Epidemiological analysis of Porcine Viral Diarrhea Pathogens in Local Area

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DOI: 10.21203/rs.2.24785/v1

SUBJECT AREAS
Virology

KEYWORDS
Porcine viral diarrhea, Luminex xTAG multiplex detection method, Clinical diarrhea sample detection, Multiple pathogen infection
Abstract

Background
Porcine viral diarrhea can cause great damage to the pig industry and high mortality to piglets. Furthermore, multiple pathogen infections and synergistic infections commonly existed in clinic. This has resulted in great difficulties in determining the main pathogenic factors, which would delay the prevention and control of diseases.

Methods
A total of 518 porcine stool specimens were collected from 9 pig herds in Shanghai, China from 2015 to 2017 and used for pathogen detection. A Luminex xTAG multiplex detection method was developed for the detection of 11 viral diarrhea pathogens, which allows for the simultaneous qualitative and quantitative detection of viral diarrhea pathogens in clinical samples.

Results
The minimum detection rate of the Luminex xTAG multiplex detection method was at least 10 times higher than the traditional PCR method. As a result, 209 (40.3%) were positive for porcine kobuvirus (PKoV), 138 (26.6%) for porcine astrovirus (PAstV), 91 (17.6%) for porcine epidemic diarrhea virus (PEDV), 78 (15.1%) for porcine sapelovirus (PSV), 68 (13.1%) for porcine sapovirus (PoSaV), 27 (5.2%) for porcine teschovirus (PTV), 25 (4.8%) for porcine deltacoronavirus (PDCoV), 17 (3.3%) for porcine rotavirus (PoRV), 16 (3.1%) for transmissible gastroenteritis virus (TGEV), 10 (1.9%) for porcine torovirus (PToV), and 9 (1.7%) for bovine viral diarrhea virus (BVDV), respectively. Furthermore, multiple infection rate of diarrhea sample was 17.57% for dual-infection, 11.58% for triple-infection, 4.63% for quadruple-infection, 0.77% for quintuple-infection, 0.58% for sextuple-infection and septuple-infection, respectively. Infection pattern of the viral diarrheal pathogens was changing, and different farm had the various diarrhea infection patterns, which proved the great importance of epidemiological surveillance and the guidance effect to clinical production. PoSaV, PoRV, PAstV, PToV and PEDV were indicated as the predominant viruses of clinical samples collected in 2017 by the quantitative analysis.

Conclusions
Here we provide a Luminex xTAG multiple detection method for viral diarrhea pathogen infection in clinical, which was more sensitive and specific than general multiplex PCR method. Furthermore, the surveillance confirmed the complicated infection status in China, which demonstrated the need for continuous surveillance and provided data for the prevention and control of viral diarrhea.

Introduction

In recent years, viral diarrhea in pig herds has led to serious problems with clinical symptoms of diarrhea, vomiting, and dehydration, thereby affecting pig growth and leading to huge economic loss [1]. This disease increases the infection rate to piglets and mortality, which could reach up to 100% [2]. Furthermore, multiplex infection and synergistic infection, which are commonly observed in clinic, pose a new challenge to disease diagnosis and control. The viruses that cause porcine diarrhea disease are diverse, including porcine epidemic diarrhea virus (PEDV) [3-5], porcine deltacorona virus (PDCoV), porcine transmissible gastroenteritis virus (TGEV) [6], porcine teschivirus (PTV), bovine viral diarrhea virus (BVDV), porcine rotavirus (PoRV), porcine sapelovirus (PSV), porcine kobuvirus (PKoV), porcine astrovirus (PAstV), porcine torovirus (PToV), porcine sapovirus (PoSaV), etc. An infection with any of these viruses can develop into similar clinical symptoms, including severe diarrhea and dehydration, and in some cases, multiplex infections of these pathogens are common. However, it remains difficult to distinguish these in clinic [4, 16-18].

