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RNA isolation with low toxic ammonium trichloroacetate

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Abstract
A method of RNA extraction with ammonium trichloroacetate (ATCA), the low toxic chaotropic agent, and the filter paper (cotton wool) for the RNA purification from ATCA-propanol-2 solution was developed. The yield and purity of total RNA isolated from plant and animal viruses, *E.coli*, from animal cells and plants were identical to those obtained by the conventional methods using toxic and hazardous agents such as guanidine thiocyanate, phenol, chloroform, etc. The quality of RNA was evaluated by the comparison of their optical and electrophoretic characteristics with those obtained after SDS-phenol deproteinization. Mass molecular diagnostics of virus and viroid infections was performed using home-made hand juice squeezer with changeable plastic shells and a vacuum manifold with mini column nests compatible with the immune plate. Use of low toxic ATCA is of particular importance for quality RNA isolation from all kinds of tissues and mass molecular diagnosis of the infecting pathogens (epiphytotics, epizootics, pandemics).

Keywords: RNA isolation, ammonium trichloroacetate for RNA isolation, virus, PSTVd DNA probing, large scale molecular diagnostics

Introduction

Isolation of quality RNA is key to effective diagnosis of infecting pathogens, especially with RNA as the genomic nucleic acid. It is especially important for viruses with RNA as the genomic nucleic acid as most of the eukaryotic viruses have RNA genome.

In the case of RNA isolation from a large number of samples from pathogens or infected species, particularly in emergencies of epizootics and epiphytotics, a universal method which exerts minimal pressure on the environment, is required. Currently, SDS-phenol/chloroform [1], guanidine thiocyanate (GTC)-phenol [2] and silica-based procedures [3, 4] are routinely used for the extractions of RNA. All these methods employ agents that are toxic that makes their extensive use hazardous for the environment.

The main objective of this work is to develop a procedure for RNA isolation that is reliable, widely acceptable, sensitive and efficient, and employs ecologically safe reagents for RNA extractions from viruses and cells.

In this communication, we illustrate the use of LTCA, a low toxic chaotropic agent for isolation of viral genomic nucleic acid from Tobacco mosaic virus (TMV) and Potato virus X (PVX), detection of Potato spindle tuber viroid (PSTVd) with the (dien)Pt-DNA probe in an infected potato [5,6] and for early diagnostics of picornavirus infections [7]. In this work we describe ammonium trichloroacetate, an agent for isolation of RNA from plant and animal viruses, *E.coli,* animal cells and plant tissues.

2. Materials and Methods

Potato infected by PSTVd was obtained from Dr. K.A. Mozhaeva (Russian Research Institute of Phytopathology). cDNA of PSTVd was amplified by RT-PCR, cloned, sequenced (GenBank accession no. EU257478) [8]. TMV U1 strain and PVX were kindly provided by Dr. V.K. Novikov (deceased). Cotton wool was locally purchased from Amelia, St.Petersburg, Russia. Silica (Cat. N112945-52-5) was procured from Sigma. Deionized double distilled water has been used throughout in this work.

2.1 Preparation of the ammonium trichloroacetate (ATCA, CCl_3COONH_4) solutions

In a fumehood, ATCA (610 g) (Reachim, Moscow, Russia, analytical grade) was placed in a thick wall glass container and approximately 350 ml (in small portions) of 25% ammonium hydroxide (Reachim, Moscow, Russia, analytical grade) was carefully added and mixed thoroughly. pH of the mixture was adjusted with ammonium hydroxide to 7.1-7.2 to give stock ATCA (the density is 1.42 g/ml, ~5.6 M).

Ammonium trichloroacetate wash solution (WB, ~3.9 M ATCA, the density is 1.3 g/ml) was prepared after the addition of 13 ml of 1 MTris-HCl (pH 7.5) and 27 ml of water to 90 ml of the stock solution ATCA.

To get lytic-dissociation buffer solution, LDB (3.9 M ATCA, 0.1 MTris-HCl, 0.025 M Na_2-EDTA, 1.5% Triton X-100, pH 7.5), 90 ml of stock solution ATCA was mixed with 13 ml of 1 MTris-HCl (pH 7.5), 6.5 ml of 0.5 M EDTA (pH 8.0), 2 ml of Triton X-100 and 18.5 ml of water.

