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Comparison of Efficiency of Different Nucleic Acid Extraction Methods for Bovine Enteroviruses in Feces

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Abstract

Bovine enteroviruses are thought to be mild pathogenic or non pathogenic viral gastroenteretis agents. However there are some cases where they are identified from cattle having different symptoms. They can also be used as markers of environmental contamination. Enterovirus family has fast replication capacity and can be used as potential vectors. For this purpose isolation and molecular studies concerning Enteroviruses are currently increasing. In this study 10 cattle feces samples were used for evaluation of 4 different extraction methods for investigation of bovine enteroviruses. These methods comprise of TRIzol LS (Life Technologies), QIAamp Viral RNA mini kit (Qiagen), NucleoSpin RNA (Macherey-Nagel) and phenol-chloroform extraction. All procedures performed in BSL-2 cabinet and RNA yield of extracts were analysed in Bio-spec (Shimadzu) spectrophotometer. This RNA templates were then tested by Verso 1 step RT PCR (Thermo Scientific) kit with spesific for Enterovirus 5'UTR primers. And observed for band formation. While QIAmp Viral RNA kit had the shortest protocol, it did not have as much sensitivity as TRIzol LS and TRIzol was determined as the most sensitive method to make extraction of bovine enteroviruses from feces.

Keywords: Bovine Enteroviruses, RT-PCR, Extraction

INTRODUCTION

Bovine enteroviruses are somewhat mild pathogens causing generally mild symptoms in cattle. But there are studies which they were isolated from animals with respiratory, digestive tract and reproductive system symptoms. Bovine enteroviruses are considered as biological markers of environmental contamination (1,2,3). These viruses are classified in *Enterovirus* genus of *Picornaviridae* family. In 9.th ICTV (International Committee on Taxonomy of Viruses) they are classified in species enterovirus E and F although their classification were named differently in previous years [8].

When starting a molecular study the first step to downstream applications is the isolation of the nucleic acid. In this case it is a RNA virus so the selection is RNA extraction. There are a number of methods and commercial kits available for this task [4,6,7]. The choice of matter is based on different factors. Such as practicality and cost value. In case of molecular studies yielded nucleic acids should also be free of PCR inhibitory substances like bile salts and complex polysaccharides [5]. Four different methods were chosen to compare the efficiency in making RNA extraction of Enteroviruses from feces. Among these methods there are two column based commercial kits where performing scientist does strictly follow the data sheet of the product. And two more flexible methods that can be modified according to scientist's needs.

First method of preference is QIAamp Viral RNA mini kit which is a column based commercial kit. Its protocol process takes about 30-40 minutes and its workflow is defined as following; the sample is first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Then conditions are adjusted to provide optimum binding of the RNA to the membrane, and the sample is loaded onto the column. After that membrane is washed away for contaminants in two steps using two different washbuffers. RNA is eluted in a RNase-free buffer to go on with further applications.

Itself being a column based method Macherey Nagel (MN)'s Nucleospin kit follows similar steps to Qiagen's kit. Basic steps being lysing, washing and elution of RNA into a clean tube. The main difference of these two kit is MN's kit is total RNA where Qiagen's kit is only viral RNA.

The main parts of TRIzol LS extraction method is homogenization, phase seperation, RNA precipitation and isolation. This method takes about 3 hours. The time this and next protocol totally takes is way more than practical column based methods.

The last method is as described by Chomczynski and Sacchi [4]. All components of this method is prepared manually making room for optimisation and lessening the cost. First sample is lysed with a denaturating solution known as Solution D then components of phenol/chloroform/sodium aceate are added. After centrifuge at 10.000 g for 10 minutes upper aqueous phase is transferred to a new tube to continue to precipitation. Then it is washed with isopropanolol and then 75% ethanol and centrifugated again. After removing excess alcohol pellet is air dried and total RNA is resuspended with RNAse free water.

MATERIALS and METHODS

Collection and Preparation of Samples

Feces samples of the study was taken from a herd which is susceptible to bovine enteroviruses. All animals were healthy looking with no clinical symtoms. 10 cattle ages changing 1 to 3 were selected and feces were taken into steril capped holders and kept at +4 C until arriving the lab. Then they were immediately frozen to -80 C to work later . First step to extraction is to prepare the feces samples, to do this feces samples were thawed and diluted in 1/10 ratio with PBS, and centrifuged at 3000 rpm for 10 min. Supernatant is then divided for extraction according to the needs of methods. Then all 10 samples were subjected to RNA extraction of 4 different methods. Samples were named with numbers 1 to 10 randomly.

