Multiplex and On-site PCR detection of swine diseases based on the microfluidic chip system

Yan Jiang

Nanjing Customs

Shan Jiang

Nanjing, Customs

Yue Wu

Nanjing Agricultural University

Bin Zhou (✉ zhoubin@njau.edu.cn)

Nanjing Agricultural University <https://orcid.org/0000-0001-7279-9489>

Kaiming Wang

Nanjing, Customs

Luyan Jiang

Nanjing Customs

Yunfeng Long

Nanjing Tech University

Gan Chen

Jiangganshan Agricultural Science and Technology Park Management Committee

Dexin Zeng

Nanjing, Customs

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Abstract

Background At present, the process of inspection and quarantine starts with sampling at the customs port, continues with transporting the samples to the central laboratory for inspection experiments, and ends with the inspected results being fed back to the port. This process had the risks of degradation of biological samples and generation of pathogenic microorganisms and did not meet the rapid on-site detection demand because it took a rather long time. Therefore, it is urgently needed to develop a rapid and high-throughput detection assay of pathogenic microorganisms at the customs port. The aim of this study was to develop a microfluidic chip to rapidly detect swine pathogenic microorganisms with high-throughput and higher accuracy. Moreover, this chip will decrease the risk of spreading infection during transportation.

Results: A series of experiments were performed to establish a microfluidic chip. The resulting data showed that the positive nucleic acid of four swine viruses were detected by using a portable and rapid microfluidic PCR system, which could achieve a on-site real-time quantitative PCR detection. Furthermore, the detection results of eight clinical samples were obtained within an hour. The detection limit of this microfluidic PCR detection system was as low as 1 copies/ μ L. The results showed that the high sensitivity and specificity of this chip system in disease detection played an important role in customs inspection and quarantine during customs clearance.

Conclusion: The microfluidic PCR detection system established in this study could meet the requirement for rapid detection of samples at the customs port. This chip could avoid the risky process of transporting the samples from the sampling site to the testing lab, and drastically reduce the inspection cycle. Moreover, it would enable parallel inspections on one chip, which greatly raised the efficiency of inspection.

Background

Polymerase chain reaction (PCR) is a molecular detection technology based on nucleic acid sequences [1]. Compared with the traditional detection methods, such as immunoassay, PCR is more time-saving, sensitive and accurate. After nearly 40 years development, the PCR molecular detection technology has become the primary method for detecting diseases caused by pathogenic microorganisms, and has been widely used in disease detection [2,3], clinical testing [4,5], food quarantine [6,7], forensic identification [8,9], customs inspection [10] and in various other fields [11–14]. The traditional PCR instrument is large in size and inconvenient to carry; and it has a higher demand for the experimental environment, a special biological laboratory and special laboratory technicians; and its reaction is in a larger volume, consumes more energy, and is not easy to integrate. These drawbacks of this instrument seriously limit its scope of use, and prevent its operation in rapid on-site real-time PCR detection [15].

Porcine circovirus type 2 (PCV2) was first discovered in Canada in 1997. It often causes weaning piglets with multiple system failure syndrome (PMWS) and was tested using quantitative PCR and antigen

capture ELISA [16]. Porcine Reproductive and Respiratory Syndrome virus (PRRSV) mainly causes reproductive disorders in pigs and first isolated in China in 1996. Nucleic acid sequence-dependent amplification technology is a new type of RNA amplification technology. Some researchers used this technology to design primers for the nsp2 gene encoded by ORF1 and established a NASBA diagnostic method [17]. Porcine epidemic diarrhea virus (PEDV) was first discovered in England in the 1970s and has spread to other European and Asian countries. Multiplex PCR is commonly used in clinical practice to differentiate between this virus, rotavirus and porcine epidemic diarrhea [18]. Pseudorabies virus (PRV) can cause reproductive disorders at any stage of pregnancy, piglets often show chin and respiratory symptoms. Some researchers have designed primers for gE and gB genes to establish gene chip detection methods to distinguish between field and vaccine virus [19].

The development of the microfluidic technology has contributed to the emergence of the microfluidic chip which is now commercially mass-produced. The microfluidic chip [20–22], also known as lab on a chip, combines and arranges various unit technologies on a small platform to implement all the biochemical laboratory experiments including sampling, sample introduction, sample preparation, reaction, product testing, and so on [23,24]. The fundamental feature of the microfluidic chip is the integration of functional modules. The major advantages of this technology are high efficiency, high density, high throughput and low consumption [25–28]. Compared with the traditional PCR method, the microfluidic chip is much smaller in size and more portable along with much less reagents. It, changed temperature more rapidly, integrated with other devices more easily, and had more moderate demands for experimental environment and lab assistants. All these advantages brought the microfluidic chip into the focus of research on rapid on-site real-time PCR detection [29–32].

