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Original Research Article

A Multiplex Assay for Simultaneous Detection of Three Retroviruses in Nonhuman Primates Based on the Luminex xTAG Platform

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Abstract: Nonhuman primates (NHPs) are widely used for investigating retroviral pathogenesis. Before experiments are conducted and breeding programs established, the animals must be judged negative for endogenous retroviruses. We developed a multiplex high-throughput detection method using multiplexed Luminex fluorescent microbeads and the xTAG system (MMxTAG). We tested three retroviral DNA detection assays for use in a single tube reaction by taking advantage of the Luminex xTAG platform. The assay showed a high sensitivity that was comparable to qPCR methods. The detection limits approached 1-10 fg/μL of plasmid DNA. We compared the specificity, efficiency and accuracy with ELISA assays and found our assay was superior in all regards. The MMxTAG performed well with clinical specimens and could selectively detect a single virus in the presence of the other two. This MMxTAG procedure can be applied to pathogen monitoring in animals and is present in a high throughput format that has low operating and development costs for protecting monkey contacts against these infectious diseases.
Keywords: Nonhuman primates; Luminex xTAG®; retroviruses.

INTRODUCTION

Nonhuman primates (NHPs) are critical to many biomedical research programs due to their similarities to humans [1-3]. Macaques used in pre-clinical studies were previously caught in the wild and harbored their natural microflora as well as pathogenic bacteria, viruses, parasites and fungi [4]. These animals are now bred in captivity for use in biomedical research programs [5]. However, the animals may still harbor novel infectious agents that can result in high morbidity and mortality as they did in wild populations [6].

Microbial surveillance programs have made much progress in excluding select pathogens from these animal colonies [6, 7]. However, simian immunodeficiency virus (SIV), simian type D retrovirus (SRV) and simian T-cell lymphotropic virus (STLV) are still present and pose significant risks to the animals as well as laboratory personnel. This is especially true in the case of developing specific-pathogen free (SPF) animals [7]. Therefore, we establish and maintain SPF colonies with the goals of improving animal health and reproduction by elimination of potential animal pathogens to (i) avoid the interference of these pathogens in biomedical research and (ii) reduce or eliminate potential sources of human occupational exposure to selected NHP viruses.

The normal laboratory procedures for detecting SIV, SRV and STLV-1 is culture in CM-174, Raji or MT-4 cells followed by immunohistochemical and immunofluorescence assays for virus identification [8-10]. ELISA or immunofluorescence assays (IFA) are required methods for excluding these pathogens in the course of selecting and monitoring SPF NHPs [11, 12]. New molecular techniques such as PCR, qPCR, high resolution melting (HRM) analysis and next generation sequencing (NGS) have made these viral screens routine. They are now incorporated into disease surveillance programs [13, 6, 14, 7, 15]. These techniques also have the potential to identify novel and uncharacterized pathogens in these laboratory animals.

Microbead-based liquid arays are widely used for antigen detection due to their relative ease of use,

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small sample volumes and multiplex platforms. Luminex microspheres have been the industry standard for microbead fluidics and the xMAP technology enables antigen capture on beads containing unique spectral addresses for up to 500 antigens simultaneously [16-20]. We have developed a Luminex xTAG assay for the simultaneous detection of three retroviruses that infect non-human primates. At the same time, it could be used more conveniently instead of PCR in the confirmatory assays, and more, it could detect the endogenous viruses to discern the false negative in ELISA assays.

MATERIALS AND METHODS

Ethical approval: All procedures performed in this study were in accordance with the ethical standards of IACUC of Guangdong Laboratory Animals Monitoring Institute which was certified by AAALAC.

Viruses and RNA extraction: SIV and SRV1-3 were cultured in CM-174 cell and Raji cell lines, respectively. RNA was extracted with Trizol reagent (Takara, Dalian). SIVcDNA and SRVcDNA were gained by reverse transcription with kit (Takara, Dalian). One of STLV-1 DNA was extracted from the ELISA-positive simian and the other named XS001 was purchased from Suzhou Xishan Biotechnology Inc., Suzhou).