Extraction Methods

Protocol of two column based kits, QIAamp Viral RNA mini kit (Qiagen), NucleoSpin RNA (Macherey-Nagel), and TRIzol LS (Life Technologies) was conducted according to datasheet of the related products. The phenol/chloroform method was conducted according to publication by Chomczynski and Sacchi [4] .

Measurement of RNA yield and purity

After samples were Yield, Purity they were analysed for RNA quantification and purity with Shimadzu Biospec-nano spectrophotometer and evaluated with the bundled software.

RT-PCR

In the light of this data to see further applications of these extracts a PCR reaction was performed using a 5'UTR region designed primer [1]. Being an RNA virus complementary DNA syntesis was needed so for practical reasons we used Verso 1 step RT PCR (Thermo Scientific) kit that has reverse transcrpitase in it. PCR cycle conditions were applied in accordance to the related publication [1].

RESULTS

Table 1 shows comparative table of results of Biospec-nano spectrophotometer. For optimal purity RNA A260/280 ratio should be 1,8-2 (4,5,6). According to data, extractions with TRIzol LS has optimal results whereas phenol/chloroform methods and Qiagens viral mini kit has little divergences from normal values of A260/A280 ratio. Nucleospin kit has some unmeaningful readings which had similar values when they are analysed again.

Table 1. Spectrophotometer readings

	TRIzol		Qiamp Viral RNA		Nucleospin		Phenol/Choloform	
Sample	**Con.**	**A260/280**	**Con.**	**A260/280**	**Con.**	**A260/280**	**Con.**	**A260/280**
1	323,95	1,808286	116,73	1,728395	26573	1,512438	28,77	1,693582
2	279,02	1,4142	121,62	1,578947	33298	1,836364	41,12	1,438252
3	363,37	1,747997	117	1,841463	20911	5	42,69	1,82866
4	112,86	1,795831	119,92	1,747126	12540	6,111111	36,97	1,825455
5	504,31	1,807927	113,94	2,121212	28491	5,2	38,69	1,818815
6	320,19	1,800944	112,26	1,8125	42405	1,651163	23,76	1,748011
7	272,32	1,81137	120,07	1,428571	24898	1,96	56,44	1,883375
8	235,25	1,788415	109,81	1,576923	16497	1,457831	30,9	1,585691
9	346,36	1,805958	118,14	1,677419	31048	5,75	48,88	1,801399
10	155,14	1,675601	121,62	1,625	30042	1,509434	31,49	1,737044

Table 2. Positivity and luminescence status of PCR reactions.

Sample	TRIzol	Viral RNA	Nucleospin	Ph/Chlo.
1	++	++	Neg	+
2	+++	++	Neg	+
3	+++	+++	Neg	+++
4	++	++	Neg	+
5	+++	+++	Neg	+++
6	+	Neg	Neg	Neg
7	Neg	Neg	Neg	Neg
8	+	Neg	Neg	Neg
9	++	Neg	Neg	Neg
10	+++	+++	Neg	+++