Pork and its processed products, as a traditional food, have become an indispensable part in people's diet [33]. China Customs, as the only pass for import and export of goods and commodities into and out of the country, is responsible for the inspection and quarantine of tens of thousands of pigs, and large amounts of pork and its processed products; which calls for rapid, efficient and safe work. At present, the process of inspection and quarantine starts with sampling at the customs port, continues with transporting the samples to the central laboratory for inspection experiments, and ends with the inspected results being fed back to the port. This process has the risks of degradation of biological samples [34] and generation of pathogenic microorganisms [35] because it takes a rather long time. Also, it does not meet the rapid on-site detection demand [36]. Therefore, development of a technology for rapid and high-throughput detection of pathogenic microorganisms at the customs port is of great significance. This study was to develop a microfluidic chip to be applied to rapid and high-throughput swine disease detection with higher accuracy and lower risk of spreading pathogenic microorganisms during transportation.

Methods

Reagents and equipment. The magnetic bead virus nucleic acid extraction kit was purchased from Suzhou Tianlong Bio-Technology Co. Ltd. The reverse transcriptase was from Vazyme Biotech Co. Ltd.

(Nanjing), and the Taq DNA polymerase was from Fapon Biotech Inc. (Zhuhai). The primers and probes used in common epidemic swine diseases detection were synthesized by Genscript (Nanjing) Co., Ltd, and the detailed sequences are shown in Appendix Table 1. The microfluidic PCR analyzer was purchased from Shenzhen Shineway Technology Corporation and the fluorescence quantitative PCR instrument was from Hangzhou Bioer Technology Co. Ltd.

Nucleic acid extraction and amplification. The nucleic acid of the pathogenic microorganisms were extracted following the instructions of the magnetic bead nucleic acid extraction kit, and the extracted DNA solution was used as the template solution for the subsequent experiments, and was stored at -20 °C in a refrigerator; or at 4°C in a refrigerator or on ice in case of immediate use in a subsequent experiment. In the nucleic acid amplification, a 25 µl reaction solution was prepared with the following components: 5 µl template solution, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 0.2 µl probe (10 µM), 0.2 µl reverse transcriptase, 0.5 µl Taq DNA polymerase, 5 µl 5X reaction buffer, and 13.1 µl sterilized water. All these components were fully mixed in a PCR tube and added to the microfluidic chip by a pipette, then PCR amplification was performed. The process of the amplification included Step One: reverse transcription at 50°C for 15 minutes; Step Two: denaturation at 95°C for 3 minutes; and Step Three: 40 cycles at 95°C for 15 seconds and at 55°C for 45 seconds.

Chip design and preparation. There were 8 reaction chambers with a volume of 2 µl each on the PCR microfluidic chip. The PCR microfluidic chips were made of silicon wafers of 500 µm thick with both sides polished and heat-resistant glass substrates of 500 µm thick by Shenzhen Shineway Technology Corporation. The PCR reaction chambers and channels on the silicon wafers were formed by dry etching using a semiconductor photolithography process. Then, sixteen holes were drilled on the silicon substrate which served as the inlets and outlets of PCR solutions, respectively. Finally, the silicon wafers were bonded to the glass substrates and the two-layer wafers were cut by a dicing saw to form independent PCR detection chips.

a standardized semiconductor fabrication process was utilized to ensure the yield rate and uniformity in quality of the chips. And the automated fabrication process could significantly reduce the cost of each single inspection by expanding the size of the silicon wafers.

The amplification detection system of the PCR microfluidic chip. A portable rapid microfluidic PCR system (SWA-01, Shenzhen Shineway Technology Corporation) was used to perform the real-time PCR amplification of the samples (Fig. 1). The dimension was 330mm×L×320mm×W×160mm×H and the weight was round 3 kg, suggesting it had a small footprint. Used in conjunction with the specially supplied reagents and the microfluidic chips, the SWA-01 PCR instrument is applicable to rapid real-time fluorescence detection of PCR in the fields of animal quarantine, food safety and public health and safety. The portable rapid microfluidic PCR instrument was mainly composed of a control system (with a touch screen graphic user interface), a power supply system, a photoelectric system, a temperature control system [35], an outer jacket part, a microfluidic chip, and a software module. The instrument had

the following advantages for on-site detection as follows: portable design, low weight, rapid thermal cycling and real time detection.