Although regular vaccine immunization has been strictly conducted, the high morbidity of diarrhea remains as a serious problem, which needs to be solved in time. Therefore, the epidemiology of diarrhea viruses need to be investigated, in order to identify the dominant viruses. Thus, the surveillance of porcine is warranted to better understand the evolution in the field. Laboratory detection methods include virus isolation, enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). However, virus isolation needs long period and complicated operations, and ELISA is mainly used for epidemiological survey. Therefore, it is important and urgent to establish a detection method with high specificity and high sensitivity, which can meet the needs of multiple pathogen detection at the same time. As the multiplex reverse transcription-polymerase chain reaction (RT-PCR) method has high sensitivity, specificity and could simultaneously detect a
variety of pathogen, it is more suitable for rapid diagnosis of multiplex infection. Therefore, in this study, a multiple detection method for viral diarrhea was developed.

The precise data about the prevalence of multiple infections and the genetic diversity of the virus in porcine and wild boars have only been reported in a limited number of countries [10, 11]. Moreover, the prevalence and multiplex infections of porcine diarrhea in Shanghai have never been studied. Therefore, the epidemiology is needed to determine the prevalence and extent of genetic diversity in these circulating strains, in order to develop vaccination programs and establish a surveillance system.

The present study focuses on the epidemiology of porcine viral diarrhea, using multiplex RT-PCR method to investigate the prevalence of multiple diarrhea viruses and identify the dominant ones. Furthermore, the multiplex infection situation was analyzed, which will pave the way to improving strategies to prevent and control virus infection in swine farms.

Materials And Methods

Specimens

A total of 518 porcine stool specimens were collected from 9 pig herds in Shanghai, China from 2015 to 2017. Pigs of all ages were sampled and 1 to 3-week-old piglets were particularly collected. Antibiotic treatment was invalid for all sampled pigs. Each sample was suspended in phosphate-buffered saline (PBS) containing 1,000 U/ml penicillin and 1,000 U/ml streptomycin and centrifuged at 12,000 r.p.m. for 10 minutes. A portion of the suspension was used for RNA extraction, while the remaining supernatants were stored at -70 °C.

Establishment of Luminex xTAG multiplex detection method

According to the conserved sequences in GenBank, DNASTar and Oligo7 software were used to design the PCR primer pairs of 11 pathogens. The M gene of PToV, M gene of PDCoV, RDRP gene of PAstV, 3D gene of PKoV, RDRP gene of PSV, 3D gene of PTV, RDRP gene of PsaV, 5' URT gene of BVDV, M gene of PEDV, N gene of TGEV and VP6 gene of PoRV were as target genes. Spacer C12 was added between the 5' end of all upstream primers and the 3' end of the anti-TAG sequence, and the 5' end of all downstream primers was biotinylated (Biotin-) tag; selecting TAG microspheres complementary to anti-TAG sequence; hybridizing biotinylated tagged amplification product with TAG microspheres,
and obtaining the hybridization product for liquid-phase chip detection, and detecting the detection result according to the MFI value.

Detection of enteric pathogens by qualitative test
According to manufacturer’s instructions, total RNA was extracted from the fecal supernatant using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). Viral RNA was converted to cDNA using reverse transcriptase M-MLV (TaKaRa, Dalian, China) in a final volume of 20 µL containing 5 µL RNA, 4 µL 5 × RT buffer, 2 µL dNTPs (10 mM), 2 µL 10 µM random primer, 0.5 µL MLV reverse transcriptase, 0.5 µL RNase inhibitor (TaKaRa, Dalian, China), and 6 µL diethylpyrocarbonate (DEPC)-treated water. The reaction was incubated at 42 °C for 1 hour, followed by incubation at 70 °C for 15 minutes. Afterwards, the cDNA was screened by polymerase chain reaction (PCR) using the method we had established.

Quantitative determination of viral nucleic acid content
In order to identify the dominant infection agents in the multiplex-infection samples, 173 diarrhea samples collected in 2017 were further quantitatively detected using the Luminex xTAG multiplex detection method. Quantitative analysis was based on the standard curves of the above 11 viral diarrhea pathogens and viral level of each pathogen was calculated the by measuring the MFI value of each sample. According to the nucleic acid content and heat map analysis, the predominant viruses in the multiplex infection samples were speculated.

