Application of digital PCR (dPCR) in the detection of Covid-19 in food

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ABSTRACT: Covid-19 detection in food is an effective solution to ensure the accurate detection rate of Covid-19. The difficulties and detection methods of food virus safety detection and the feasibility of digital PCR detection technology are analyzed. The main parameters and characteristics of dPCR technology and other PCR technologies are compared. The application of dPCR technology in the detection of food viruses and pathogenic bacteria, the application of dPCR technology in the preparation and purity verification of Covid-19 RNA reference material, and the steps and methods of dPCR technology in food testing Covid-19 were expounded. Compared with traditional detection methods, digital PCR technology has great advantages in virus detection limit and stability. dPCR will develop towards high flux and automation, and achieve the absolute quantification of multiple target sequences at low cost. It will help to play a crucial role in the detection of covid-19 in food.

1 Introduction

With the continuous spread of covid-19 epidemic, the host transmission network of F will become more complex, and many common poultry and livestock will inevitably be infected and spread the virus. Recently, the food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) jointly issued the interim guidelines for food enterprises to prevent and control the covid-19 pneumonia covid-19 and food safety. The guideline makes it clear that Covid-19 is not a food safety issue, but it has brought great impact and changes to the food industry. Food production, supply and consumption is a complex network system, each link must establish and implement effective good health practices and food safety management system. It is necessary to fast wild animals and increase the detection of food virus after the common poultry infected with virus can not be avoided for the time being [1,2]. In order to better detect the presence of coronavirus in food, it is necessary to integrate clinical diagnostic technology into virus detection methods, and constantly innovate and optimize the detection technology, so as to ensure the accurate detection rate of coronavirus.

2 Difficulties and methods of food virus safety detection

At present, there are the following difficulties in the detection of virus carried by food. First of all, the composition of different food is quite different. The pretreatment process of removing food impurities will greatly reduce or even lose the amount of virus carried by food, resulting in false negative detection results. Secondly, the concentration of viruses from food sources is often low, and some viruses have strong activity in vitro, so it is easy to cause infection of operators in the process of virus enrichment before detection. Thirdly, some viruses can be adsorbed on food surface for a long time without losing infectivity, which will cause cross contamination of food and reduce the reliability of positive results. In addition, in view of the characteristics of sars-cov-2 with diverse potential hosts and complex transmission network, and the route of food contamination caused by sars-cov-2 is still unclear, the detection of sars-cov-2 in food will be more difficult. The traditional methods used in food virus detection mainly include electron microscopy, cell culture, enzyme-linked immunosorbent assay (ELISA) and nucleic acid hybridization. Most of these methods have obvious limitations and can not be applied to the detection of Covid-19. Electron microscopy detection requires 10⁵-10⁶ virus particles, and the titer of virus carried by food is often difficult to reach the detection limit of this method. Cell culture method needs to culture Covid-19

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virus in vitro. This process needs to be carried out in P3 biosafety laboratory, and the high affinity between the spike protein of Covid-19 virus and human ACE 2 receptor brings great risk of infection to operators. Therefore, cell culture method is not suitable for food detection of Covid-19 virus. ELISA has the advantages of simple operation, time saving and high efficiency, but the sensitivity of this method is low, and it is difficult to meet the detection requirements of Covid-19 [3-5]. To sum up, the above traditional food virus detection methods are difficult to meet the high-efficiency detection of Covid-19 in food, so we need to refer to the existing clinical detection methods of Covid-19 to select the appropriate virus detection technology.