Primers design: PCR primers for detection of individual viruses were designed using standard PCR conditions to generate ~ 200 bp amplicons (Table 1). These primers amplified SIV gag, SRV p27 and STLV-1 env genes. A highly conserved region of the Simian actin gene (Genbank: NM_001033084) was used as an internal control for identification of simian species as previously described [15]. Coupling of DNA to the microspheres used a commercial kit (Luminex) following the procedure provided by the manufacturer. The sequences of the primers and xTAG sequences are shown in Table 1. Primers were synthesized by Shenggong Biotechnology (Shanghai, China).

MMxTAG Protocol: (1) PCR amplification. Multiplex PCR reactions for amplification of the SIVgag, SRV p27 and STLV-1 env genes were carried out using a commercial PCR kit following instructions provided by the manufacturer (Qiagen, Shanghai,China). The mixture contained 1× reaction master mix, 0.2 μM of each of the xTAG and biotinylated primers and 2.0 μL template. The PCR amplification program was 95 °C for 5 min; 33 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; followed by a final elongation step at 72 °C for 5 min. (2) Hybridization. Luminex MagPlex-xTAG microspheres (Luminex, Shanghai, China) were equilibrated to room temperature, vortexed for 20s and then sonicated for 10s. Four different MagPlex-xTAG microspheres with different anti-xTAG sequences were

diluted to have 50 of each microsphere set per μL in sterilized 1 × Tm Hybridization Buffer (0.2 M NaCl, 0.1 M Tris, 0.08% Triton X-100, pH 8.0).The mixture was prepared so that 1000 microspheres of each set were contained in each reaction. Streptavidin and R-Phycoerythrin Conjugate (SAPE, Invitrogen, Carlsbad, CA, USA) was diluted to 1 μg/ml in 1× Tm Hybridization Buffer. For each hybridization reaction 20 μL MagPlex-TAG microspheres (Luminex, Shanghai, China), 5 μL of each PCR reaction (or H_2O control) and 75 μL of reporter solution (Luminex, Shanghai, China) were combined and the mixture was hybridized at 45° C for 30 min. (3) Signal detection. Hybrid complexes were analyzed for each bead type and the fluorescence intensity (MFI) was measured using a Luminex 200 instrument and analyzed using xPONENT 3.1 software included with the instrument (Luminex). The sample volume was set to 60 μL with a minimum of 100 beads per target with a gate of 7500 to 13500, and the plate heater was adjusted to the hybridization temperature, 45°C. The background reactions included all reaction components except that an equal volume of water replaced the DNA. The MFI of a positive signal was defined as four times the reference negative.

The evaluation of sensitivity and specificity: Sensitivity and specificity was determined by cloning the tagged PCR amplicons from each gene into plasmid vector pMD19-T. Recombinant plasmids were introduced into Escherichia coli strain DH5αby transformation for the production of pure plasmid DNA. Plasmids were serially diluted and coupled to beads as outlined above. These served as quantification standards. Specificity was determined by using non-matching templates in control reactions.

Compared with ELISA: Nineteen blood samples were collected and DNA was extracted from whole blood using a commercial kit (Tiangen Biotech co.LTD., Beiing). Serum from the samples was used for comparative analysis using a commercial ELISA kit (Xishan Biotechnology).

Application to clinical samples: Clinical samples were collected from the simian-bred farms, precursor genomic DNA was extracted with a commercial kit (Tiangen Biotech co.LTD. , Beiing), then was analyzed with MMxTAG platform.

RESULTS

Analytical sensitivity and specificity: We initially tested the quality of our detection method by adding single template test NA to the Luminex bead triplex reaction mixture. We achieved sensitivities approaching 100ag/uL using the triplexed beads. A mixture of all three plasmid DNAs gave detection sensitivities approximately 1fg/uL (Fig. 1). Control electrophoretogram of multiplex PCR products revealed

results similar to these (Fig. 2). When the Luminex reaction mixtures contained only a single specificity bead, the sole positive MFI value was that of the corresponding added DNA. When the cDNAs were mixed and added to the triplex reaction, all cDNAs could successfully be detected without interference from the others (Table 2).