2.2 Cellulose method for RNA isolation from the ATCA solutions

2.2.1 *Preparation of cellulose adsorbent*

To prepare cellulose adsorbent, 2x2 mm square pieces of filter paper (Baver, Tver, Russia) were saturated with 10 ml of 50 mM Tris-HCl, 10 mM Na_2-EDTA, pH 9.0-9.2 buffer and vortexed vigorously to produce hair springs cellulose. Cellulose pulp was diluted with 40 ml of water, an excess of the liquid phase was discarded and cellulose was suspended

(70 mg/ml) in 10 mM Tris, 1 mM Na_2-EDTA, 0.02% NaN_3, pH 7.0-7.4. Small aliquots of the stock suspension were kept at room temperature in the dark for months.

2.2.2 *Filter paper capture of RNA*

Usually, the cleared RNA ATCA-extract was mixed with LDB containing various ATCA concentrations (see *Results*), then cellulose stock suspension up to final concentration 2 mg/ml and two volumes of propanol-2 were sequentially added. The mixture was first allowed to stand for 10 min to adsorb RNA and then loaded on the minicolumn. The liquid was slowly drained, cellulose was washed with 0.5 ml of 70% propanol-2 and the column was dried a little to evaporate propanol-2 under aspirator vacuum. RNA was eluted from cellulose 2-3 times with water, precipitated with 3 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 4.7), washed with 70% ethanol, dried under vacuum and dissolved in water.

For rapid isolation of viral RNA in large quantities, a sterilized cotton wool (Amelia, St.Petersburg, Russia) instead of filter paper was used. In aqueous solution, 100 mg of swollen cotton wool occupies a volume of 1 ml. Cleared cell ATCA-extracts (*see* Results) were mixed with propanol-2 to its final concentration of 70% and opalescent mixtures were loaded onto mini columns with cotton wool. The liquid was allowed to flow through (30 drops/min) and each column was washed with 4 ml of 70% propanol-2 and then dried under low vacuum for 10 min. Bound RNA was eluted 3 times with 1-2 ml (in total) of water. All operations were performed at reduced pressure of a water aspirator. The purified RNA preparations have absorption readings $A_{260}/A_{280} \geq 1.9$ and $A_{230}/A_{260} \geq 2.2$ in water solution.

2.3 Silica-ATCA method for RNA isolation

Preliminary prepared amorphous silica suspension [3] was washed at a slow rate of centrifugation twice with 100 µl of WB, two times with 50 µl of 70% ethanol and 50 µl of acetone and then vacuum dried.

1 g of fresh or frozen plant tissue was homogenized with a mortar and pestle at room temperature in 6 ml of LDB containing 3.9 M of ATCA and kept for 20 min. Extracts were centrifuged for 5 min at 10000 g to give cleared cell ATCA-extract, centrifugation time was included in the total time of incubation. Then 300 µl of amorphous silica suspension and 1.5 ml of LDB were added to 400 µl of the cleared cell extract, and the mixture was incubated at room temperature for 15 min before centrifugation at 12,000 rpm for 10 min. Precipitated silica gel was transferred to 0.6 ml microcentrifuge tube with pierced bottom and this mini spin column was placed in 1.5 ml collection tube. The gel was washed twice with 0.5 ml of wash buffer (WB), two times with 0.5 ml of 70% ethanol, 0.5 ml of acetone and then vacuum dried. RNA was eluted from silica by 200 µl of water at 56ºC for 10 min, and the eluate was collected by centrifugation at 12,000 rpm for 2 min. This elution step was repeated. Finally, RNA was precipitated with 3 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 4.7), washed with 70% ethanol, dried to remove ethanol and dissolved in 20-25 µl of water. This RNA preparation was suitable for the DNA probing and RT-PCR analysis.

