Rapid detection of three rabbit pathogens by use of the Luminex x-TAG assay

Miaoli Wu1,2, Yujun Zhu2, Feng Cong1, Dan Rao2, Wen Yuan1, Jing Wang1, Bihong Huang2, Yuexiao Lian2, Yu Zhang2, Ren Huang3* and Pengju Guo4*

Abstract

Background: Domestic rabbits, especially New Zealand white rabbits, play an important role in biological research. The disease surveillance and quality control are essential to guarantee the results of animal experiments performed on rabbits. Rabbit hemorrhagic disease virus, rabbit rotavirus and Sendai virus are the important pathogens that needed to be eliminated. Rapid and sensitive method focus on these three viruses should be established for routine monitoring. The Luminex x-TAG assay based on multiplex PCR and fluorescent microsphere is a fast developing technology applied in high throughput detection. Specific primers modified with oligonucleotide sequence/biotin were used to amplify target fragments. The conjugation between oligonucleotide sequence of the PCR products and the MagPlex-TAG microspheres was specific without any cross-reaction, and the hybridization products could be analyzed using the Luminex 200 analyzer instrument. Recombinant plasmids were constructed to estimate the detection limit of the viruses. Furthermore, 40 clinical samples were used to evaluate the efficiency of this multiplex PCR based Luminex x-TAG assay.

Results: According to the results, this new method showed high specificity and good stability. Assessed by the recombinant plasmids, the detection limit of three viruses was 100 copies/μl. Among 40 clinical specimens, 18 specimens were found positive, which was completely concordant with the conventional PCR method.

Conclusions: The new developed Luminex x-TAG assay is an accurate, high throughput method for rapid detection of three important viruses of rabbits.

Keywords: Rabbit hemorrhagic disease virus, Rabbit rotavirus, Sendai virus, Multiple PCR, Luminex x-TAG

Background

Domestic rabbit (Oryctolagus cuniculus), especially New Zealand white rabbit, has attracted more and more attention in biomedical, immunological and pharmaceutical research, because of its intermediate size and phylogenetic proximity to primates [1]. It played an important role in production of antibodies, eye research as well as cardiovascular disease [2, 3]. Rabbit is one of the most commonly used experimental animals and must be free of some important pathogens.

The first outbreak of rabbit hemorrhagic disease (RHD) caused by the rabbit hemorrhagic disease virus (RHDV) occurred in 1984 in Jiangsu Province, China and spread all around the world rapidly [4]. It’s an acute and mostly fatal contagion in both domestic and wild rabbits, characterized by acute necrotizing hepatitis and hemorrhage [5]. Actually there are three different clinical features, the pre-acute, acute and sub-acute forms [6]. Among which the sub-acute form causes no clinical symptoms and rabbits will recover within 2~3 days [7]. Rabbit rotavirus (RRV) infection was the major cause of mild to severe diarrhea in rabbits [8]. The rotavirus isolated from infected rabbits belongs to Group A rotaviruses (RVAs), which also infect humans and other animals. It’s a highly contagious mild virus and disseminated by fecal-oral route [9–11]. Although the infection rate of RRV is high, most infections are subclinical. However, co-infection with other bacteria or viruses may cause severe enteritis and the excretion by the infected rabbits will become the contaminate source and cause new infection. Sendai virus (SV), also known as a murine
parainfluenza virus type 1, belongs to *Respirovirus, Paramyxoviridae* family. It causes transmitted respiratory tract infections in a variety of animals [12]. Unlike rodents, rabbits are not sensitive to SV, and the infection will only cause fever but not respiratory tract contagious in rabbits.

Despite the asymptomatic infection and low mortality of rotavirus and Sendai virus infection, the existence of these two viruses will affect the quality of experimental animals and severely interfere with the results of animal experiments on them [13]. To improve the quality of rabbits and ensure the accuracy of animal experiments, RHDV, SV and RRV are the required inspection items ruled by the national quality standard of China.

