template. The PCR cycling parameters were as follows: 30 cycles of 94°C for 30 s, 60°C for 1min, and 72°C for 1.5 min, with a initial hot start at 94°C for 5 min and a final extension at 72°C for 5 min. The PCR products were detected by 2% agarose gel electrophoresis. Additional healthy and stereotyped blue fox genomic DNA samples were used to test the RAPD marker, each group including 30 individuals. Once the RAPD fragment presented as polymorphic between the two sample pools, the band of amplified DNA corresponding to this segment was excised from the agarose gel, purified using an Agarose Gel DNA extraction Kit and cloned into the PMD-18T vector. The sequences obtained were analyzed using the BLAST program for a similarity search.

Design of primers and analysis of the SCAR marker

The SCAR primers (sense: 5' AACGGCCAGTGATTCGA 3' and anti-sense: 5' CATTACCAGGCATCCATTCC 3') were designed against the RAPD fragment sequence using Primer 5 software. The PCR conditions used for SCAR amplification were similar to those that were used in the RAPD analysis. Amplification products were all resolved electrophoretically on 2% agarose gels.

LAMP analysis of genomic DNA

Based on the candidate fragment of RAPD marker A10, six primers (Outer primers F3 and B3, forward inner primer FIP, reverse inner primer BIP, and loop primers LF and LB) were designed by PrimerExplorer4 (Fujitsu, Tokyo, Japan; http://primerexplorer.jp/e/). The location and sequence of each primer are summarized in Table 1.

LAMP was performed in a total 25 mL reaction mixture containing 0.8 mM each of FIP and BIP, 0.2 mM each of the outer primers, 0.4mM each of LF

Table 1. Six LAMP primers specific for self-biting behavior.

Primer	Sequence (5'-3')
F3	CGGGTGATTTCCGGTTTGA
B3	ACGCAGCCATGATCTTCTTG
FIP(F1c +F2)	TCCAGCGTCACCGTCACGCGTCCGAAGAGCCGTGAGG
BIP(B1c + B2)	AAGGCAGTCGGCATGGGCGGCGGGATCAACCGCAAT
LF	GTTGGGAAGACCGATAAGAGC
LB	GGGGATCGCGGCTGTTTCT

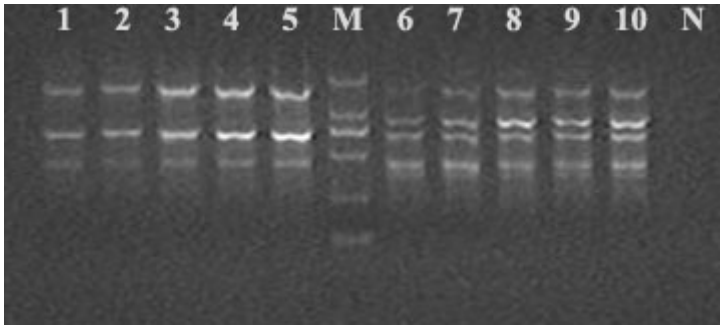

Fig. 1. PCR amplified DNA fragments from health and self-biting mink genetic DNA using A10 primer M: Marker 1∼5 the health mink; 6∼10 self-biting mink.