2.4 Adsorption of RNA on silica from guanidine thiocyanate solution

A sample extraction was done by grinding of 100 mg of plant tissue with 0.5 ml of guanidine thiocyanate (GTC) solution (9 M GTC**,** 0.1 M Tris-HCl, pH 7.0, 1.5% Triton X-100, 25 mM Na_2-EDTA) in 1.5 ml tube at room temperature. The crude plant extract was clarified by centrifugation for 3 min at 12,000 rpm. GTC (1.5 ml) solution and 300 µl of earlier constituted silica gel [3] were added to 400 µl of the clarified extract, the mixture was vortexed and the silica was precipitated at 12,000 rpm for 15 min. The precipitate was washed twice with 1 ml of 9 M guanidine thiocyanate**,** 0.1 M Tris-HCl, pH 7.0, followed by 0.5 ml of 70% ethanol, once with 0.5 ml of acetone and dried for 5-10 min at 56ºC. RNA was extracted twice with 200 µl of water at 56ºC (5-10 min) and collected by centrifugation at 12,000 rpm for 2 min. 3 M sodium acetate (0.1v) (pH 4.8-5.0) and 3 volumes of ethanol were added to the supernatant to precipitate RNA at -20ºC. Collected by centrifugation, the preparation of RNA was dissolved in 20-25 µl of water and stored at -20ºC.

2.5 Isolation and purification of viral RNA from TMV and PVX

To 100 µl of purified TMV in water (18 mg/ml), 900 µl of LDB containing different concentrations (from ~3.5 M to 1 M) of ATCA was added, mixed and incubated for 5, 10, 20 or 30 min at room temperature. Propanol-2 (2.5 ml) was added to each 1 ml sample obtained. The mixtures obtained were loaded on mini columns (prepared earlier) with cellulose or cotton wool. Adsorbents in each column were dried a little under low vacuum to remove excess of liquid. The viral RNA was eluted 3 times with water (Table 1).

PVX RNA was isolated from the virus suspension at concentration of 6 mg/ml. To 100 µl of the virus suspension in 5 mM phosphate buffer (pH 6.8), 900 µl of 3.9 M ATCA solution in 0.1 M Tris-HCl, pH 7.5, 25 mM Na_2-EDTA, 1.5 % Triton X-100 was added, stirred by vortex shaker for several seconds, incubated for 15 min at room temperature and centrifuged at 12,000 rpm for 5 min. PVX RNA was precipitated from the supernatant with 2 volumes of ethanol.

2.6 Isolation and purification of total RNA from *Nicotiana glutinosa* and *Solanum tuberosum*

1 g of fresh or frozen leaf tissue of *Nicotiana glutinosa*was ground to fine powder with a mortar and pestle at room temperature with 6 ml of LDB containing various concentrations of ATCA (see *Results*). Aliquots of 1.5 ml each were picked up from the suspension and incubated for 5, 10, 20 or 30 min at room temperature, and centrifuged at 10000 g for a maximum of 5 min. About 1 ml of the clarified extract was mixed with 2.5 ml of propanol-2 and opalescent mixtures were loaded onto columns with cotton wool. Further purification of RNA was processed essentially as described for TMV.

Similarly, 100 mg of fresh or frozen *Solanum tuberosum* sample (leaf, seedlings, tube plant) and 0.5 ml of LDB were ground to fine powder with a mortar and pestle at room temperature to produce the cell extract. The clarified cell ATCA-extract was obtained by centrifugation at 12,000 rpm for 3 min at room temperature, 1.25 ml of propanol-2 was added and RNA samples were stored at -20ºC or purified as for *Nicotiana glutinosa.*

2.7 Preparation of samples for analysis of infected plants

The manual juicer with removable shells was used to obtain samples for analysis of potato infected with viruses and viroid. Previously, the cellulose sorbent was washed with LDB (~3 M ATCA). Cellulose sorbent (100-200 µl) was transferred into 0.6 ml centrifuge mini tubes with a small hole at the bottom. This was inserted into 1.5 ml centrifuge mini tubes-collectors.
About 5-10 µl of the extract 30-50 mg of the leaf tissue were immediately mixed with 16-32 µl LDB, sustained for 10 minutes at room temperature, loaded onto the sorbent in the mini spin-columns, and quickly washed with 100-200 µl LDB (~3M ATCA) in Eppendorf centrifuge at low speed to remove the solution. The mini spin columns were washed then with 70% alcohol, and the RNA was eluted into tubes-receivers with 20-30 µl of water. Collected RNA samples were analyzed with specific DNA probes on the nitrocellulose membrane or by RT-PCR technique.

