Article @ Virology

Development and Evaluation of a Rapid DNA Preparation Method for PCR-based DNA Virus Detection

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ABSTRACT

We describe a simple, rapid and resource-saving method of DNA preparation from cultured cells, sera and animal tissues for PCR-based DNA virus detection. The method does not require the proteinase K, ethanol or phenol/chloroform used in conventional methods, and the entire procedure is performed in the same tube, reducing possible cross contamination between samples and the expense of laboratory ware. The protocol utilizes guanidine HCl and sodium dodecyl sulfate successively to lyse cells and dissociate proteins from nucleic acid at high temperature, and precipitates SDS and proteins at low temperature while reducing guanidine HCl concentration sufficiently to permit PCR-based virus detection. This method is extremely low cost, high sensitivity and provides a quick and effective way for clinical and laboratory virus detection, and is especially useful for simultaneous analysis of a large number of samples.

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Abbreviations: DDW, Double Distilled Water; SDS, Sodium Dodecyl Sulfate;
G-HCL, Guanidine HCl;G-S, Guanidine HCl-SDS;
PCR, Polymerase Chain Reaction; PCV2, Porcine Circovirus 2;
PPV, Porcine Parvovirus; PBV, Porcine Bocavirus

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More and more new porcine DNA viruses like Porcine circovirus 2 (PCV2)[1], Swine Torgue teno virus TTV[2] and Porcine Bocavirus (PBV)[3, 4] were found and with highly prevalence among pig herd. These newly found viruses cause huge economic loss every year. To understand the virus circulation in the herd, molecular epidemiology investigation from a mount of samples by PCR is mostly used. DNA extraction is the first step in DNA virus detection by the polymerase chain reaction (PCR). A reliable and applicable protocol for the extraction of DNA from a number of species would be very useful for large scale screening of materials for the presence of the virus and would be the first step towards, arresting its spread. The conventional phenol/chloroform DNA extraction method is toxic, time-consuming, and utilizes protease digestion, organic solvent extraction and alcohol precipitation. While a number of improved methods [5-8] have been developed, they require multiple steps which make them inconvenient for processing large numbers of samples. To speed virus detection with a large number of samples, we developed a new DNA isolation method, avoiding the phenol/chloroform extraction steps and eliminating multistep tube transfers. DNA-containing supernatants produced by this method can be used for PCR-based virus detection in cultured cells, sera and animal tissues.

Materials and Methods

1.Samples and viruses

Three samples, organs from pig infected with PCV2, were used as model samples for method development. Additional fifty-three samples were assayed by two methods: 18 cell cultures, 24 tissues (kidney, spleen and/or lymph gland mixed together) for PCV2 and 11 cell cultures for PPV. Virus stock (PCV2 and PPV) utilized in this study were isolated by our lab and organ samples were from different farms. These samples were stored in −80 ºC.

2. Methods

2.1 Optimal condition for DNA extraction

One gram of each model sample with 1ml lysis buffer was grinding using the Mixer Mill MM 400 (Retsch, Germany) for 4 min at 30HZ and centrifuged at 4 ºC for 5mins at speed 4000 rpm/min. Super--natant of each sample was transformed into new EP tube and diluted for 10 times using for DNA extraction.

Guanidine HCl and sodium dodecyl sulfate (SDS) are dissolved in sterile DD water with concentration ranged from 1-5M and 5%-25%, respectively. To get the appropriate concentration of two reagents, cross method analysis were used. Briefly, 0.1ml each sample supernatant was added into 25 1.5ml EP tubes numbered from 1-25. Number 1-5, 6-10, 11-15, 16-20 and 21-25 were added

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into 10 μL G-HCL solutions with concentration of 1M, 2M, 3M, 4M and 5M. The tubes were totally vortexed and assigned into 5 groups as follow: Group1 with tube 1, 6, 11, 16, 21; Group2 with tube 2, 7, 12, 17, 22; Group3 with tube 3, 8, 13, 18 and 23; Group4 with tube 4, 9, 14, 19 and 24; Group 5 with tube 5, ,10, 15, 20 and 25.