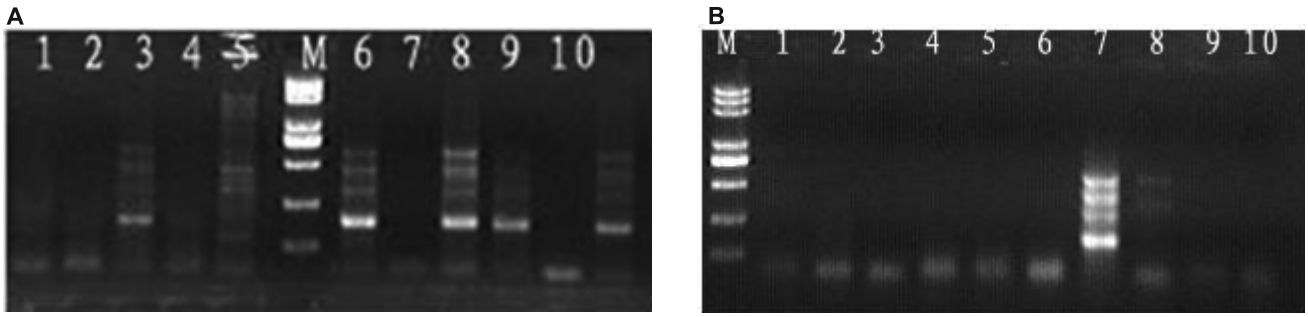

Fig. 2. Amplification of SCAR in two groups' mink
Fig. 2A. 1-10 the self-biting mink; Fig. 2B. 1-10 the health mink; M: 100 bp DNA marker.

and LB, 1.2 mM dNTPs, 1.0 M betaine (Sigma, St. Louis, MO), 8 mM $MgSO_4$, 1 μL Bst DNA polymerase (New England Biolabs, Beverly, US) and 2 μL the extracted template DNA (Nagamine et al. 2001, Nagamine et al. 2002a, Fukuta et al. 2003, Gunimaladevi et al. 2004). The reaction temperature was optimized and LAMP was carried out for 40 min and terminated at 80C for 2 min. LAMP products were subjected to electrophoresis on a 2% agarose gel. Healthy and stereotype behavior individuals (15 of each) from another mink farm were tested by SCAR and LAMP, and sensitivity of the detection was compared between these two methods. A χ^2 test in a 2×2 table was performed to determine whether a relationship exists between the SCAR and LAMP markers of the two groups. SAS8.0 software was used to analyze these data.

Results

RAPD-PCR analysis

High-molecular weight genomic DNA was isolated from fresh blood samples. To identify DNA markers linked to the stereotype behavior of the mink, 100 RAPD primers were screened using pooled total DNA from both the healthy and stereotyped groups of animals. The random primer A10 amplified an approximately 1000 bp fragment (denoted RA10-1000) from the stereotype behavior group. This fragment was not detectable in the healthy group and was thus regarded as being specific to stereotype behavior (Fig. 1). The RA10-1000 fragment was excised from the agarose gel, purified, and sequenced. The sequence was found to be 959 bp in length and exhibited a 73% similarity to the Canis Brucella.

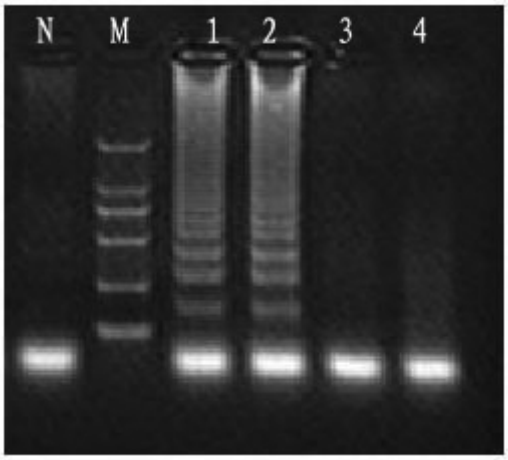

Fig. 3. Specificity of the LAMP Reaction for detection of self-biting disease of mink M, DL2000; Lane 1-2, DNA of self-biting mink; Lane 3-4, DNA of health mink; N, Negative control. All the products were electrophoresed on 2% agarose gels.

Table 2 Distribution among health and self-biting mink by SCAR marker and LAMP.

	With SCAR marker	Without SCAR marker （（total））	χ^2
SCAR-A10 Health group Sick group	23.33% 70%	76.67% 30%	0.0481
LAMP Health group Sick group	5% 100%	95% 0%	<0.001

Conversion of the RAPD marker into a SCAR marker

Based on the RA10-1000 fragment sequence, a pair of primers designated S-A10 was designed to convert the RAPD marker into a single locus SCAR marker. As expected, the primer pair amplified a 573 bp fragment from genomic DNA of the stereotype behavior group, but not from the healthy animals. The validity of the SA-10 primer pair was tested using the DNA of mink from another farm (Fig. 2). The χ^2 test indicated significant difference ($p<0.05$) between the two groups.