2.8 SDS-phenol extraction of RNA from plant samples

About 50 mg of plant tissue was homogenized in 0.25 ml of extraction buffer (100 mM Tris-HCl (pH 7.2), 1% SDS, 1 mM EDTA) and incubated in a water bath for 5 min at 100ºC to denature proteins and nucleic acids. It was then incubated in ice water for 5 min and the RNA containing supernatant was obtained by centrifugation for 5 min at 10,000 rpm. An equal volume of phenol/chloroform (9:1 v/v) saturated with 0.1 M Tris-HCl (pH 7.5) was added to the supernatant and nucleic acids were thoroughly deproteinized until no protein is visible in the interphase of the organic and aqueous phases. The total RNA was precipitated by ethanol, dissolved in water and stored at –20ºC.

2.9 Crude RNA preparation by ammonium trichloroacetate from ascites Krebs II cells

Seven days murine ascites Krebs II carcinoma cells were rinsed in sucrose buffer (8% sucrose, 10 mM Tris-HCl, 1 mM $MgCl_2$, pH 8.0). The rinsed cells may be frozen in liquid nitrogen and stored at -70ºC. About 1 ml of hypotonic buffer (10 mM Tris-HCl, 15 mM NaCl, 1 mM $MgCl_2$, pH 8,0) was added to 100 mg of the cells, and the cell suspension was kept for 20 min in ice/water bath for osmotic shock. The cells were then homogenized in Dounce homogenizer (20 tractions) and centrifuged for 10 min at 6000 rpm at 4ºC, a pellet of nuclei was formed. An equal volume of LDB solution was added to the cleared supernatant obtained and the mixture was vortexed and incubated in ice/water bath for 30 min. Then RNA was precipitated after addition of equal volume of propanol-2 overnight at 0°C and collected by centrifugation. The precipitate was washed with 70 % ethanol, dried in a vacuum desiccator and dissolved in water. RNA UV-spectra was recorded on Hitachi U-2800A spectrophotometer.

2.10 Extraction of total RNA by ammonium trichloroacetate from *Escherichia coli* (crude preparation)

Frozen *E. coli* cells XL-1 (20 mg) were suspended in 200 µl of ice-cold solution (200 µg/ml egg lysozyme, 10 mM Tris-HCl, 0.5 mM Na_2-EDTA, pH 8) and kept on ice for 20-30 min, then 800 µl of LDB solution was added and immediately mixed at room temperature. The cell lysate was incubated for 20 min and centrifuged at 12,000 rpm (~12,000 g) for 5 min.
Two volumes of ethanol were added to the supernatant and a bulky precipitate was separated by centrifugation. The precipitate was washed twice with 70 % ethanol, dried in a vacuum desiccator for 10 minutes and suspended in 50 µl of water. RNA concentration was calculated using the equation A_{260} reading of 1.0 is equivalent to ~40 µg/ml of single-stranded RNA. The RNA preparations were analyzed by agarose gel electrophoresis in the presence of ethidium bromide [1].

3. Results

In this work, ATCA has been used for RNA extraction and purification from cells of prokaryotes and eukaryotes, and viruses by using filter paper or cotton wool. The method is highly suited for the processing of large number of samples for obtaining crude preparations of cellular RNA, highly suited for the followed detecting the target RNA.