Results
Establishment of Luminex xTAG multiplex detection method
Based on a single detection system, multiple detection systems were multiplex to establish a Luminex xTAG multiplex detection method for the simultaneous detection of 11 diarrheal viruses. The optimal of hybridization system and reaction conditions were as follows: 20µL microsphere working solution, 5µL PCR amplification product, and 75µL SAPE report buffer; the results of optimal hybridization temperature was 42 °C, and the best hybridization time was 30 min. The Luminex xTAG assay method specificity was test, which showed that each primer pair had good specificity and there was no cross-reaction between the primer pairs. The sensitivity test of the Luminex xTAG detection method showed that the minimum detection lines of this method were: PTV of $3.12 \times 10^3$ copies/µL, PKOV of $2.92 \times$
10^3 \text{ copies/µL}, \text{PDCoV of } 2.79 \times 10^3 \text{ copies/µL, PSV of } 3.37 \times 10^3 \text{ copies/µL, PSaV of } 2.7 \times 10^3 \text{ copies/µL, PAstV of } 3.02 \times 10^3 \text{ copies/µL, PToV of } 2.65 \times 10^3 \text{ copies/µL, PoRV of } 2.57 \times 10^3 \text{ copies/µL, PEDV of } 1.74 \times 10^3 \text{ copies/µL, BVDV of } 2.41 \times 10^3 \text{ copies/µL, TGEV of } 2.75 \times 10^3 \text{ copies/µL. The minimum detection rate was at least 10 times higher than the traditional multiplex PCR method. Specific tests showed that each primer pair had good specificity and there was no cross-reaction between the primer pairs.}

**Viral pathogens infected in diarrhea stools were diversified**

A total of 518 porcine stool specimens from five districts in Shanghai were detected using multiplex RT-PCR method. Results revealed that all 11 viral diarrhea pathogens could be detected in feces and the top three positive pathogens were PKoV, PAstV and PEDV. The specific positive rate was as follows: 209 (40.3%) samples were positive for PKoV, 138 (26.6%) samples were positive for PAstV, 91 (17.6%) samples were positive for PEDV, 78 (15.1%) samples were positive for PSV, 68 (13.1%) samples were positive for PoSaV, 27 (5.2%) samples were positive for PTV, 25 (4.8%) samples were positive for PDCoV, 17 (3.3%) samples were positive for PoRV, 16 (3.1%) samples were positive for TGEV, 10 (1.9%) samples were positive for PToV, and 9 (1.7%) samples were positive for BVDV, respectively (Table 1 & Fig. 1). Therefore, complicated pathogen composition existed in clinic, which emphasized the importance of monitoring the fluctuant infection spectrum to guide the clinical production.

| Virus   | Positive numbers | Positive percent |
|---------|------------------|------------------|
| PKoV    | 209              | 40.30%           |
| PAstV   | 138              | 26.60%           |
| PEDV    | 91               | 17.60%           |
| PSV     | 78               | 15.10%           |
| PoSaV   | 68               | 13.10%           |
| PTV     | 27               | 5.20%            |
| PDCoV   | 25               | 4.80%            |
| PoRV    | 17               | 3.30%            |
| TGEV    | 16               | 3.10%            |
| PToV    | 10               | 1.90%            |
| BVDV    | 9                | 1.70%            |

Multiple infections were commonly seen in clinic

A total of 182 co-infections were identified, with positive rate of 35.14%. Dual-infection had the
highest detection rate of 17.57% (Table 2). High positive rate (11.58%) of triple-infection was also observed, while the positive rate of quadruple- and quintuple-infection was 4.63% and 0.77%, respectively. Most surprisingly was that six (0.58%) or seven viral diarrhea pathogens (0.58%) were simultaneously detected from one diarrhea stool, indicating a complex diarrhea pathogen infection pattern and pathogenesis in clinic. As we can see (Fig. 2), dual-infection with PKOV and PASTV had the highest positive rate (5.79%), followed by co-infection with PKOV and POSAV (1.93%). Triple-infection with (PKOV + POSAV + PASTV) was the most common, while triple-infection with (PSV + PASTV + PEDV) had the second high rate 1.74%, followed by triple-infection with (PKOV + PASTV + PEDV) (1.16%). It showed that PKoV and PAsTV infections had continuously increased in recent years, which indicated that they might play synergistic roles in the pathogenesis of diarrhea disease.