Results

The sensitivity test using plasmid sample. In the present study, we first tested the sensitivity of the PCR reagents in the amplification on the traditional PCR instrument using the standard plasmid sample. The plasmid sample was 10-fold serial diluted and was detected on the fluorescence quantitative PCR instrument, Bioer, Hangzhou. As shown in Fig. 2, the result showed that the detection limit of the PCR reaction was 1 copies/ μL ; and the linear correlation coefficient of its dynamic range and the Ct value was 0.9992. It could hence be concluded that the reagent of the PCR detection met the requirements for on-site rapid screening of positive nucleic acids in terms of the detected sensitivity and dynamic range.

The detection limit testing using plasmid sample. We then chose four principal swine viruses, including PRRSV, PEDV, PRV and PCV2 (positive viral nucleic acid was offered by Nanjing Agricultural University), which are often found in related inspection at the customs, and we synthesized the plasmids of these viruses as positive references. The concentrations of the primers and probes, and the reaction temperatures were optimized by means of the plasmid amplifications on the PCR microfluidic chips. The microfluidic chip had 8 chambers, each chamber accommodating 2 μL of the PCR reaction solution. The volume of the amplification system was reduced that was helpful in the rapid PCR process. The rapid heat transfer kept the activity of the Taq DNA polymerase at the maximum level during the rise and fall of temperature. The microfluidic chip with silicon as its substrate further accelerated the heat transfer during the PCR thermal cycle, because of silicon's excellent thermal conductivity. It was obvious that the amplification reaction's fluorescence intensity on the chip presented itself in a typical S-shaped curve, and the negative control chamber, in which sterilized water was used as the template, did not show any amplification, indicating that the specificity of the primers and the probe, and the optimization of the amplification temperature were all appropriate here.

The initial concentration of the four plasmids was 10^9 copies/ μL . All the plasmids were 10-fold serial diluted to the final concentration of 1 copies/ μL , and every concentration was tested. As shown in Fig. 3, the sensitivity test of the four positive plasmids were 10 copies/ μL for PRRSV, 10 copies/ μL for PEDV, 100 copies/ μL for PRV and 1 copy/ μL for PCV2, respectively, which would meet the requirement for detection in terms of the virus load obtained from the clinical samples of the animals.

The specificity testing using positive nucleic acid. Besides the sensitivity test, we also verified the specificity performance of the PCR detections on the microfluidic chip. Based on the positive nucleic acids of four viruses isolated from the clinical sick pigs, which performed the typical symptoms of reproductive disorders, multi-system failure of weaned piglets, and watery diarrhea. We chose one sample as the target for the specificity test, and the other three samples were used as the control. The PCR amplifications were performed on one chip with one reaction chamber filled with the target sample, the rest of the chambers filled with the other three samples as well as other two porcine-transmitted viral

agents including porcine transmissible gastroenteritis virus (TGEV) and porcine rotavirus (PRVA). As shown in Fig. 4, the result showed that the four PCR protocols all had a very high specificity, because only the target sample presented a significant amplification curve and the control samples had no amplifications. The fast amplification process of the PCR system contributed not only to the high specificity of the PCR amplification but also the short duration in the non-required temperature zone.

The detection performance of the microfluidic PCR system using positive nucleic acid. Finally, we verified the PCR microfluidic chip detection performance using the positive nucleic acid of swine pathogens. Every two chambers on the chip were used for the reaction of one clinical sample, and one chip accommodated four samples in parallel reactions. At the same time, we also performed the detection for the viruses of four swine diseases on a traditional tube-based PCR instrument for parallel reference. As shown in Fig. 5A, each of the four samples had a significant S curve with a varied Ct value (17.5 for PRRSV, 33.5 for PEDV, 18.5 for PRV and 16.5 for PCV2, respectively). The Ct values of the four swine diseases tested on chip-based and tube-based PCR platform showed no significant differences. The differences in the Ct values were due to the variety in the virus abundance of each of the original positive nucleic acid. The magnitude of the fluorescence intensity increment in the different positive nucleic acid at the end of the reaction was due to the different amplification efficiency of primers and probes for different target gene sequences. However, the Ct value was usually used as a criterion when determining the positive or negative nature of the sample. The sample with a Ct value smaller than 35 could be considered as positive threshold, and the sample with a Ct value greater than 35 needed to be re-examined.