3.1 Isolation and purification of RNA using adsorption chromatography on cellulose

The efficiency of the viroid and viral RNAs preparations to adsorb from the ATCA water-alcohol mixtures onto several types of fibrous cellulose: filter paper, ash-free cellulose filters of different texture, and Whatman 3MM paper, as accessible and low-cost material, was examined. It was found that the common filter paper and cotton wool appeared to be the best adsorption materials for RNA binding from trichloroacetate solutions in comparison with various cellulose fabrics of another origin. Whatman 3MM paper was slightly worse for this procedure.
To purify multiple samples of RNA, adsorption, washing and elution of RNA were carried out in 1 ml hypodermic syringes that were settled in the top part of a home-made vacuum cassette for 48 mini column nests and filled with cellulose slurry (0.3-0.5 ml) or 100 mg cotton wool under low vacuum of aspirator (Fig. 1).

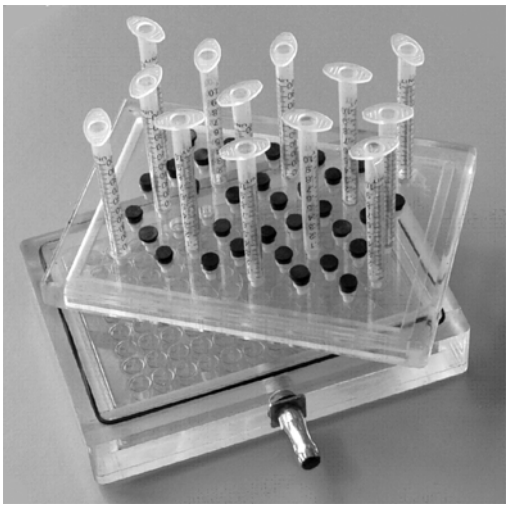

Fig 1: Vacuum manifold for mini chromatography. Prior to chromatography, the standard 96-well immune plate was placed in the lower chamber of the manifold to collect the eluates. The upper plate has conical holes, in which minicolumns are fixed. Holes (and tips of syringes) are matched with wells of the immune plate. Free holes are plugged with rubber stoppers to achieve appropriate vacuum. The rubber gasket makes sure a seal between the upper and lower chambers.

Using this technique, a large number of samples may be handled in a short span of time.

3.2 Isolation and purification of the viral RNA from TMV

Prior to RNA sorption, cellulose or cotton wool was washed with LDB, containing different concentration (from ~3.5 M to ~1 M) of ATCA, then with 4 ml of 70% ethanol. The adsorbent was dried a little under low vacuum to remove excess of liquid. In tables 1a-1d**,** the dependence of the extraction conditions on the efficiency of the TMV RNA extraction is compared. While using LDB (~1 M ATCA) (Table 1d), there is a significant reduction in the yield of RNA and the deterioration of its A_{260}/A_{280} ratio.

Table 1 (a-d). Purification of the viral RNA from TMV.

Table 1a.

	Time of incubation with LDB (~3.5 M ATCA), min											
	5			**10**			**20**			**30**		
	Eluate fractions											
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
RNA concentration, µg /ml	19	24	12	22	53	43	131	45	15	21	63	13
Fraction volume, µl	250	900	950	250	900	950	350	625	800	250	950	1000
Quantity RNA in the fraction, µg*	48	.03	72	56	31	3	46	28	12	54	29	3
Total RNA, µg	87			90			86			87		
Ratio A260/A280	2.1	2.2-	-	2.1	2.0	-	2.1	2.1	2.0	2.1	2.0	-
Percentage of the conventional yield**	97			100**			95***			96***		

Table 1b

	Time of incubation with LDB (~3 M ATCA), min											
	5			**10**			**20**			**30**		
	Eluate fractions											
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
RNA concentration, µg /ml	95	22	12	93	53	21	188	54	7	183	37	13
Fraction volume, µl	350	900	900	125	1000	1000	150	900	850	175	900	900
Quantity RNA in the fraction, µg*	34	20	10	12	53	21	28	48	6	32	33	12
Total RNA, µg	64.2			85.0			82.3			76.9		
Ratio A260/A280	2.3	2.2	2.2	2.3	2.2	2.2	2.3	2.3	2.3	2.3	2.2	2.2
Percentage of the conventional yield**	71			94			91***			85***		