3 Feasibility analysis of digital PCR detection technology

3.1 Advantages of digital PCR in virus detection

With the development of molecular biology technology, the quantitative detection method of nucleic acid is also constantly updated. At present, the methods of nucleic acid quantitative detection mainly include amplification technology, branch chain DNA signal amplification technology and real-time fluorescence quantitative PCR technology. In recent years, the new method of nucleic acid quantification, digital PCR, has been widely used in the absolute quantitative detection of samples, which provides a new method for nucleic acid quantification [6-8]. Digital PCR (dPCR) is a standard PCR reaction system, which is distributed to thousands of small PCR reaction systems. As far as possible, each PCR reaction hole contains "1" or "0" templates, and then PCR amplification is carried out on the PCR instrument; According to the presence and absence of fluorescence signal in PCR results, the number of copies of samples was counted. Finally, the copy number of samples was obtained by Poisson statistical method. From the first in vitro amplification of DNA in 1971 to the invention of the third generation PCR technology, dPCR in 1999, PCR technology has been developed rapidly and comprehensively, and has been widely used in the detection of biology, and has shown its advantages in virus detection [9,10]. In recent years, with the maturity of microfluidic technology, digital PCR technology based on microfluidic technology has been developed rapidly. Combining microfluidic technology with digital PCR technology can avoid the complicated sample dispersion operation, and form large-scale independent reaction units automatically and high-throughput in a short time. It can simplify operation, shorten the experimental cycle,

and significantly improve the accuracy and sensitivity of quantitative analysis results.

As a new generation of nucleic acid quantitative technology, dPCR can achieve absolute quantitative of micro target gene without interference of matrix. Microfluidic technology has gradually become the main technology platform of digital PCR analysis system. In the quantitative analysis process, digital PCR does not need to rely on the standard curve without reference materials, and has high accuracy, and the sensitivity is better than traditional methods and qPCR methods, which can reach 10 times or higher than that of qPCR; In a certain range of tests, the linear relationship is good, the standard deviation is small, the accuracy is high, and the repeated experimental results of different laboratories or testing institutions fluctuate little [11,12]. As one of the most reliable methods in molecular quantitative detection, it has been studied in the fields of copy number quantification, microbial detection, inspection and quarantine, medical diagnosis research, gene detection, resource and environmental science, and has great potential in food related detection. At present, the research has used digital PCR to quantitatively adenovirus, hepatitis B virus, HIV virus, influenza A virus, etc. The results show that the micro drop PCR can detect the samples effectively and monitor the virus load more accurately.

3.2 Comparison of digital PCR and other PCR techniques

Polymerase chain reaction (PCR) is a widely used method in molecular biology research, mainly including PCR, real-time fluorescent quantitative PCR (qPCR), digital PCR, reverse transcription PCR (RT-PCR), etc. [13]. qPCR is a commonly used qualitative and quantitative gene analysis technology, but this method needs to use standard products, draw standard curve and use cycle threshold to analyze the results, which not only increases the workload and detection cost, but also causes certain errors in the actual detection process [14-17]. Compared with the traditional qPCR technology, digital PCR technology has high sensitivity, accuracy and specificity, especially suitable for detecting rare gene mutation, subtle copy number variation and absolute quantification of nucleic acid. As a new technology, digital PCR technology has been widely used in many fields such as precision medicine, microbiological detection and food safety [17-19]. At present, a number of commercial digital PCR instruments have appeared in the market, which has a positive role in further expanding and deepening the application of digital PCR technology. The comparison between dPCR and other PCR techniques is shown in Table 1.

Table 1 Comparison of dPCR, qPCR and PCR techniques

	dPCR	qPCR	PCR
Quantitative method	absolute quantitative	Relative quantitative / absolute quantitative	Qualitative / semi quantitative
Principle	The PCR reaction system was	The amount of amplified products	The end point PCR results

	separated and amplified by PCR. The copy number of samples was directly obtained by counting the number of positive and negative reactions.	was proportional to the fluorescence signal intensity. The samples were quantified by the cyclic threshold and standard curve of the reaction.	were obtained by gel electrophoresis.
Scope of application	Absolute quantification of viral load; Copy number variation analysis; Detection of rare mutations; Gene expression was quantified; Second generation sequencing library analysis.	Detection and quantification of pathogens; Relative detection of gene expression; Single nucleotide polymorphism analysis; The detection of tumor markers.	Establishment of cDNA library; Detection of pathogens; Phylogenetic and evolutionary studies; DNA mutation; Diagnosis of diseases; Genotyping, etc.
Amplification efficiency	No impact	Influence	Influence
Dynamic range	4-6 logs	6-9 logs	< 2 logs
Advantage	Absolute quantification, no need of standard curve, high sensitivity and stronger tolerance to PCR inhibitors.	The detection has wide dynamic range, wide application range and low cost.	It has the advantages of simple operation, low detection cost, wide application range, and can meet therequirements of various detection.
Inferiority	The dynamic range of detection is small, false positive is easy to occur, and the cost is high.	The amplification efficiency is easily affected by PCR inhibitors.	The agarose gel involves the insertion of dyes into toxic nucleic acids with low sensitivity.