The traditional methods for pathogen identification include etiology diagnosis, serological diagnosis as well as molecular diagnosis [14, 15]. According to the laboratory animal microbiological quality control standards of China, the recommended test methods for these viruses mainly are the etiology and serological diagnosis. Both of them are time-consuming and laborious, compared with molecular diagnostic techniques. Polymerase chain reaction (PCR) with high sensitivity and specificity is widely used in pathogenic identification [16, 17]. Reverse transcription-PCR (RT-PCR) and quantitative reverse transcription-PCR (RT-qPCR) assays had been developed for monitoring of rabbit hemorrhagic disease virus, Sendai virus as well as rabbit rotavirus [18–20]. However, the restricted throughput limited the application of PCR, even the multiplex real-time quantitative PCR could not detect more than five pathogens in one reaction. The development of rapid and sensitive multiplex diagnostic method was extremely important for rabbit health monitoring. Compared with conventional PCR methods, the Luminex technology was a high-throughput, rapid, sensitive and labor-saving multiplex assay [21]. Conjugation of microbeads with different fluorescent dyes could differentiate as much as 100 targets in a single reaction. This technology offered a variety of applications in pathologic diagnosis [22–24].

In this study, we developed a multiplex PCR-based MagPlex-TAG assay for simultaneous detection of rabbit hemorrhagic disease virus, rabbit rotavirus and Sendai virus.

**Methods**

**Virus and vaccine**

The combined rabbit viral hemorrhagic disease and *Pasteurella multocida* vaccine and rabies vaccine were purchased from the animal epidemic prevention and control center in Tianhe district (Guangzhou, China). The nucleic acid of classical RHDV strain was kindly offered by Shanghai Veterinary Research Institute. The Sendai virus, *Salmonella typhimurium*, *Helicobacter bilis* (H.b), *Helicobacter rodent* (H.r), *Escherichia coli* (E.coli) and the nucleic acid of rabbit rotavirus, rabbit adenovirus as well as rabbit coronavirus were preserved by our laboratory.

**Sample collection and DNA/RNA extraction**

All the clinical samples, including 24 fecal samples, 10 tissue samples and 6 nose swabs, were provided by a rabbit farm from Qingdao (Shandong Province, China) and preserved at −80 °C. The nucleic acid extraction was performed by the automatic nucleic acid extraction instrument (Tiangen Biotech, Beijing, China) according to the manufacture’s instruction. All the nucleic acid were stored at −80 °C before used.

**Primers design**

Specific primers of the three viruses were designed based on the conserved VP60 gene, N gene and VP4 gene of rabbit hemorrhagic disease virus, Sendai virus and rabbit rotavirus respectively (Table 1). Each forward primer was modified with a unique oligonucleotide “TAG” sequence at the 5’ terminus, which was used to couple with the magnetic fluorescent microsphere. All the reverse primers were biotinylated at the 5’ terminus for fluorescent detection with streptavidin-R-phycocerythrin (SAPE). The forward primers and oligonucleotides were best match by the Tag-It Oligo Design Software v.3.00 (7B052). All the primers were synthesized and purified by high-performance liquid chromatography (HPLC) (Sangong, Guangzhou, China).

**Multiplex PCR amplification**

Three sets of primers were confirmed by the simplex PCR reactions before the multiplex PCR (mPCR)

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**Table 1** Sequences of primers used in the Luminex x-TAG assay

| Primer | Sequence | Target gene | Accession number | Genome location | Product size (bp) |
|--------|----------|-------------|-----------------|----------------|-----------------|
| RHDV-F | CTTCACAAAATAACCCATCACA | VP60 | KF494951.1 | 285~309 | 161 |
| RHDV-R | CCAACCTGTCACATCACA | N | | |
| RRV-F | ATGGTTCGCTTGTGTCTTAGTTG | VP4 | U62152.1 | 303~325 | 251 |
| RRV-R | ATGCGTTGGGTGTAGTTCCTGTA | | | |
| SV-F | TGACAACAAAACCAGTAGAACCAC | | | |
| SV-R | ACCATAGGTGCAACACAGCCATTCA | | | |

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amplification. The mPCR was performed using the one-step RT-PCR Kit (Qiagen, Valencia, CA; Cat.no.210212) with a total volume of 50 μl containing 100 ng of template DNA/RNA. The mPCR amplification conditions were as follows: 50 °C, 30 min for reverse transcription, followed by activation at 95 °C for 15 min. Then, DNA amplification was carried out by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by final extension at 72 °C for 10 min. All the samples were tested in triplicate and the assays were run with negative control.

