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Short communication

Detection of multiple specific adventitious viruses in viral gene therapy products using multiplex PCR coupled with capillary electrophoresis

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Adventitious agents, comprising unintentionally introduced microorganisms in the production of biological products, pose a significant challenge in ensuring the safety of gene therapy products. The revised International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guildline Q5A(R2) from September 2022 highlights the inclusion of viral vector-based gene therapy products in safety discussions, emphasizing controls in material sourcing, testing, and viral clearance [1]. Detecting adventitious virus contamination is complex due to the unique characteristics of gene therapy products and the limitations of routine testing methods. The US Food and Drug Administration (FDA) recommends incorporating routine and specific virus detection methods, including those outlined in various pharmacopeias. Existing control methods have limitations, prompting the need for highly sensitive and broad-spectrum detection approaches. Unlike traditional biological products, gene therapy products primarily consist of live viruses, necessitating methods that distinguish between the main virus and adventitious viruses. Current virus detection techniques, such as polymerase chain reaction (PCR) , sequencing, mass spectrometry, and DNA microarrays [2–4], have their drawbacks. The PCR coupled with capillary electrophoresis (PCR-CE) system combines the high sensitivity of PCR with the capability of capillary electrophoresis for multi-target detection, enabling a sensitive and broad-spectrum viral detection solution through the special design of primers. Applied extensively in animal disease control, the PCR-CE system is now introduced for the first time in detecting adventitious viruses in biological products.

The PCR-CE based experimental procedure established by this research consists of three steps: sample pretreatment, nucleic acid amplification, and capillary detection. The pretreatment includes the release/extraction of nucleic acids from the sample and RNA reverse transcription. During nucleic acid amplification, different annealing temperatures were used at various PCR stages to enhance detection sensitivity and specificity (Fig. 1A).

Some representative virus types were selected for analysis, including viruses with a high risk of human infection, viruses associated with gastrointestinal infections such as hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis C virus (HCV), high carcinogenic risk viruses like human papillomavirus (HPV) and Epstein-Barr virus (EBV), herpes viruses including herpes simplex virus (HSV), human cytomegalovirus (HCMV), human herpesvirus type 6 (HHV-6), human herpesvirus type 7 (HHV-7), and human immunodeficiency virus (HIV). Additionally, three high-risk viruses originating from animal sources—simian immunodeficiency virus (SIV), porcine parvovirus (PPV), and porcine circovirus (PCV)—were also included in the analysis, as animal cells or culture materials derived from animals may be used during virus production. The 13 selected viruses were high-risk adventitious viruses in gene therapy products, and most were required to be controlled in the pharmacopoeia.

For the purpose of control, a virus-specific fragment was ligated to a pM18-T plasmid vector after gene synthesis to serve as a positive control, thereby mitigating biosafety risks. For DNA viruses, the specific DNA sequences of the virus were directly utilized. In the case of RNA viruses, the vector was connected using the DNA sequence that corresponds to the particular RNA fragment. However, the DNA template of an RNA virus does not accurately represent the reverse transcription process. To address this, an RNA pseudovirus can be created, which not only reduces biosafety risks but also simulates the nucleic acid extraction and RNA reverse transcription processes of actual viruses. The pseudovirus technology has been extensively applied across various research