Ten microliter SDS solutions with a concentration of 5%, 10%, 15%, 20% and 25% were added into tubes in Group 1, 2, 3, 4, and 5, and the tubes were vortexed thoroughly. After placement in boiling water for 3mins, 100 μL cold (2-4 ℃) DDwater was added, followed by centrifuging at 10,000g for 5mins, and the resulting supernatant was then ready for PCR analysis. Furthermore, the volume of water added into the sample-GHCL-SDS mixture was evaluated with volume as follows: 40, 60, 80, 100, 120, 140,160, 180, 200, 500, 800 and 1,000μL.

2.2 PCR amplification and Application

To evaluate DNA extracted by G-S method, PCR were performed. Aliquots of 1μl supernatants extracted by the G-S method were mixed with primers (2 μl), dNTP (2μl; 25mM), DNA polymerase (2.5U; TaKaRa), 10x DNA buffer (2.5μl; TaKaRa) and ddH2O to a 25 μl final volume. Standard PCR amplifications were performed as follows: 5 min at 94°C, 34 cycles of 30secs at 94°C, 30 secs at 65/55 °C, and 30 secs at 72°C, followed by a 5 min hold at 72°C. The PCR products were detected by 1% agarose gel electrophoresis.

Besides, DNA of 53 additional samples was extracted with the optimal condition of G-S method and by standard phenol/chloroform methods. Initial volume of the each of sample was 100μL and final volume of supernatants /DNA by G-S and phenol/chloroform methods are 200 μL and 50 μL, respectively. DNA was amplified through standard PCR assay with 2μl supernatants extracted by the G-S method and 0.5μl DNA extracted by the phenol/chloroform method. Product length of PCV2(PCV2F: accagcgcacttcggcagcggcag, PCV2R: gcgggccaaaaaaggtacagttcc) and PPV (PPVF: actctcagctactgcagcat, PPVR: tgcattattaaccatctactccat) is 797 bp and 503 bp, respectively.

2.3 Concentration test and Quantitative PCR

Determination of the nucleic acid concentration of 5 samples' DNA (PCV-2 nucleotides positive according to the above results) extracted by these two methods were performed through software 2000 Nanodrop (ThermoFisher SCIEN--TIFIC). One microliter of each DNA was tested and the concentration was showed as microgram per microliter.

For further comparing the sensitivity of DNA extracted by G-S and phenol /chloroform method， the copies of the target gene were calculated using SYBR

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Green (Biotool, Houston, USA) based absolutely quantitative PCR (qPCR). A plasmid preparation containing the entire PCV2 genome (EU095020; 1.65×10^{10} copies/μL) was 10-fold serially diluted from 10^3 to 10^7 and used as a template to draw a standard curve. The PCR amplification volume containing: 10μL 2×SYBR Green Master Mix (High ROX), 0.2μL of each primer (PCV2qFp: ctagaaacaagtggtggga tgttac, PCV2qRp: cattccaacggggtctgattgctg), 0.5 μL DNA by phenol/chloroform method or 2μL DNA by G-S method, dH2O added to 20 μL. The qPCR thermal cycling protocol (ABI StepOneTM 2.1, US) was as follows: initial denaturation for 5min at 95℃ followed by 40 cycles of 15 s at 95℃, 30 s at 60℃.

2.4 Statistics

Differences of the total DNA content and copies number of the target gene in the DNA by two methods were calculated by Independent-Samples T test using the IBM® SPSS Statistics 19 software (IBM, Armonk, NY, USA). Confidence interval was set as 95%, and a *P* value less than 0.05 was considered significant.