Comparison of MMxTAG and ELISA for detection of three retroviruses: We performed blind testing and compared the MMxTAG and ELISA procedures for nineteen Simian blood samples (Table 3). The majority of samples were negative by both methods with three exceptions: Sample DWBSIV was SIV positive and samples 0921930C and 0922906C were STLV positive. However, the latter sample (0922906C) was also SIV positive only with the ELISA

procedure. This sample was judged negative by qPCR (data not shown).

Application to clinical specimens: We next examined thirty-nine clinical whole blood specimens containing EDTA as anticoagulant. These had all been previously determined to be negative by ELISA by the animal facility laboratory. The samples from two different primate colonies were collected and used for DNA extraction. We found two positive STLV samples and the findings were corroborated by qPCR (data not shown). Two positive control samples (DWBSIV and DWBSRV) that had been previously infected with SIV and SRV, respectively, were determined positive. The control STLV DNA (sample XS001) was determined negative (Table 4).
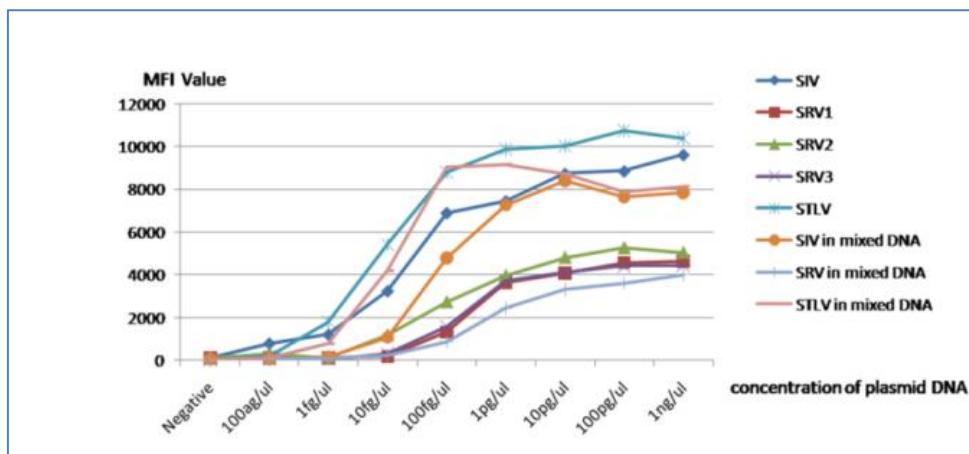

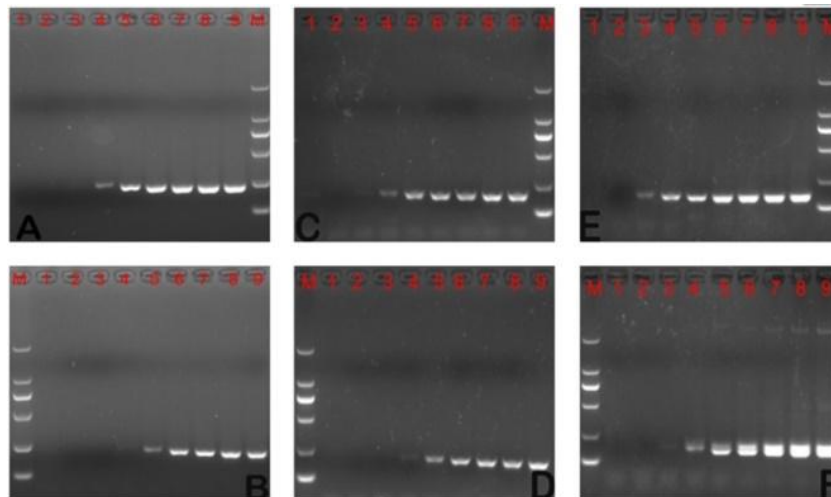**Fig-1: The sensitivities of the single plasmid DNA and mixed DNA of SIV/SRV/STLV**