**Luminex assay**
The PCR products were conjugated with MagPlex-TAG microspheres, which were pre-coupled with “anti-TAG” sequence. The working mixture containing 2500 of each target microspheres was diluted with 1× Tm Hybridization Buffer (0.2 M NaCl, 0.1 M Tris, 0.08% Triton X-100, Ph 8. 0, filter sterilized). For each reaction, 5 μl of amplified product or distilled water, 75 μl of SAPE solution and 20 μl of the working MagPlex-TAG microsphere mixture were well mixed together before incubated in a thermocycler for 30 min at 45 °C. The Luminex 200 analyzer was applied to analyze the products after the hybridization reaction.

**Data analysis**
The data analyzed by the Luminex xPONENT software were reported as median fluorescence intensity (MFI). For background calculation, negative controls contained all the hybridization components except target DNA, were set in each experiment. The cutoff value of the three target pathogens were obtained from all the negative PCR controls. Specifically, the cutoff value was defined, for each bead set as the mean of the MFI values of negative controls + 3 SD (Standard deviation).

**The evaluation of specificity**
To evaluate the specificity of the x-TAG assay, the *Pasteurella* (Pas.), *Salmonella typhimurium* (S.ty), *Helicobacter bilis* (H.b), *Helicobacter rodent* (H.r), *Escherichia coli* (E.coli), rabbit coronavirus (RCoV), rabbit adenovirus (RAV) and rabies virus (RV) nucleic acids were tested with previously indicated primers. Positive and negative controls were simultaneously tested. All the products were further analyzed on the Luminex 200 analyzer after hybridization.

**The evaluation of sensitivity**
The PCR products of RHDV (161 bp), RRV (251 bp) and SV (148 bp) were cloned into the pGEM T easy vector (Promega, Madison, USA). The plasmids were in vitro transcribed (IVT) by RiboMax™ Large Scale RNA production system T7 (Promega, Madison, USA) according to the manufacturer’s instructions. 40 units RNase-free DNase™ (Promega, Madison, USA) enzyme was used to remove plasmid DNA. Trizol LS reagent (Invitrogen, Carlsbad, CA) was used for RNA isolation according to the manufacturer’s instructions. The RNA concentrations were estimated by spectrophotometry. Serial ten-fold dilutions of RNA standard were performed as templates to determine the sensitivity of the x-TAG assay, and the results were confirmed in triplicate.

**Clinical samples detection**
A total number of 40 clinical samples including nose swabs, faeces and tissues were tested by both Luminex x-TAG assay and conventional PCR assay.

**Results**

**Specificity analysis of the Luminex x-TAG assay**
The specificity of the three primer pairs was confirmed using the unrelated nucleic acid as PCR templates. There was no cross-amplification during the test and the fluorescence signals were observed only in the corresponding positive controls (Fig. 1).

**Sensitivity of the Luminex x-TAG assay**
The sensitivity of the Luminex x-TAG assay was examined by testing serial ten-fold dilutions of RNA standard, and distilled water was used as negative control. The results showed that the detection limits of the three viruses were 10² copies/μl (Fig. 2). The MFI value and the corresponding concentration were detailed in Table 2.

**Reproducibility and stability analysis**
To further assess the Luminex x-TAG assay, three parallel reactions were carried out with standard RNA at concentrations of 1 × 10⁵ and 1 × 10⁶ copies/μl. The
coefficient of variation of the intra-assay and inter-assay were displayed in Table 3.