Conversion of the RAPD marker into a LAMP marker

LAMP primers were designed by RA10-1000 fragment and their system was perfected. The most superior system of LAMP consisted of 2.5 μL 10×buffer, 1.2 mM dNTP, 1.0 M betaine, 8 mM Mg^{2+}, 1 μL Bst DNA polymerase, and 2 μL the extracted template DNA. The reaction condition of the LAMP is 62.1°C for 1 h and heating at 80°C for 2 min. In order to evaluate the specificity of the primer, healthy mink and self-biting mink genome was amplified by the LAMP assay. The result showed that self-biting mink genome sequence was detected, but not the healthy mink genome sequence (Fig 3). The χ^2 test indicated significant difference ($p<0.01$) between the two groups (Table 2), the specificity of primer for self-biting mink was proved. It was also proven that LAMP can be used to detect self-biting disease.

Discussion

Farmed mink are known for showing stereotypical behavior and tail biting under standard housing conditions. There are several factors influence the stereotypical behavior, such as genetic, environmental, physiology, and endocrinology (Mason 1994, De Bellis et al. 1999, De Bellis et al. 1999, Gunnar 2000, Schore 2002, Vinke et al. 2002, 2004, Garner et al. 2004, Jeppesen et al. 2004). In the wild, fur animals seldom show the stereotypical behavior. But when they are farmed in the cage, fur animals perform poor adaptability to living environment and emergence of stereotyped behaviors (Leena Ahola 2007). Studies of animal emotions are important approaches to ensuring animal welfare in applied ethology. Moe (2006) studied anticipatory behavior, which

may be useful for the development of indicators of positive emotional states and, thus, positive welfare in farmed silver foxes. Sun et al. (2013) found that SBB in minks is a behavior associated with intermittent excitation, which is closely related to Ca^{2+} influx from extracellular flow to intracellular. Although stereotypies appear to be strongly related to environmental conditions or welfare, it is commonly observed that only a proportion of individuals develop stereotype under identical conditions, and the occurrence of stereotyping animal may be of genetic origin. The tendency to perform stereotypy is heritable in bank voles (Schoenecker and Heller 2000), and different strains of the same species housed under identical conditions may show strikingly different stereotypy levels (e.g. mice: Nevison et al. 1999, mink: Jeppesen et al. 2004). A similar positive correlation between the occurrence of stereotypies in parents and their offspring has been indicated in other species (Hansen 1993). Research has shown that sensitivity to the development of self-biting behavior is affected by the genetic background of an individual (Li et al. 2008, Liu et al. 2011).

RAPD is a widely used DNA marker in genetic polymorphism detection of whole genome, which provides ease of use and fast and reproducible results. The RAPD method is easy to operate, but poor in repeatability. So the self-biting specific marker we identified was converted into SCAR marker and LAMP. Although SCAR marker is effective and the value of χ^2 is less than 0.05, but 23.33% of health mink were detected. Compared with SCAR marker, LAMP is more sensitive to diagnose the disease. As a novel molecular-based technique, loop-mediated isothermal amplification (LAMP) reaction has been developed, which can amplify nucleic acids with high specificity, sensitivity and rapidity under isothermal conditions (Notomi et al. 2000). During the reaction, the DNA is amplified as multiple stem loops that appear as several bands of different size when visualized by agarose gel electrophoresis. In addition, the presence or absence of the target pathogen can be observed directly using stains with intercalating properties (Notomi et al. 2000) or by assessing the amount of white precipitate formed from magnesium pyrophosphate (Mori et al. 2007).

Conclusions

In summary, we used two methods to detect self-biting disease. LAMP-based method is rapid, sensitive, specific, and can detect sick animal quickly. The initial findings of this work are very promising and suggest that this test should be evaluated further in different laboratories and with a larger animal cohort. Although limited numbers of clinical samples were used in the present study, it has been shown that LAMP could be a convenient tool for detecting self-biting animals because it requires minimal laboratory facilities and is relatively simple and inexpensive to perform.

Acknowledgements

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