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SHORT COMMUNICATION

Simplified RNA Extraction from Trichostrongylus Infected Sheep Blood to Amplify Ovine Interleukin-6 Gene

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ABSTRACT
A high quality RNA (407-420µg/mL) was extracted from blood of sheep infected with *Trichostrongylus* spp., by using RBC's lysis buffer followed by TRIzol reagent. Agarose gel electrophoresis showed clearly, discrete ribosomal RNA with no apparent degradation. The purified RNA samples were used successfully in reverse transcription reaction (RT) and produced cDNA was used in PCR reaction to amplify gene fragment of the ovine IL-6. A high quality amplification product of expected length of 651bp DNA was obtained. The procedure of extracting RNA was found to be simple, reliable and consistent, suitable for RT-PCR.

INTRODUCTION

The detection of nucleic acid sequences is a widely used identification technique in most of the recent diagnostic and research laboratories. The target nucleic acid sequences are amplified by polymerase chain reaction (PCR) and the products (amplicons) are detected in ethidium bromide-stained gels or by colorimetric enzyme immunoassay. In order to amplify nucleic acids (DNA or RNA), these molecules need to be extracted and separated from other components of micro-organisms, tissues, cellular materials, biological fluids etc that could inhibit amplification reactions (Wiedbrauk et al., 1995; Schepetiuk et al., 1997). A standard method to extract RNA is the phenol-chloroform extraction and ethanol precipitation technique. Other techniques used for the purification of RNA include silica beads, proteinase-K, TRIzol reagent and guanidine isothiocyanate (Chomczynski and Sacchi, 1987, 2006; Boom et al., 1990; Davis and Boyle, 1990). Additionally, numerous RNA extraction kits are also available commercially. All these techniques have their own merits and demerits. The present paper reports an improved method for RNA extraction from whole blood using lysis buffer followed by TRIzol reagent.

MATERIALS AND METHODS

Extraction of RNA

Fresh whole blood from five sheep infected with *Trichostrongylus spp.* were collected in EDTA coated tube (BD vacutainer) and used for the extraction of RNA. One millilitre whole blood was mixed with 5 mL RBC's lysis buffer {0.17M Tris (hydroxymethyl-aminomethane) and 0.16M Ammonium chloride, 1:9} (Hudson and Hay, 1991) and incubated on ice for 15 minutes, vortexed 2 times during incubation time, so that the cloudy suspension became translucent. It was then centrifuged at 10,000 rpm for 15 minutes at room temperature. The supernatant was discarded and the pellet was again dissolved in 1.5 mL RBC's lysis buffer by gentle pipetting. The suspension was then transferred into a mini-centrifuge tube and centrifuged at 10,000 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet was dissolved in 1 mL TRIzol reagent (Invitrogen™, USA) by pipetting followed by vortex until it was completely dissolved. It was incubated for 5 minutes at room temperature. A total of 200 µL chloroform was added, shaken by hand and then vortexed to dissolve completely. After incubation for 10 minutes at room

temperature, it was centrifuged at 16,000 rpm for 15 minutes to collect the aqueous phase. A 500 µl isopropyl alcohol was added to the aqueous phase, mixed gently and incubated for 10 minutes at room temperature. After that, the suspension was centrifuged at 16,000 rpm for 10 minutes. After discarding the supernatant, 500 µl of ethanol (70 %) was added into the micro-pellet at the bottom and centrifuge for 16,000 rpm for 10 minutes. All the liquid from the tube was removed carefully and air dried for 15 minutes at room temperature followed by the addition of 20 uL DEPC treated water to dissolve RNA by pipetting few times. The extracted RNA was stored at -20C in the freezer till further use.

Estimation of RNA quality

RNA concentration was measured at A_{260} (SmartSpec 3000; BioRad, USA) and purity was estimated by the absorbency ratio $A_{260}/A_{280.}$ Integrity of RNA samples were appraised on 1% agarose electrophoresis gel (Sigma®, USA).

Amplification of ovine IL-6

The extracted RNA samples were used in the RT-PCR reaction in order to amplify the ovine IL-6 gene. The reaction mixture contained 5 µL RNA, 2 µl each primer (Invitrogen™, USA) and 1.3 U of Strata Script reverse transcriptase (Invitrogen™, USA) in a final volume of 50 µl was incubated at 42ºC for 30 minutes, followed by 10 minutes at 68ºC.

Five microliters of the resultant cDNA was used in PCR reaction with using Taq DNA Polymerase (Qiagen Inc., USA). After denaturation for 5 min at 95ºC, the DNA was amplified for 35 cycles at 95ºC for 30 second, 55ºC for 30 second, and 72ºC for 30 second with the final extension for 5 minutes at 72^{o}C, in a final volume of 50 µl.

The primers used were 5' CCA GGA TCC CAG CTA TGA ACT CCC TCT TC 3' (sense) and 5' GGA GAA TTC GCT ACT TCA TCC GAA TGA CTC 3' (antisense). PCR products

(5 µl) were loaded on a 1% agarose electrophoresis gel, stained with ethidium bromide to visualize a band with an expected length of 651 bp under UV illuminator.

RESULTS AND DISCUSSION

The choice of appropriate RNA extraction methods is considered a critical step for the successful and valid use of molecular techniques like RT-PCR and PCR. A standard method of RNA purification is the phenol-chloroform extraction and ethanol precipitation technique. This method is complicated, time-consuming, and requires extensive use of disposable plastic ware to prevent cross-contamination (Shafer et al., 1997). Silica RNA extraction method is the other technique which requires complicated buffers, numerous centrifugations and careful pipetting to remove the RNA solution from the silica beads. The additional pipetting or handling increased the risk of losing the RNA; so it is considered to be the least efficient method for RNA extraction (De Paula et al., 2003). A modified version of the guanidine isothiocyanate method is also used for RNA extraction but it is a time consuming requiring 20-24 hours.

Proteinase K is considered to be an efficient method for RNA extraction, since the use of proteinase-K solution followed by phenol-chloroform extraction reduces the concentration of residual protein and membrane components that could inhibit *Taq* polymerase activity. However, additional safety measures are required for the handling and disposing of phenol. Further, standardized commercial methods using the kits, although more time efficient, result in an approximately ten fold less sensitivity as compared to the standard technique (Fanson et al., 2000; Knight et al., 2010).

TRIzol reagent is considered to be the most satisfactory and consistent for extracting total RNA suitable for RT-PCR analyses (Chadderton et al., 1997). In the present study, moderate concentration (407-420 µg/mL) of high quality RNA was purified from whole sheep blood by using lysis buffer followed by TRIzol reagent.

The quality of RNA in this study was confirmed in several ways. First, the A_{260}/A_{280} absorbance ratio was ranged from 1.92-1.97 indicating that RNA was relatively free of protein and polysaccharides contamination. Second, 1% denaturing agarose gel electrophoresis clearly showed discrete ribosomal RNA with no apparent RNA degradation. Third, the RNA was successfully used in RT-PCR and the generated cDNA was used in PCR to amplify the ovine IL-6 gene. A 651bp high quality amplified product was detected by electrophoresis on 1 % agarose gel.

It was concluded from our results, that the RNA extraction with TRIzol method can be improved by using RBC's lysis buffer to get high quality RNA suitable for RT-PCR.

Authors' contribution

All authors contributed equally in preparing this manuscript.

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