Table 2
The co-infections of clinical samples. A total of 182 co-infections were identified, with positive rate of 35.14%. Different multiplex infections had been shown in the table.

| Complex infection | Positive NO. | Positive percent |
|-------------------|--------------|-----------------|
| Dual-             | 91           | 17.57%          |
| Triple-           | 60           | 11.58%          |
| Quadruple-        | 24           | 4.63%           |
| Quintuple-        | 4            | 0.77%           |
| Sextuple-         | 3            | 0.58%           |
| Septuple-         | 3            | 0.58%           |

Viral infection spectrum in one farm

In tracking the annual viral diarrhea tests in one farm from 2015 to 2017, it was observed that the prevalence of the viral diarrhea pathogens also changed over time (Fig. 3). In 2015, PEDV had the highest positive rate of 45.83%, while the second highest was PKoV (33.33%). However, in 2016, PKoV became the most popular pathogen with a very high positive rate of 68.67%, and this was closely followed by PAsTV (32%) and PSV (24%). The PEDV positive rate significantly decreased, which was merely 12%. In 2017, PAsTV had the highest detection rate of pathogens (52.50%), followed by PKoV (40%) and PEDV (17.5%). It indicates that diarrhea disease is no longer mainly caused by three major pathogens (PEDV, TGEV and PoRV), which hints that other pathogens may play synergistic roles in the pathogenesis of diarrhea disease.

According to the present surveillance results, the farm strengthened its prevention and control of infectious diseases. As observed in the recent years, there was a decrease in enteric pathogens,
proving the great importance of epidemiological surveillance and the guidance effect to clinical production.

Different farms show variable infection spectrum
The composition of enteric pathogens among different farms was analyzed (Fig. 4 & Fig. 5). In farm A, eleven kinds of pathogens were detected including multiple co-infections (dual-, triple-, quadruple- quintuple- and sextuple-infection). In farm B, only eight kinds of pathogens were identified including dual- and triple-infection. It was concluded that the pathogen composition of a specific farm was unique. Therefore, different prevention and control measures should be carried on based on the monitored pathogens.

Dominant infection agents were analyzed by quantitative test
Using the Luminex xTAG multiplex detection method in this study, 173 diarrhea stools from the same farm were chosen to analyze the predominant virus. As a result, 11 viral diarrhea pathogens were detected, and the positive detection rate of PEDV, PKoV, PoRV, PSV, BVDV, PoSaV, PToV, PAstV, TGEV, PDCoV, and PTV was 32.4%, 26.0%, 14.5%, 6.9%, 6.4%, 11.6%, 2.9%, 26.0%, 0.6%, and 1.73%, respectively (Table 3). The concentration of different pathogens in each sample was presented as different colors in a heat-map (Fig. 6). Therefore, the pathogen content in each sample could be estimated intuitively. Different colors represent different concentrations of pathogens. The closer the color was to black, the lower concentration was present; and the closer the color was to red, the higher concentration was present in the sample.

Table 3
The detection of various pathogens by Luminex xTAG multiplex detection method. 173 diarrhea samples collected in 2017 were further quantitatively detected using the Luminex xTAG multiplex detection method.

| Virus  | PEDV  | PKoV  | PAstV | PoRV  | PSaV  | PSV   | BVDV  | TGEV  | PToV  | PTV   | PDCoV |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Positive percent | 32.40% | 26.00% | 26.00% | 14.50% | 11.60% | 6.90% | 6.40% | 3.50% | 2.90% | 1.70% | 0.60% |