Fig-2: The electrophoretogram of multiplex PCR products

A- plasmid DNA of SIV, B- plasmid DNA of SRV1, C- plasmid DNA of SRV2, D- plasmid DNA of SRV3, E- plasmid DNA of STLV, F-mixed plasmid DNA of SIV/SRV1/SRV2/SRV3/STLV

Table-1: PCR primers designed for SIV, SRV, STLV and Simian actin gene

Luminex xTAG Bead No.	Primers	Sequence (5'-3') [a]
67	SIV-tagF	ATCTCAATTACAATAACACACAAA-spacer18-ACGACCCGGCGGAAAGAAAAGT
	SIV-tagR	Biotinylated-TGCACCAGATGACGCAGACAGT
15	SRV-tagF	TACTTCTTTACTACAATTTACAAC-spacer18-AYGGGGCTACTGCYCCATA
	SRV-tagR	Biotinylated-GCCATTACCKGCYTGTTGATT
56	STLV-tagF	CTTAAACTCTACTTACTTCTAATT-spacer18-GTGCCAATCATGGACCTGCC
	STLV-tagR	Biotinylated-TCCTGGAGCGTCGATTAGAAGG
19	Actin-tagF	ATACTTTACAAACAAATAACACAC-spacer18-CCTCCCTGGAGAAGAGCTACGA
	Actin-tagR	Biotinylated-ATGCCACAGGACTCCATGCC

a: The sequences that are italicized and underlined are anti-xTAG sequences

Table-2: Specificity of single- and multiple-specificity of xTAG beads

Bead NO. Target	single xTAG bead				Multiple xTAG beads			
	67(SIV)	15(SRV)	56(STLV)	19(Actim)	67(SIV)	15(SRV)	56(STLV)	19(Actim)
Water	- [b]	-	-	-	-	-	-	-
SIV cDNA	+ [c]	-	-	+	+	-	-	+
SRV1 cDNA	-	+	-	+	-	+	-	+
SRV2 cDNA	-	+	-	+	-	+	-	+
SRV3 cDNA	-	+	-	+	-	+	-	+
STLV DNA	-	-	+	+	-	-	+	+
Mixed DNA [d]	+	+	+	+	+	+	+	+
Genomic DNA [e]	-	-	-	+	-	-	-	+

b: Negative (-); c: Positive (+); d: Mixed DNA was mixed with SIVcDNA, SRVcDNA and STLV DNA; e: Genomic DNA from the blood of a SPF simian.

Table-3: A comparison of ELISA and MMxTAG procedures for detecting SIV, SRV and STLV in blood samples

Sample Name	SIV		SRV		STLV	
	ELISA	MMxTAG	ELISA	MMxTAG	ELISA	MMxTAG
BS001	-	-	-	-	-	-
BS002	-	-	-	-	-	-
BS003	-	-	-	-	-	-
BS004	-	-	-	-	-	-
BS005	-	-	-	-	-	-
BS006	-	-	-	-	-	-
BS007	-	-	-	-	-	-
BS008	-	-	-	-	-	-
BS009	-	-	-	-	-	-
BS010	-	-	-	-	-	-
BS011	-	-	-	-	-	-
BS012	-	-	-	-	-	-
BS013	-	-	-	-	-	-
BS014	-	-	-	-	-	-
BS015	-	-	-	-	-	-
0921930C	-	-	-	-	+	+
0922906C	+	-	-	-	+	+
XS001	-	-	-	-	ND [f]	-
DWBsiv	+	+	-	-	-	-

f: Not determined.