+ weak luminescence

++ average grade luminescence

+++ strong luminescence

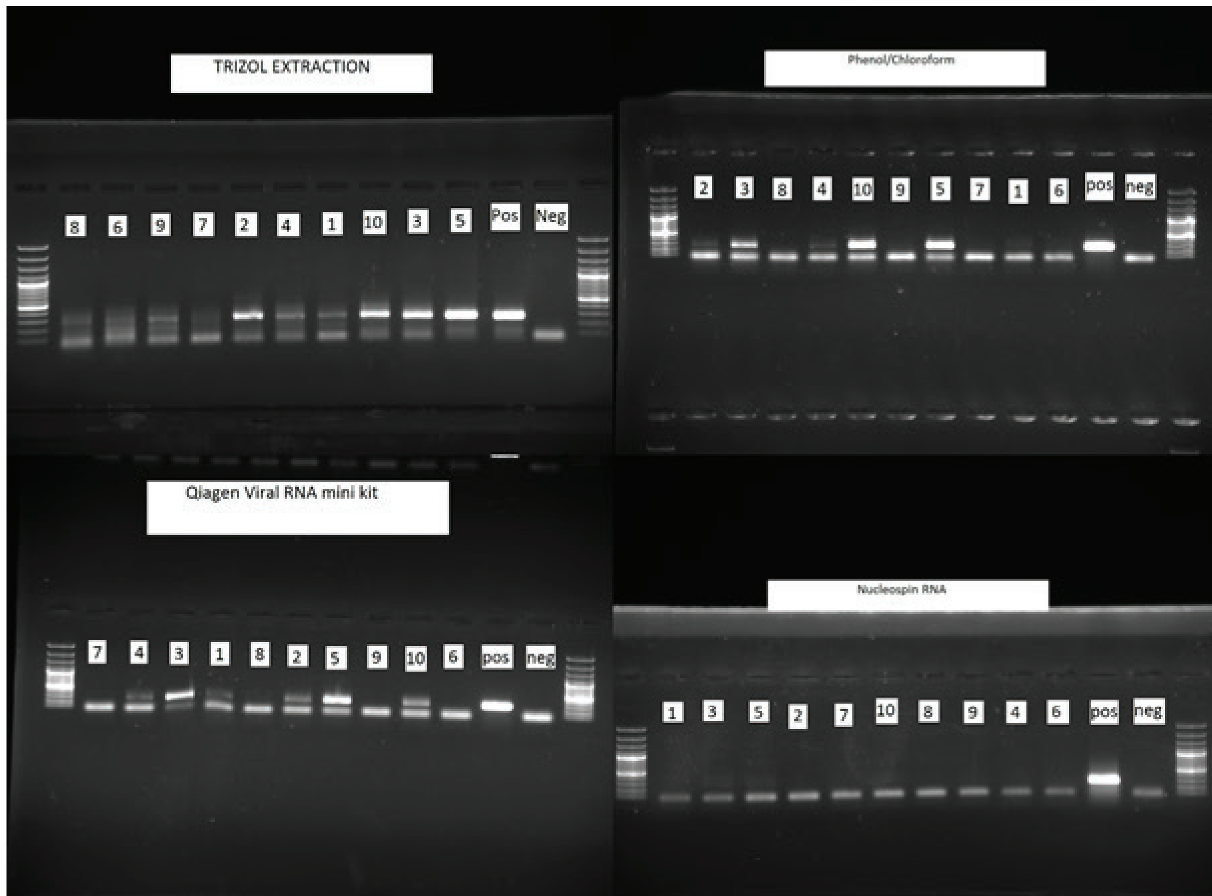

Figure 1. Gel images of four extraction methods

After performing RT-PCR, amplicons were evaluated with agarose gel electrophoresis and various luminescent bands were detected. Results are shown in Table 2 and gel images are shown in Figure 1.

DISCUSSION

When we evaluate the nucleic acid yields, as shown on Table 1, the quantity of RNAs are detected variable among extraction methods. Viral RNA mini kit is only spesific for viral RNA whereas with other methods total RNA is extracted. Values for total RNA were extractions expected to be higher than viral RNA method, but this was not the case. When purity of the extracted RNA is evaluated by examining A260/280 values, we detected no major diversion of normal range for TRIzol method and minor diversions for phenol/chloroform and viral RNA kit. Some readings of Nucleospin kit was erratic. In general all A260/280 values seemed applicable to molecular studies. It is evaluated that the values for extraction methods are consistent within same methods, but RNA yields differ among different methods. Only Nucleospin kit has unmeaningful values. As the cause was not clear, all extracts were carried out with RT-PCR to see further applications.

When agarose gel images are examined, we see there are 9 samples for TRIzol, 6 samples for viral RNA, and 6 samples for phenol/chlorofom is consistent with the positive control's band. Densitiy of the bands were graded and most luminescent bands were seen on TRIzol extracts indicating this is the most sensitive method to use for extracting bovine enterviruses from feces. We observed no spesific band for Nucleospin method. When the spectrophotometric values being erratic is taken into account this spesific method does not seem a convinient method for BEV extraction.

The column based methods are practical and less time consuming but their sensitivity is not as high as TRIzol according to our findings. When sensitivity is the most important factor, as in any diagnostic study, we conclude that TRIzol method has best overall values for BEV extraction from feces.

It is worth considering that all our samples were taken from subclinical/healthy looking animals and these results may vary when samples are taken from clinically infected animals.

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