Table 1c

	Time of incubation with LDB (~2 M ATCA), min											
	5			**10**			**20**			**30**		
	Eluate fractions											
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
RNA concentration, µg /ml	44	27	16	222015			432820			503023		
Fraction volume, µl	250900950			3509501000			250950950			220950950		
Quantity RNA in the fraction, µg*	112415			81915			112919			112922		
Total RNA, µg	51			42			60			62		
Ratio A_{260}/A_{280}	1.981.971.95			1.871.941.97			2.01.951.96			1.972.02.0		
Percentage of the conventional yield**	56			47***			66			69		

Table 1d

	Time of incubation with LDB (~1 M ATCA), min			
	5	**10**	**20**	**30**
	Eluate fractions			
	123	**123**	**123**	**123**
RatioA_{260}/A_{280}	1.621.641.42	1.461,511.53	1.451.461.51	1.601.541.40

*calculated as per standard equation A_{260} (1.0) = ~40 µg/ml of single-stranded RNA.
**[9].

*** RNA yield decrease with increasing time of incubation of extracts with ATCA may explain data scattering of four independent experiments (~6%) of the RNA isolation.

The data presented in Table 1 indicate that to obtain approximately 100 µg of the purified RNA preparation, the optimal conditions for helical rod-like tobacco mosaic virus deproteinization have to be considered as a treatment with LDB (~3.5 M ATCA) for 10 min at room temperature and elution of RNA with ~1.1 ml of water from the column packed

with 100 mg of cotton wool or cellulose. Electrophoretic analysis of the RNA released from TMV treated by ATCA at different concentrations of ammonium tricloroacetate is illustrated in Fig. 2.

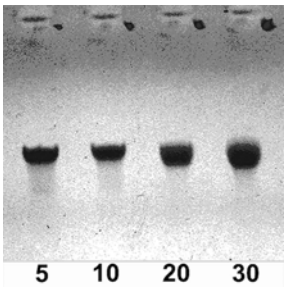

Fig 2: Agarose gel electrophoresis of the TMV RNA eluted from cotton wool with water. Numbers below show time (in min) of deproteinization of TMV with LDB (3.9 M ATCA).. Aliquots from the first fractions of the 5, 10, 20 and 30 min eluates (see Methods) were analyzed. RNA staining was done with ethidium bromide.

3.3 Comparison of the ATCA-cellulose method with the phenol/chloroform-detergent method of the virus RNA isolation

Treatment of *Potato virus X* with ~3.5 M ATCA for 5 min leads to dissociation of virus and release of viral RNA molecules (Fig. 3).

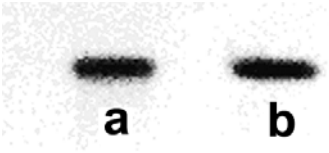

Fig 3: Agarose electrophoresis of the PVX RNA samples obtained by deproteinization of PVX with (a) 3.9 M ATCA and (b) SDS-phenol/chloroform.

Electrophoretic characteristics of PVX RNA isolated by standard SDS-phenol/chloroform and ATCA-cellulose methods were compared and found very similar. The same results were obtained for TMV RNA.

3.4 Analysis of a large number of samples

The most laborious and time-consuming procedure was preparation of samples. For ease of operation, multiple tissue samples were handled with a home-made hand juice squeezer provided with washable and sterilized shells from hard Plexiglas (Fig.4).

Fig 4: Home-made hand juice squeezer with changeable and sterilized plastic shells. 50–60 mg of the potato leaf tissue is cut into strips, placed in the cavity of left shell and then the right convex shell is set into the cavity. Both shells have terminal recesses that while combined form terminal wells. Combined shells are put into the squeezer and cell juice is pressed out into shell wells and picked up with a micropipette.

The shells have wells at both sides to collect cell tissue extract. It takes 1-1.5 minute to get 2-10 µl of plant extract, enough for a diagnostic assay, with this tool from 50-100 mg of potato, tomato, cucumber soft fresh or frozen tissue. Extract is immediately mixed with the excess of the powerful chaotropic agent and RNA was purified from *N. glutinosa* as described below.