Table-4: Results of clinical specimen testing by MMxTAG

Sample	actin	SRV	STLV	SIV	Sample	actin	SRV	STLV	SIV
CS1	+	-	-	-	CS24	+	-	-	-
CS2	+	-	-	-	CS25	+	-	-	-
CS3	+	-	-	-	CS26	+	-	-	-
CS4	+	-	-	-	CS27	+	-	-	-
CS5	+	-	-	-	CS28	+	-	-	-
CS6	+	-	-	-	SC1	+	-	+	-
CS7	+	-	-	-	SC2	+	-	-	-
CS8	+	-	-	-	SC3	+	-	-	-
CS9	+	-	-	-	SC4	+	-	-	-
CS10	+	-	-	-	SC5	+	-	-	-
CS11	+	-	-	-	SC6	+	-	-	-
CS12	+	-	-	-	SC7	+	-	-	-
CS13	+	-	-	-	SC8	+	-	-	-
CS14	+	-	-	-	SC9	+	-	-	-
CS15	+	-	-	-	SC11	+	-	-	-
CS16	+	-	-	-	SC12	+	-	+	-
CS17	+	-	-	-	DWBSIV	+	-	-	+
CS18	+	-	-	-	DWBSRV	+	+	-	+
CS19	+	-	-	-	XS001	+	-	-	-
CS20	+	-	-	-	H_2O	-	-	-	-
CS21	+	-	-	-	plasmid DNA	ND	+	+	+
CS22	+	-	-	-	DNA mixture	+	+	+	+
CS23	+	-	-	-	Blank[g]	-	-	-	-

g: Water control sample.

DISSCUSSION

In this study, we established a multiplex assay for the simultaneous detection of three retroviruses based on Luminex xTAG platform, and evaluated its performance with clinical specimens. The MMxTAG assay has been previously shown to be pathogen-specific and with sensitivity equal to or exceeding PCR or qPCR procedures [21, 19]. The assay accuracy was improved due to the greater sample sizes using the MFI of individual beads, allowing for greater statistical significance. This assay also allows for simultaneous detection of multiple nucleic acid sequences in a single reaction, and saves time while lowering the cost and work associated with single tube detection [22]. Moreover, it solves the shortcoming that traditional multiplex PCR presents in the design of different sized amplification fragments. In addition, the Luminex assay has been tested with up to 100 different templates in a single reaction tube that will lead to more advances in high-throughput detection [23]. Multiplex PCR coupled to the Luminex xTAG platform is currently in place for pathogen detection, SNP genotyping and disease screening [24, 25]. There is a multiplex micro-bead immunoassay (MMIA) as an alternative to PCR for detecting up to six viruses in primates using antibody detection. The assay has a greater sensitivity than ELISA does but the bead-coupling procedure to recombinant viral antigens and synthetic viral peptides greatly increased the assay cost [16, 18].

Identical volumes of sample and mixed plasmid DNA were used in each test, which was equivalent to a four-fold dilution. Therefore, the sensitivity of a single test was greater than the quadruplex detection for the plasmid DNA in our experiments. Interestingly, the control STLV DNA we purchased gave a negative result and we cannot currently account for this. However, only two clinical samples were detected as STLV-positive and this result was consistent with previous findings using these same samples [26].

CONCLUSIONS

In China, with the continuous development of simian breeding programs, the management level is becoming standardized and many enterprises have their own quarantine facilities and personnel. Therefore, a rapid method for the determination of viral infections can screen out infected animals before they are subjected to a lengthy quarantine process. Given that ELISA results may include false positives, the MMxTAG method can be a favorable addition to their testing procedures while saving time and money and protect monkey contacts against these infectious diseases. We developed a multiplex PCR-based on Luminex xTAG platform that is a high-throughput, sensitive and specific method that can be easily adapted to any animal laboratory.

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Author's contribution

ZH L, L T, XX L, J W, YJ Z, and XQ Y made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data. ZH L was involved in drafting the manuscript or revising it critically for important intellectual content. Y Z and R H gave final approval of the version submitted for publishing. All authors read and approved the final manuscript.

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