Application to clinical samples
The Luminex x-TAG assay was applied for clinical sample detection, and confirmed by both the conventional PCR and sequencing analysis. The assay detected genetic material of RHDV \((n = 3)\), SV \((n = 15)\) and RRV \((n = 10)\) in the clinical samples. Among which, the co-infection of RHDV +SV \((n = 2)\), RHDV+RRV \((n = 3)\), SV + RRV \((n = 7)\) was observed. Only 2 samples were detected with triple infection (Table 4). All the samples were detected by conventional PCR method and the results were in accord with the Luminex x-TAG assay.

Discussion
Although conventional PCR had been used for RHDV, SV and RRV identification, there is no multiplex-assay for simultaneous discrimination of these viruses. Multiplex-PCR assays allow for detection of different targets in one reaction. The interpretation of the mPCR results is based on the sizes of amplicons. It limits the multiplicity in the single reaction and fails to meet the requirements of high-throughput detection. The Luminex x-TAG assay based on PCR products coupled with fluorescent encoding microsphere is a high-throughput, accuracy technique, which is widely used in pathogenic diagnosis [25]. However, there is a lack of studies that apply and investigate x-TAG assay in the veterinary field.

In this study, we described the development and validation of the Luminex x-TAG assay for monitoring of

| Table 2 | The MFI values of different standard RNA concentrations |
|---------|---------------------------------------------------------|
| Sample concentration | RHDV Mean MFI | RHDV SD | SV Mean MFI | SV SD | RRV Mean MFI | RRV SD |
| 10^8 copies/μl | 8159 | 196 | 9621 | 215 | 8324 | 245 |
| 10^7 copies/μl | 7639 | 134 | 8421 | 195 | 7502 | 227 |
| 10^6 copies/μl | 5256 | 221 | 7026 | 143 | 6242 | 194 |
| 10^5 copies/μl | 4211 | 115 | 5673 | 136 | 5103 | 187 |
| 10^4 copies/μl | 3106 | 194 | 4168 | 185 | 3965 | 278 |
| 10^3 copies/μl | 2154 | 164 | 3075 | 204 | 2837 | 243 |
| 10^2 copies/μl | 1518 | 103 | 1946 | 184 | 1264 | 149 |
| 10^1 copies/μl | 572 | 117 | 543 | 125 | 496 | 138 |
| Background | 174 | 29 | 267 | 35 | 185 | 26 |
| Cut-off value | 1000 | 1000 | 1000 |

Table 3 | The reproducibility and stability analysis of the Luminex x-TAG assay |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Virus concentration (copies/μl) | Intra-assay/MFI CV(%) | Inter-assay/MFI CV(%) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| RHDV 1 × 10^8 | 8086 | 8381 | 8012 | 2.4 | 8321 | 8075 | 8112 | 1.62 |
| 1 × 10^8 | 4081 | 4284 | 4271 | 2.73 | 4405 | 4312 | 4165 | 2.81 |
| SV 1 × 10^9 | 9414 | 9629 | 9821 | 2.23 | 9632 | 9563 | 9412 | 2.21 |
| 1 × 10^8 | 5723 | 5775 | 5518 | 2.4 | 5583 | 5695 | 5354 | 3.13 |
| RRV 1 × 10^8 | 8441 | 8052 | 8478 | 2.94 | 8446 | 8249 | 8056 | 2.36 |
| 1 × 10^8 | 5218 | 5194 | 4907 | 3.67 | 5048 | 5241 | 5339 | 3.71 |