Results

1. The optimal condition for DNA extraction of G-S method

The amplification results of the DNA extracted from 3 model samples by G-S method were shown in the Fig 1. For the model sample1 (Fig1-1), all groups have signal detected and the signal value in Group 1(Fig1-1a, 5% SDS solution with 1-5M G-HCL) and Group2 (Fig1-1b, 10% SDS solution with 1-5M G-HCL) are weak than Group3(Fig1-1c), 4(Fig1-1d),and 5(Fig1-1e). While the signal value in Group 3, 4 and 5 are quite closely to each other. For the model sample 2 and 3, similar results were obtained which was no signal value in Group 1 and the signal in Group 5 was much higher than other groups. Besides, sample 3 under the condition of 2M G-HCL and 25% SDS solution was still negative amplification. Take into account these results, concentration of G-HCL and SDS solution was set as 3M and 5%, respectively.

The water volume added into the sample-GHCL-SDS mixture was evaluated. According to the results of amplification (Fig1-1f, Fig1-2f, Fig1-3f), the amplification signal of DNA in the tubes with water volume over 100 μL (including 100 μL) were much higher than those lower 100 μL. The final water volume added into the sample-GHCL-SDS mixture was 100μL.

2. Standard PCR and Application

DNA of fifty-three samples was amplified by standard PCR, and results showing that (Fig.2A-2b) positive rate by G-S and phenol/chloroform method was 92.5% (49/53) and 85%(45/53), respectively.

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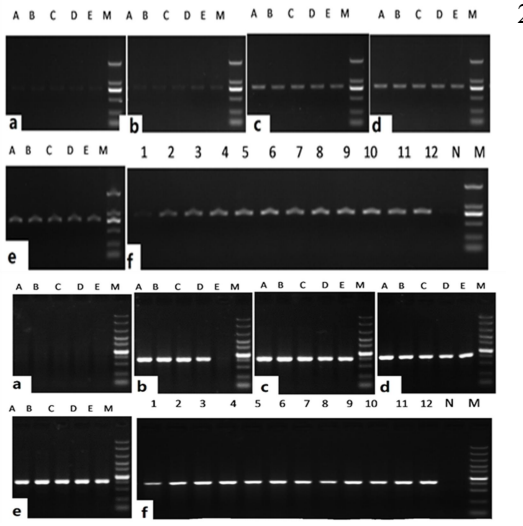

Fig.1.Optimization of Gua-HCL and SDS solution concentration a, b, c, d and e are concentrations at 1M, 2M, 3M, 4M and 5M of Gua-HCL solution, and A, B, C, D and E are concentrations at 5%, 10%, 15%, 20% and 25% of SDS solution. f for water volume added into the Sample-Gua-SDS mixture, and 1-12 means 40, 60, 80, 100, 120, 140,160, 180, 200, 500, 800 and 1,000μL