The quantitative analysis speculated that the five viruses (PoSaV, PoRV, PAstV, PToV, and PEDV) were the predominant viruses in the multiplex infection sample. They not only could be detected in multiple co-infected samples, but also were relatively high in co-infected samples. Therefore, in virtue of the quantitative analysis, the detection results will be more clearly visible and targeted prevention or therapy may be carried out in the pigsty, paving the way to instructing the clinical production.
Discussion
Porcine viral diarrhea disease still seriously endangers the development of the pig industry, and leads to significant economic losses for pig farmers worldwide [7]. Clinically, the complexity of the disease has increased. In some cases, multiplex infections with two or more viruses are common, which seriously interfere with the clinical diagnosis [8–12]. It has been speculated that the incidence of diarrhea would decline due to the vaccine prevention of PEDV, TGEV and PoRV triplets. However, diarrhea continued to threaten pig farms. Expect for these three major porcine viral diarrhea pathogens (PEDV, TGEV and PoRV), other viral diarrhea pathogens have also been reported in recent years [13–20]. In particular, the situation of multiplex infections has become more serious, resulting in increased pressure in the prevention and control of porcine diarrhea. Although the correlation between emerging viruses and diarrhea has not been clearly discussed, these co-infections have indeed enhanced the severity of diarrhea in the present study. Therefore, in order to accurately differentiate the infections in clinical specimens and prevent the transboundary spread of porcine viral diarrhea disease, it is necessary to conduct pathogen monitoring in clinical production. Currently, PCR-based methods have been proven to be convenient and highly sensitive for detecting porcine diarrhea-associated viruses [2, 21]. The multiple PCR method for testing 4 or 7 kinds of diarrhea pathogens was established in the laboratory of the investigators, and were applied for clinical detection [21, 39]. However, complicated infections require a more accurate detection method. Therefore, we further developed Luminix xTAG high-throughput detection method for viral diarrhea pathogens in pigs, which has the advantages of high flux, wide range of detection, and small sample size. Furthermore, this is suitable for the large-scale screening of clinical samples, and is especially suitable for the multiplex infection detection of samples. Using Luminix xTAG technology, the standard curves for the above 11 diarrheal pathogens were established, and the content of each pathogen was calculated by measuring the MFI value of each sample. By analogy, the viral load can be determined for each sample, and finally, the pathogen with the highest risk of infection in each pig farm was analyzed, which would help guide the formulation of immunity and control measures in pig farms. In addition, this can also intuitively identify the maximum level of pathogens in each sample
Based on the cluster analysis software.

It was considered that animals co-infected with more than one enteric virus experienced increased intestinal epithelium damage and/or viral replication, which results in more severe diarrhea [10].

Forty samples of diarrhea in piglets in Sichuan Province were tested and five samples (12.5%) of multiplex infections of PKoV, PAstV and PToV were identified [12]. Chang Tiecheng et al. [13] tested 165 samples obtained from 42 pig farms, and reported that 2 of 42 pig farms were infected with PEDV and TGEV, accounting for 4.76%. Furthermore, seven pig farms were infected with PEDV and PoRV, accounting for 16.67%, and two pig farms were infected with three viruses, accounting for 4.76%.

This was consistent with the present results, in which there were serious multiplex infections in these pathogens, and seven pathogens were even detected in a sample. However, no new vaccines for diarrhea pathogens had been developed and applied to pig farms. Furthermore, there have been instances of co-infections in sows, even though these are usually asymptomatic. This may explain the persistence of viruses within the herd, and facilitation of vertical transmission.

Since PKoV has the highest infection rate and co-infection rate, further investigations should be conducted to research its characteristics and pathogenic mechanism. Since the first report of PKoV in Hungary [22] and China [23], it has been confirmed that PKoV was widely present in several countries, and plays an important role in diarrhea outbreak in pigs [24–28]. The statistical analysis of the PKoV positive rate between diarrheic and healthy pigs, as well as a survey for other enteric pathogens in diarrheic pigs, suggested that PKoV may play a role as a causative agent of gastroenteritis in pigs [25]. Recent studies have revealed the genetic diversity and possible pathogenic role of PKoV in conjunction with other pathogens in piglets [13, 25].

PAstV is widely distributed worldwide, and is highly prevalent among piglets with or without diarrhea. PAstV was first identified by electron microscopy from the feces of piglets with diarrhea in 1980 [11]. A survey of PASTV infection in pig farms in Japan revealed that the positive rate of PAstV was 82.9% [29]. Similarly, the high prevalence (79.6%) of the astroviruses in pigs between 2005 and 2007 was reported in Canada [30]. In South Korea, 25/129 (19.4%) domestic pigs and 1/146 (0.7%) wild boar fecal samples were tested positive for PAstV [31]. Multiple PAstV types were identified in many cities
in China [32–37]. The co-infection of different genotypes in the same pig was commonly observed, while in an individual pig a high genetic diversity was observed for viruses that belong to the same PAsTV genotype [37]. PAsTV exists in at least five distinct lineages (PAsTV1-PAsTV5) within the genus Mamastrovirus. The phylogenetic analysis revealed that PAsTV1, PAsTV2 and PAsTV3 were more closely correlated to AstVs from humans and other animals, when compared to each other, indicating the past cross-species transmission and zoonotic potential of these PAsTV types [30, 38].
The pathogen composition of different farms varied. Therefore, clinical control should be based on pathogen monitoring. Although infection was frequent in the winter season on farms in the temperate climate, diarrhea infection occurred throughout the year in porcine farms. Furthermore, the pathogen composition was more complex, and multiple infections more frequently emerged. Therefore, seasonal prevention measures are more important to control the diarrhea. The etiology of porcine diarrhea is complex. This might be influenced by bacteria, in addition to viral factors, and the interaction between bacteria and viruses might also contribute to the complexity of the disease. A single pathogen might not be the main cause of diarrhea, however, the unique relationship among different pathogens need further research. The specific mechanisms of the diarrhea pathogens for porcine diarrhea diseases remain unknown. However, the potential hazards can not be ignored, and these should be detected and prevented at an early stage.