Table 4 | Screening results for 40 clinical samples for Luminex x-TAG assay |
| Sample | RHDV | SV | RRV | Sample | RHDV | SV | RRV |
|---------|------|----|-----|--------|------|----|-----|
| F1 | – | + | + | F21 | – | + | – |
| F2 | – | – | – | F22 | – | – | – |
| F3 | – | – | – | F23 | – | – | – |
| F4 | – | + | – | F24 | – | + | + |
| F5 | – | + | – | T1 | – | + | – |
| F6 | – | – | – | T2 | + | – | + |
| F7 | – | – | – | T3 | – | – | + |
| F8 | – | – | – | T4 | + | + | + |
| F9 | – | + | – | T5 | + | + | + |
| F10 | – | – | – | T6 | – | + | + |
| F11 | – | – | – | T7 | – | + | + |
| F12 | – | – | – | T8 | – | – | – |
| F13 | – | – | – | T9 | – | – | – |
| F14 | – | – | – | T10 | – | + | – |
| F15 | – | – | + | N1 | – | – | – |
| F16 | – | – | – | N2 | – | – | – |
| F17 | – | + | – | N3 | – | – | – |
| F18 | – | – | – | N4 | – | – | – |
| F19 | – | + | + | N5 | – | + | + |
| F20 | – | – | – | N6 | – | – | – |

f faecal samples, T tissue specimens, N nose swab
+ + +: strong positive (MFI > 5* cut-off)
+ +: positive (3* cut-off < MFI < 5* cut-off)
+: weak positive (cut-off < MFI < 3* cut-off)
-: negative (MFI < cut-off)
RHDV, SV, and RRV. The new method showed good specificity, no cross-reaction with other tested pathogens. The detect limit of the three viruses was $10^2$ copies/μl. 40 specimens were tested using both Luminex x-TAG assay and conventional RT-PCR, and the results of different methods are consistent with each other. These findings suggest that the developed x-TAG assay based on multiplex PCR for screening three pathogens is applicable.

To further optimize the assay, we performed it on different hybridization conditions. The results indicated that 45°C was the optimal hybridization temperature and the addition of 1% BSA in the 1x Tm Hybridization Buffer could remarkably reduce the background MFI values.

A large scale of negative samples mainly SPF samples should be tested to determine the cut-off value of each viruses. To guarantee the accuracy of the test and avoid false positive/negative results, MFI values of specimen that is close to the threshold should be double-checked by monoplex PCR or sequencing.

**Conclusion**
The multiplex assay is an efficient alternative to monoplex RT-PCR and greatly reduces the number of reactions required. In this report, the establishment of this effective system will allow precise detection and identification of RHDV, SV, and RRV. This approach might be the promising rabbit quality control methods. Besides, more target pathogens could be integrated into the established assay for better utilization.

**Abbreviations**

col: Helicobacter bilis; Hr: Helicobacter rodent; NTC: Non template control; Pa.s.: Pasteurella multocida; RAV: Rabbit adenovirus; RCov: Rabbit Coronavirus; RHD: Rabbit hemorrhagic disease; RHDV: Rabbit hemorrhagic disease virus; RRV: Rabbit rotavirus; RV: Rabies virus; RVAs: Group A rotaviruses; S.ty.: Salmonella typhimurium; SV: Sendai virus

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**Availability of data and materials**
All data generated or analysed during this study are included in this published article.

**Authors’ contributions**
Conceived and designed the experiments: YZ, RH and P.J.G. Performed the experiments: MLW, YJZ, DR. Sample collection: BHH and YXL. Analyzed the data: JW. Contributed to the writing: FC and YW. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**
Not applicable

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interest.

**Author details**
1 Guangdong Provincial Key Laboratory of Laboratory Animals, Guangzhou 510633, China. 2 Guangdong Laboratory Animal Monitoring Institute, Guangzhou, China. 3 Guangdong laboratory animals monitoring institute, Guangzhou 510633, China. 4 Guangdong key laboratory of laboratory Animals, Guangzhou, China.

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**References**

1. Peng X. Transgenic rabbit models for studying human cardiovascular diseases. Comp Med. 2012;62(6):472–9.

2. Weber J, Peng H, Rader C. From rabbit antibody repertoire to rabbit monoclonal antibodies. Exp Mol Med. 2017;49(3):e305.

3. Webre JM, et al. Rabbit and mouse models of HSV-1 latency, reactivation, and recurrent eye diseases. J Biomed Biotechnol. 2012;2012:612316.