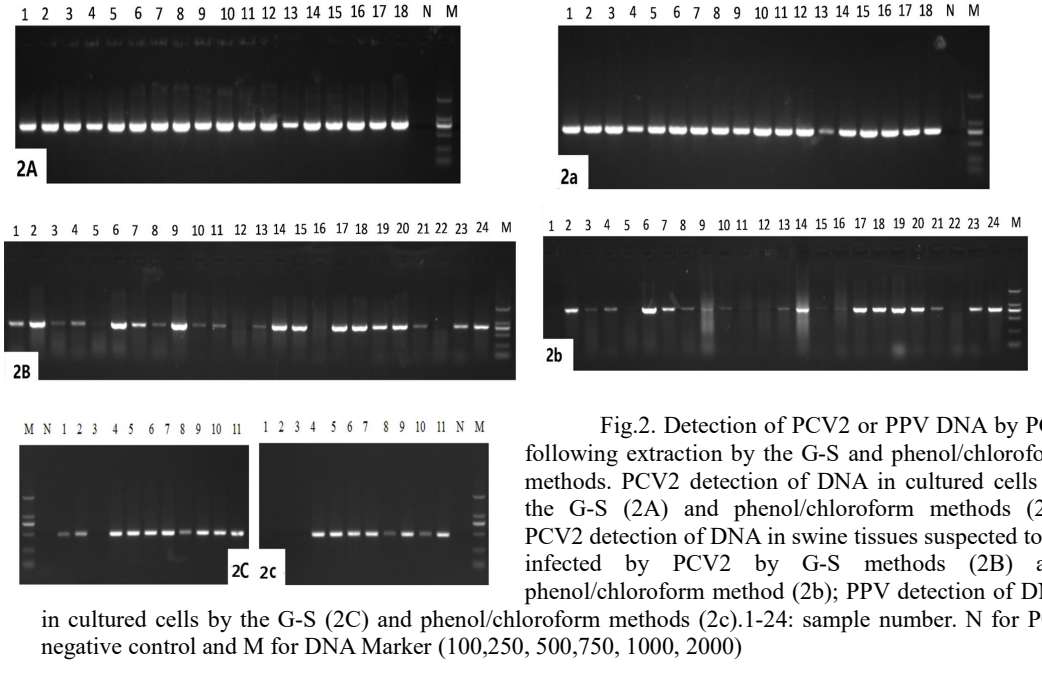

Fig.2. Detection of PCV2 or PPV DNA by PCR following extraction by the G-S and phenol/chloroform methods. PCV2 detection of DNA in cultured cells by the G-S (2A) and phenol/chloroform methods (2a); PCV2 detection of DNA in swine tissues suspected to be infected by PCV2 by G-S methods (2B) and phenol/chloroform method (2b); PPV detection of DNA in cultured cells by the G-S (2C) and phenol/chloroform methods (2c).1-24: sample number. N for PCR negative control and M for DNA Marker (100,250, 500,750, 1000, 2000)

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For 18 PCV2 cell samples, the PCR results were all positive and the signal of the 13 sample by G-S method was higher than phenol/chloroform methods, while others were with the same signal value. For organ samples, the number of positive amplification was 21/24 and 20/24 by G-S and by phenol/chloroform methods, respectively.

Samples with different signal were grouped: samples with high signal by G-S were:2, 6, 9, 13, 14, 15, 17, 18, 19 and 20, while by phenol/chloroform method were: 6, 17, 18, 19 and 20. Samples with medium signal by G-S method were:1, 7, 23 and 24, while by phenol/chloroform method were:2, 7, 14, 23 and 24. Samples with low signal by G-S method were:3, 4, 5, 8, 10, 11, 13 and 21, while by phenol/chloroform method are:3, 4, 5, 8, 9, 10, 13, 15, 16 and 21. Negative samples by G-S method were: 12, 16 and 22, while by phenol /chloroform method were: 1, 11, 12 and 22.

3. Concentration test and Quantitative PCR

Nucleic acid concentration of 5 samples extracted by two methods was calculated and the results were listed in the table 1. The total content was calculated and it was a little higher the G-S method than by the phenol/chloroform method, although no significant differences (p=0.397). Both 260/280 and 260/230 ratio of the DNA

Table 1: Nucleic acid concentration extracted from samples by G-S and Phenol/chloroform method

Sample ID	Nucleic Acid Conc(ng/μl)		Total content (μg/100μL sample)		260/280		260/230	
	Phenol	G-S	Phenol	G-S	Phenol	G-S	Phenol	G-S
9	6930.6	1924.6	346.53	384.92	1.89	1.83	2.16	1.91
24	6169.6	1602.6	308.48	320.52	1.84	1.81	2.21	1.98
57	9992.8	2844.7	499.64	568.94	1.87	1.89	2.16	1.97
66	3707.6	1167.4	185.38	233.48	1.86	1.85	2.21	2.03
87	3123.5	1792.4	156.175	358.48	1.89	1.85	2.04	1.96