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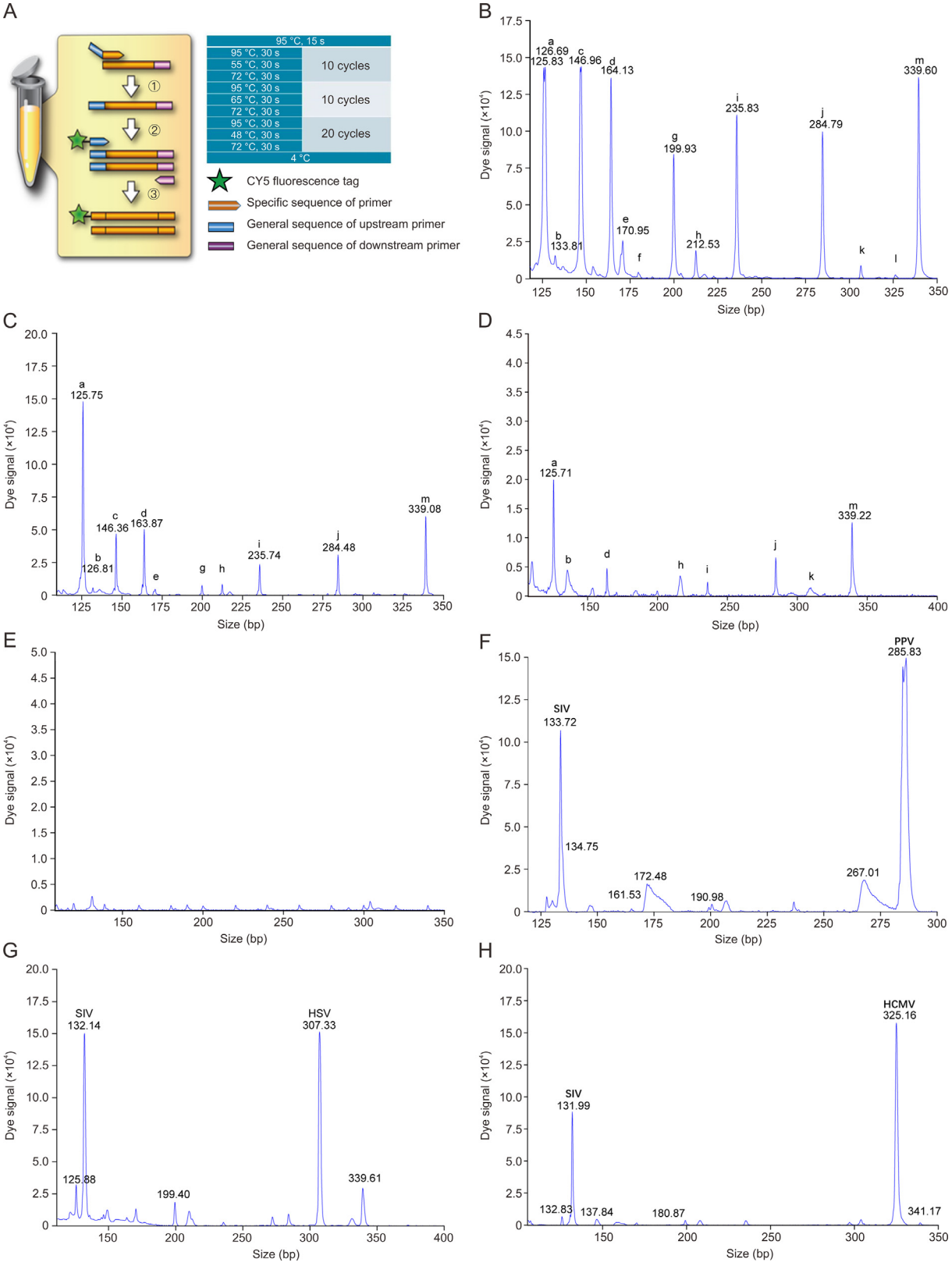