4 Application of PCR in food pathogen virus detection

At present, the common methods of virus detection are mainly to detect the protein or specific nucleic acids by immunoassay. As the latest molecular biological detection technology, dPCR is not necessary to establish standard curve and not affected by matrix effect, and it is suitable for quantitative detection of virus molecules, especially low copy virus. Since the commercialization of dPCR products, the application of dPCR to the detection of viruses, especially the quantification of viral load has been a hot topic in recent years. DPCR, influenza virus, enterovirus, bluetongue virus, porcine epidemic diarrhea virus and infectious spleen and kidney necrosis virus have been quantitatively analyzed in the literature. In order to compare the performance of RT-qPCR and rt-dPCR, Coudray meiner and others used the two methods to detect Norwalk virus and hepatitis A virus in water and lettuce. Through sensitivity analysis, RT-PCR can detect lower virus copies; In order to further analyze the tolerance of complex matrix, the results of labeling lettuce samples showed that the recovery of RT-PCR was significantly higher than that of rtqpcr. Pavšič The results showed that the results of direct detection were more repetitive and closer to the real load of the virus [20,21]. Lin Yingchi et al. Established a method of digital PCR to detect hepatitis A virus in shellfish and berry. Methods after the samples were enriched by protease K digestion polyethylene glycol method, RNA was extracted by high purity virus nucleic acid kit, and then the hepatitis A virus was detected by digital PCR. Results the method has a typical amplification, good repeatability and stability. The sensitivity of detection of hepatitis A virus in strawberry samples is 25.30 ccid50/20 g, that of raspberry samples is 6.32 ccid50/20 g, and that of shellfish samples is 12.54

ccid50/2g, indicating that it is highly sensitive. Zhang Li Li et al. Established the ddPCR technology as the key index to accurately detect the SIV DNA load in the cells and tissues of the animal model of AIDS, and used for real-time dynamic evaluation of hidden virus in the storage. Methods according to the mature qPCR method, the reaction conditions of the microdrop PCR were optimized to determine the optimal annealing temperature; The detection range of ddPCR was determined by the detection of 10 times diluted PGEM sivgag477 plasmid standard. By several DNA samples, the stability of the method is judged, and the absolute quantitative method of SIV virus DNA based on micro drop PCR is established. It can effectively quantitatively the viral DNA load in the virus storage samples in cells and tissues, greatly improving the accuracy of the model application evaluation [24].

5 Steps and methods of dPCR technology for Covid-19 detection in food

The main steps of micro drop PCR are as follows:

5.1 Sampling method for surface samples of food and edible agricultural products

5.1.1 Water / seafood (including live aquatic animals) surface

In addition to the external surface of the animal, the swab should also be applied in the natural pores such as oral cavity, gill, cloaca and shell; For the aquatic products that have been divided, the cross section of aquatic products should also be smeared.

Take the disposable long handle swab soaked with sterile normal saline or PBS, or open the sampling

reagent package, take out the sampling swab, put the swab into the sampling solution of the sampling tube, apply the swab horizontally and vertically at the sampling position selected on the packaging surface for more than 5 times.

Insert the swab into the sampling tube filled with 3.0ml sampling solution. When the swab is about 1cm from the bottom of the sampling tube, bend and break the swab handle, cover the cover of the sampling tube, record and complete the sampling.

5.1.2 Surface of animal products

For the carcass of animals, the surface of the carcass should be smeared and sampled. Besides the external surface of animals, the smearing scope of the sampling swab should also be deep into the oral cavity and other detectable cavities; For segmented animal products, eggs and animal fats, the samples should be directly smeared on the surface.

5.1.3 Plant food surface

Smear and sample the surface including the surface gap.