Conclusions
In summary, here we provide a Luminex xTAG multiple detection method for viral diarrhea pathogen infection in clinical, which was more sensitive and specific than general multiplex PCR method. The high infection rate supported that viral diarrhea infections were epidemic in pigs in China. In addition, multiple infections were observed and a maximum of seven major pathogens were detected in one stool sample, which demonstrated the complicated status of porcine diarrhea disease. Therefore, in order to prevent this disease in the future, attention should be given to the relevant research on these pathogens and relevant vaccines, and the expansion of the vaccine reserve, in order to prepare for the outbreak of a new round of porcine diarrhea disease in advance.

List Of Abbreviations
Porcine kobuvirus (PKoV), porcine astrovirus (PAstV), porcine epidemic diarrhea virus (PEDV), porcine sapelovirus (PSV), porcine sapovirus (PoSaV), porcine teschovirus (PTV), porcine deltacoronavirus (PDCoV), porcine rotavirus (PoRV), transmissible gastroenteritis virus (TGEV), porcine torovirus (PToV), bovine viral diarrhea virus (BVDV), phosphate-buffered saline (PBS), diethylpyrocarbonate (DEPC), polymerase chain reaction (PCR), enzyme linked immunosorbent assay (ELISA), reverse transcription-polymerase chain reaction (RT-PCR).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare no competing financial interests.

Funding

This work was supported by the National Key Research and Development Program of China (Grant No. 2018YFD0500102) and the Shanghai Agriculture Applied Technology Development Program, China (No. 2019-3-2).

Author Contributions

H.L. designed the research, Y.S., B.L, J.T., and J.CH. performed research; Y. S., B.L. and H.L. analyzed data; Y.S., B.L., and H.L. wrote the paper.

Acknowledgments

Not applicable.

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Figures
The positive percent of different enteric viruses from 2015 to 2017 in Shanghai. A total of 518 porcine stool specimens from 5 districts in Shanghai were detected by multiplex RT-PCR method.
Figure 2

Dual- and Triple-infections of different enteric viruses. Various combinations of co-infections were identified.
Figure 3

Pathogen spectrum of a farm in Chongming District. The porcine stool specimens from Chongming district in Shanghai were detected by multiplex RT-PCR method every year from 2015 to 2017.
Figure 4

**Farm A**

| Virus | PKOV | PASTV | PSV | POSAV | PEDV | PORV | TGEV | PTOV | PTV | PDCOV | BVDV |
|-------|------|-------|-----|-------|------|------|------|------|-----|-------|------|
| Positive percent | 68.67% | 32% | 24% | 24% | 12% | 11.33% | 8% | 2.67% | 1.33% | 1.33% | 0.67% |

**Farm B**

| Virus | PEDV | PSV | PKOV | PTV | PDCOV | PASTV | BVDV | PTOV |
|-------|------|-----|------|-----|-------|-------|------|------|
| Positive percent | 37.74% | 24.53% | 20.75% | 16.98% | 7.55% | 7.55% | 3.77% | 1.89% |

![Graph showing virus detection in Farm A and Farm B.]

**Figure 4**

Detection of enteric viruses in different farms. The composition of enteric pathogens among different farms was analyzed.
Co-infections of different farms. Different co-infections were analyzed in different farms.
Figure 6

The Heatmap for quantitative results of some samples. 173 outbreaks of diarrhea in swine from 2017 were tested by the Luminex xTAG multiplex detection method. Only part data were present in the figure.