Fig. 1. Experimental principle, sensitivity verification, and results of real sample measurement. (A) Principle of viral nucleic acid amplification and conditions of multiplex polymerase chain reaction (PCR). Each primer consists of two parts, a general sequence, and a specific sequence. Process ①: The general part of the primers was added to both ends of the target sequence. Process ②: The product of process ① was amplified and complementary-paired with the tagged general primers in the reaction buffer. Process ③: The PCR amplification products were generated using fluorescent tags. A gradient annealing temperature was set during the amplification reaction to improve the sensitivity of the reaction. (B–E) The sensitivity test results for 13 viruses. The 13 target viral genes detected at 1000 copies (B), ten target genes detected at 100 copies (C), Eight target genes detected at 10 copies (D), and none target gene detected at 1 copy (E). (F–H) The peaks of simian immunodeficiency virus (SIV) pseudovirus and real viruses porcine parvovirus (PPV) (F), herpes simplex virus (HSV) (G), and human cytomegalovirus (HCMV) (H) were found at the corresponding molecular weight position after reverse transcription. a: Epstein-Barr virus; b: simian immunodeficiency virus; c: hepatitis B virus; d: human herpesvirus type 7; e: human immunodeficiency virus; f: human papillomavirus; g: hepatitis A virus; h: human herpesvirus type 6; i: hepatitis C virus; j: porcine parvovirus; k: herpes simplex virus; l: human cytomegalovirus; m: porcine circovirus.

domains, demonstrating its capability to detect a range of viruses [5]. The pseudovirus was instrumental in ensuring the quality of the reverse transcription process for any adventitious RNA viruses that might be present in the sample, with false-negative outcomes being ruled out. Simultaneous procedures for sample pretreatment, PCR, and capillary electrophoresis detection were conducted for the SIV pseudovirus and the samples. The detection of the characteristic SIV peak in the capillary electrophoresis pattern confirmed the success of the reverse transcription process (Fig. S1).

Due to the need to amplify multiple targets in the PCR system, the primers should not interfere with each other when designing primers. Moreover, the length of each PCR product should be different (more than 3 bp to be easily distinguished). A connector of general sequences should be added at the 5′ end of the upstream and downstream specific primers to enable separation and detection of each PCR product via capillary electrophoresis. Also, there should be a short sequence in the PCR system (pre-added to the 5X PCR buffer) that is complementary to the general sequences and connected to CY5 fluorescence group. This short sequence can add a fluorescent signal to the PCR product during amplification for detection using the capillary fluorescence detector (Fig. 1A). Conserved sequences among different virus-subtypes (as many as possible) should be selected when designing specific primers for each pathogen. However, conserved sequences are not always available. Therefore, degenerate bases can be used when some bases in the primer sequences are different or specific primers for different subtypes can be designed if it is difficult to find suitable primers. The primer information for the 13 viruses involved is available in Table S1.

During method validation, this study conducted specificity, sensitivity, repeatability and exclusivity verification, and the results showed that all 13 viruses were able to peak at their specific positions (Fig. S2). Among them, the detection sensitivity for 8 viruses was at 10 copies per reaction, for 3 viruses was at 100 copies per reaction, and for 2 viruses was at 1000 copies per reaction (Figs. 1B–E and Table S2). The repeatability and exclusivity verification for HBV as a representative were both satisfactory (Figs. S3 and S4).

Three virus samples (PPV, HSV, and HCMV) were mixed with SIV pseudovirus in equal volumes for nucleic acid extraction to verify the actual testing capability of this scheme. Each sample peak was detected at the corresponding molecular weight position after reverse transcription, PCR, and capillary electrophoresis. SIV pseudovirus signal was also detected (Figs. 1F–H). These results indicate that the established scheme can detect various adventitious viruses in practical applications.

The study's innovations lie in the PCR-CE technology's application, offering heightened sensitivity and specificity amid a background of main virus interference. This high-throughput method reduces detection time, workload, and sample usage. Introducing pseudovirus control enhances experimental reliability, particularly for RNA viruses. This research addresses a critical gap in efficiently detecting high-risk adventitious viruses during production, holding significance for both safety assessment and clinical applications.

CRediT authorship contribution statement

Guangyu Wang: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Lingli Xu:** Investigation, Conceptualization. **Lei Yu:** Writing – review & editing, Resources. **Xinchang Shi:** Writing – review & editing, Data curation. **Xi Qin:** Writing – review & editing, Data curation. **Yong Zhou:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2024.101096.

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