Discussion

The aim of this study is to develop a fast and on-site swine disease detection solutions to decrease the transit time for customs clearance. Point of care testing could be the optimal choice for the on-site detection and PCR technology as the gold standard for infectious disease detection has been applied. However, the current PCR performance still need sample transportance to testing lab and the resulting data could not been get in less than 3 h. Therefore, a portable and miniaturized PCR detection platform is needed to develop. Herein, the microfluidics chip based PCR testing solution along with short testing duration, multiple testing capability and instrument portability was established for diagnostic assay.

Compared to traditional lab-based PCR detection that must be finished in the central lab, our portable PCR tester could realize an on-site detection for swine diseases and a rapid result observation. In addition, four swine pathogens could be detected on a single chip simultaneously. A rapid thermal cycling capability of the PCR tester with the help of microheater realized a shorter detection time, which is helpful for a fast custom clearance and also for an efficient disease control to decrease the further spread risk of infection disease. In order to realize an on-site molecular test, many factors need to be considered, including testing duration, instrument portability, operation complication, contamination and so on. A real time PCR method may avoid the contamination of this process and need no more offline analysis because of. a miniaturization of the optical detection module for a fluorescent detection. Around 1-

10copies/uL detection limit on chip could meet a fast screening demand and has a fine accordance with that of the traditional PCR instrument. As for the specificity of this method, no cross reaction occurred when more than one PCR detection performed in a single chip well, suggesting that the PCR reagent component had a good compatibility with the chip surface. Additionally, a fast thermal cycling could increase the PCR enzyme amplification efficiency. Therefore, this chip is helpful for the further development of a multiple optical channel instrument for high-throughput multiplex PCR detection. In the future, a more high-throughput chip that could realize a more than 16 samples testing on a single chip will be developed. The further study needs to develop a automated sample preparation system for nucleic acid extraction and purification, which will decrease the on-the-spot detection duration.

Conclusions

In this study, the microfluidic PCR detection system had successfully detected 10 copies/ μ L of PRRSV, 10 copies/ μ L of PEDV, 100 copies/ μ L of PRV and 1 copies/ μ L of PCV2, which showed good sensitivity and specificity as for the samples of positive nucleic acid. All the detection were completed within 1 h. The microfluidic PCR detection system established metted the requirement for rapid detection of samples at the customs port [36,37]. In the case of the infectious diseases of swine, application of this chip would effectively help the on-site inspection to be carried out. Thus, it could avoid the risky process of transporting the samples from the sampling site to the testing lab and drastically reduce the inspection cycle. Moreover, it would enable parallel inspections on one chip, which greatly raised the efficiency of inspection. All these provided a strong technical support for rapid customs clearance. In the future, the PCR microfluidic chips with more reaction chambers (16 and 32 channels) will be developed to accommodate more samples for simultaneous detection, along with higher throughput, greater efficiency and accuracy.

Abbreviations

PCR: Polymerase chain reaction

Declarations

Competing Interests

The authors declare that they have no competing interests.

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Availability of data and materials

Raw data is available from the corresponding author on reasonable request.

Authors' contributions

YJ designed and directed the study. SJ,LYJ,YFL and YJ perform the experiment ,collect results and analyze data. YW and BZ draft the first vision of the manuscript. KMW, GC and DXZ collect clinical serum samples. BZ critically revised the manuscripts. All the participants carefully reviewed this vision manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable Competing interest.

Author details

1 Animal ,Plant and Food Inspection Center, Nanjing Customs;

2 MOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China

3 Jinggangshan Agricultural Science and Technology Park Management Committee

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Table 1

Due to technical limitations Table 1 is available as a download in the Supplementary Files.

Table 1. The sequences of the primers and probes of the four swine diseases.

F=the forward primer; R=the reverse primer; P=the probe.

Figures

Figure 1

(a) The portable rapid microfluidic PCR system; (b) Size comparison of the microfluidic PCR instrument (within the red dotted box) with the traditional large PCR instrument (Roche 480); (c) Demonstration of the portable design of the instrument.

Figure 2

(a) The sensitivity test of the portable rapid microfluidic PCR system; (b) The linear correlation curve of the dynamic range.

Figure 3

Sensitivity test of the PCR protocols of the four swine disease viruses.

Figure 4

Specificity test of the four swine disease viruses on the chip.

Figure 5

Parallel detection results of four clinical samples of swine diseases on the 8-chamber microfluidic chip (a) and on traditional tube-based PCR instrument (b) .

Supplementary Files

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