The disposable long handle sampling swab soaked with sterile normal saline or PBS was smeared on the surface of each part vertically and horizontally for 5 times, and then the sampling swab was rotated.

Cut off the hand contact part and put it into the sampling tube with 3.0ml sampling solution.

For acidic or soft fruit (such as strawberry) surface sampling. The specific method was as follows: 25 g sample was randomly selected and transferred to a sterile bag, 40ml the buffer solution (Tris matrix 12g, glycine 3.8g, beef extract 10g, dissolved in 1000 ml water, pH9.5) was added, and the room temperature was shaken for 20minutes. The suspension was transferred to a clean 50ml centrifuge tube for subsequent RNA extraction.

5.1.4 Inner and outer packaging surface

If the surface area is less than 100cm^2 , take all the surfaces; If $1000\text{cm}^2 \geq \text{surface area} \geq 100\text{cm}^2$, take 100cm^2 , if the surface area is greater than 1000cm^2 , it can be sampled for many times, and the total sampling area is not less than 10% of the surface area. Focus on the frequently contacted parts.

5.2 RNA extraction of samples

RNA extraction kit was used for RNA extraction of samples, and the samples were dissolved in rnasefree water.

5.3 Sample and reaction system preparation

The one-step reverse transcription micro drop digital PCR kit used in the micro drop digital PCR experiment includes PCR reaction solution, reverse transcriptase and DTT (dithiothreitol).

5.4 Droplet formation

Qx200 micro droplet generator can generate 8 samples of micro droplets at a time, and it takes about 2 minutes to generate 8 samples of micro droplets.

5.5 Preparation of PCR reaction

After the transfer of the droplets, in order to avoid pollution, the sealed 96 well plate was placed on Eppendorf PCR instrument for PCR amplification.

5.6 The results were analyzed by quantasoft software

The number of positive, negative and total droplets detected by the droplet reader in the selected reaction well can be viewed. The total number of droplets in a single reaction pore is an important index to evaluate the quality of the experiment. Generally, the average total number of droplets in each reaction is more than 12000, so the experimental reaction is better; If the number of micro drops is less than 10000, it indicates that the number of micro drops generated in the experiment is small, so the experiment needs to be repeated.

5.7 Precautions for experimental operation

5.7.1 Optimization of annealing temperature for micro drop digital PCR

The annealing temperature is the most important parameter affecting the PCR reaction, which has an important impact on the specificity of the reaction. Too low annealing temperature may cause non-specific amplification, and too high annealing temperature will reduce the sensitivity of the reaction. In order to determine the optimal annealing temperature, according to the annealing temperature range required by the reagent, it is necessary to detect the annealing temperature and select the optimal annealing temperature.

5.7.2 Determination of detection range of micro drop dPCR

In order to determine the detection order of micro drop digital PCR, RNA nucleic acid standard was used for detection. The detection range of RNA nucleic acid standard was quantified by UV spectrophotometer. The experiment was repeated for three times[22,23].

6 Conclusion

Compared with traditional detection methods, digital PCR technology has great advantages in detection limit, stability and so on, and has great potential in food quantitative and qualitative analysis. As a new detection method, dPCR has some inherent limitations and shortcomings. However, with the development of

manufacturing technology, the optimization of digital PCR instrument and the professional and systematic training of testing personnel, digital PCR technology can provide more possible methods and solve more problems for scientific research and practical application in the future. In the future, dPCR will build a highly integrated dPCR detection platform, with only one instrument to complete the whole detection process. While ensuring the accuracy of the system, dPCR will develop towards the direction of high-throughput and automation, and realize the absolute quantification of multiple target sequences at the same time with low cost[24,25]. With the improvement of dPCR detection technology and the continuous development of commercial instruments, the accuracy and sensitivity of dPCR will be higher and higher, which provides an efficient and feasible means for food safety detection and food quality control, and is of great significance for the formulation of food detection standards and the prevention of foodborne diseases.

Acknowledgments

This work was supported by the Characteristic innovation project (NATURAL SCIENCE) of regular college in Guangdong Province (2018ktszx270).

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