4. Abrantes J, et al. Rabbit haemorrhagic disease (RHD) and rabbit hemorrhagic disease virus (RHDV): a review. Vet Res. 2012;43:12.

5. Schwensow NI, et al. Rabbit hemorrhagic disease virus: persistence and adaptation in Australia. Evol Appl. 2014;7(9):1056–67.

6. Nystrom K, et al. Histo-blood group antigens act as attachment factors of rabbit hemorrhagic disease virus infection in a virus strain-dependent manner. PloS Pathog. 2011;7(8):e1002188.

7. Xu ZJ, Chen WX. Viral haemorrhagic disease in rabbits: a review. Vet Res. 1989;13(3):205–12.

8. Fushuku S, Fukuda K. Examination of the applicability of a commercial human rotavirus antigen detection kit for use in laboratory rabbits. Exp Anim. 2006;55(1):71–4.

9. Baker DG. Natural pathogens of laboratory mice, rats, and rabbits and their effects on research. Clin Microbiol Rev. 1998;11(2):231–66.

10. Schoondermark-van de Ven E, et al. Rabbit colony infected with a bovine-like GSP[11] rotavirus strain. Vet Microbiol. 2013;166(1–2):154–64.

11. Martella V, et al. Lapine rotaviruses of the genotype P[2,2] are widespread in Italian rabbitries. Vet Microbiol. 2005;111(1–2):117–24.

12. Matveeva OV, et al. Mechanisms of Oncolysis by paramyxovirus Sendai. Acta Virol. 2013;57(2):16–16.

13. Kerr PJ, Donnelly TM. Viral infections of rabbits. Vet Clin Exot Anim Pract. 2013;16(2):437–68.

14. Meng GF, et al. Seroprevalence of Encephalitozoon curiculi and toxoplasma gondii in domestic rabbits (Oryctolagus cuniculus) in China. Korean J Parasitol. 2015;53(6):759–63.

15. Liu J, Kerr PJ, Strive T. A sensitive and specific blocking ELISA for the detection of rabbit calicivirus RCV-A1 antibodies. Virol J. 2012;9:182.

16. Matthee SQ, et al. Isolation and characterization of a novel Betacoronavirus subgroup a coronavirus, rabbit coronavirus HKU14, from domestic rabbits. J Virology. 2012;86(10):5481–96.

17. Le Gall-Recule G, et al. Emergence of a new lagovirus related to rabbit haemorrhagic disease virus. Vet Res. 2013;44:481.

18. Bugele WA, Horvath CM. MicroRNA profiling of Sendai virus-infected AS49 cells identifies miR-203 as an interferon-inducible regulator of IFIT1/ISG56. J Virol. 2013;87(16):9260–70.

19. Matthee SQ, et al. Comparative quantitative monitoring of rabbit hemorrhagic disease viruses in rabbit kittens. Virol J. 2014;11:109.

20. Bonica MB, et al. Complete genome analysis of a rabbit rotavirus causing gastroenteritis in a human infant. Viruses. 2015;7(2):844–56.

21. Jiang L, et al. Simultaneous detection of nine key bacterial respiratory pathogens using Luminex xTAG(R) technology. Int J Environ Res Public Health. 2017;14(3):E223.

22. Santos HL, et al. LUMINEX(R): a new technology for the simultaneous detection of five Entamoeba spp. commonly found in human stools. Parasit Vectors. 2013;6:69.

23. Chen R, et al. Bead-based suspension array for simultaneous differential detection of five major swine viruses. Appl Microbiol Biotechnol. 2015;99(2):919–28.
24. Ocheretina O, et al. Use of Luminex MagPlex magnetic microspheres for high-throughput spoligotyping of Mycobacterium tuberculosis isolates in port-Au-prince, Haiti. J Clin Microbiol. 2013;51(7):2232–7.
25. Zhou L, et al. Simultaneous detection of five pathogens from cerebrospinal fluid specimens using Luminex technology. Int J Environ Res Public Health. 2016;13(2):193.