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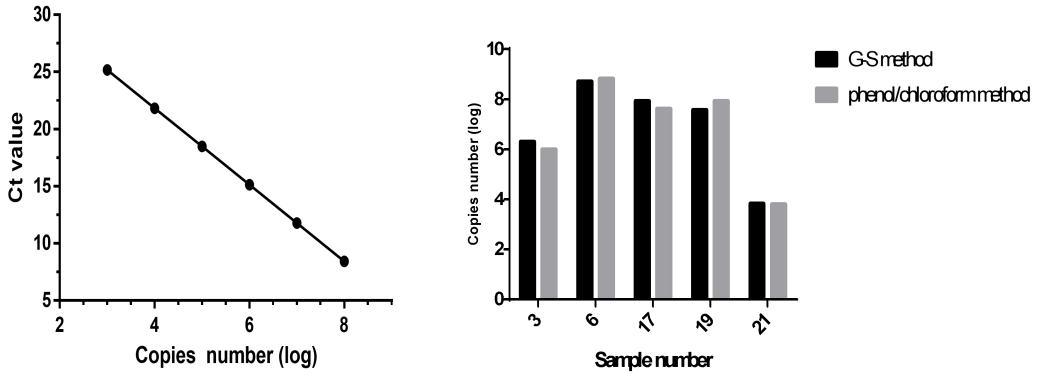

Fig. 3 Standard curve (3A) and samples copies number by two methods (3B).
Standard curve was drawn with plasmids following 10-flod serials dilution ranged 1.65×10^{3} - 1.65×10^{8} copies per microliter. Comparing of PCV2 DNA by qPCR with DNA extracted by the G-S (black bar) and standard phenol/chloroform (gray bar) methods

extracted by phenol/chloroform method were all a little higher than G-S method. The 260/280 ratio of both methods was located in the range of 1.81-1.89 which indicates there was no protein left and the purity of the DNA by two methods was quite high. The 260/230 ratio of all the DNA extracted by phenol/chloroform method was over 2.0 while 4 of 5 by G-S method were over 2.0 and the sample 5 was 1.96. The significance was calculated and no differences (p=0.91).

Discussion

In the present study, we developed a DNA extraction method based on two reagents: guanidine HCl and SDS. To evaluate the stable of G-S method, we investigated the DNA content, concentration and purification comparing to phenol/chloroform method. The concentration of the DNA extracted by G-S method is lower than phenol/ chloroform method, but the total content by G-S method is a little higher than phenol/chloroform method. There were no significant differences according to conventional and quantity PCR. The methods could be used for DNA extraction.

The new method utilizes two protein denaturants successively. High concen--trations of guanidine HCl are known to have powerful cell lytic and protein denaturing properties. SDS is also a potent detergent that can lyse cells and disrupt protein non-covalent bonds, thereby causing denaturation and loss of native conformation. However, when mixed with guanidine HCl at room temperature, SDS is precipitated, which is why the mixture must be heated to

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maintain the SDS in solution. During incubation of the mixture in boiling water, DNA-protein and protein-protein inter--actions dissociate, and cooling result in the deposition of denatured proteins and most of SDS-guanidine HCl. Added water must be at least 10x the volume of guanidine HCl (or SDS) to dilute SDS andguanidine HCl and the remaining SDS sufficiently to ensure the PCR amplification reaction.

While the conventional phenol/ chloroform method and many commercial DNA extraction kits enrich the DNA concentration, the G-S method dilutes the DNA in samples. Based on application results by standard PCR amplification, the G-S method was slightly more sensitive (Fig.2). While from the data in Fig.3, the G-S method is significantly more sensitive than the phenol/chloroform method. The reason is not only the quantity of DNA extracted by the G-S method is higher than by phenol/ chloroform, but less interference factors which remaining to be determined. Additionally, the G-S method has obvious advantages. Because it does not require proteinase K and the entire procedure is performed in one tube, the possibility of cross contamination during tube transfers is eliminated. Furthermore, the procedure requires less time (less than 30 min for ≤24 samples), and is relatively environmentally friendly since it eliminates the need for ethanol and toxic reagents such as phenol or chloroform, as well as reducing the need of plastic laboratory ware. The method uses only small amounts of guanidine HCl and SDS (both inexpensive and readily available) making it particularly suitable for PCR-based viral DNA isolation, even for those laboratories with limited facilities.

Acknowledgments

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