For viroid infection, suitability of RNA samples obtained by ATCA technology was successfully examined by molecular diagnostics of test-tube potatoes from the collection of the Lorh Potato Research Institute [10, 11]. Viroid cDNA was obtained by RT-PCR from viroid infected test-tube potato, cloned as pGEM-3Z(PSTVd)$_2$[5, 8] and labeled with (dien)Pt as described [12]. Three techniques of total RNA preparation, i.e. SDS-phenol, silica-capture–guanidine thiocyanate[4] and ATCA-cellulose, for detection of PSTVd by (dien)Pt-DNA probing were compared and found to yield similar results [6].

3.5 Extraction and purification of RNA from *Nicotiana glutinosa* and *Solanum tuberosum*

As mentioned above, total RNA was also extracted and purified from *N. glutinosa*.The yield and the characteristics of the *N. glutinosa* RNA isolated with LDB (~3.5 M ATCA) and LDB (~3 M ATCA) are presented in Table 2.

Table 2 (a-b) RNA isolation from Nicotiana glutinosa

Table 2a

	Time of incubation with LDB (~3.5 M ATCA), min											
	5			10			20			30		
	Eluate fractions											
	1	2	3	1	2	3	1	2	3	1	2	3
RNA concentration, µg /ml	35	20	11	47	29	13	71	31	8	50	18	4
Fraction volume, µl	365	950	950	250	1000	1000	250	1000	1000	350	1000	950
Quantity of RNA in the fraction, µg*	13	19	10	12	30	13	18	31	8	17	18	4
Ratio A_{260}/A_{280}	1.78	1.78	1.78	1.92	1.82	1.82	1.96	1.87	1.65	1.96	1.87	-
Total RNA, µg	42			53			56			40		

Table 2b

	Time of incubation with LDB (~3 M ATCA), min								
	10			20			30		
	Eluate fractions								
	1	2	3	1	2	3	1	2	3
RNA concentration,µg /ml	52	12	3	79	25	4	45	9	4
Fraction volume, µl	500	1000	1000	250	1000	1000	500	1000	1000
Quantity RNA in the fraction, µg*	26	12	3	20	25	4	23	9	4
Ratio A_{260}/A_{280}	2.0	1.93	-	2.1	1.91	-	2.0	1.93	-
Total RNA, µg		41			50			36	

Use of 1 and 2M ATCA did not yield satisfactory results (unacceptable A_{260}/A_{280} ratio) (data not shown). Thus, the best results for *N. glutinosa*.and *S. tuberosum* RNA extractions were obtained for ~3.0 M ATCA extraction for ~15 min. Decrease in the amount of RNA extracted after 30 min exposure of the cell extract with ATCA may be attributed to greater aggregation of denatured proteins and other biopolymers which associate with about one third of the RNA to form precipitate during clarification of the extract by centrifugation prior to cellulose chromatography.

Gel electrophoresis of total RNA isolated from potato leaf tissue by ATCA technique is presented in Fig. 5. Identical electrophoresis picture was obtained with ATCA-silica method. Because of its simplicity, cotton wool column method for the plant RNA isolation was found highly suitable.

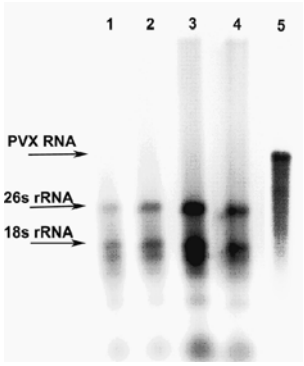

Fig 5: Electrophoresis in 1% agarose gel of the potato leaf RNA extracted with LDB containing different concentrations of ATCA: (1) 1 M, (2) 2 M, (3) 3 M and (4) 3.9 M ATCA, respectively. Each time RNA was isolated from 100 mg of plant tissue (see Methods) and dissolved in 50 µl of water. About 15 µl of the sample RNA solution was loaded on the gel. (5) PVX RNA isolated by the SDS-phenol/chloroform method and stored frozen in water at -20ºC for a long time was used as marker.

3.6 Isolation of crude RNA with ammonium trichloroacetate from ascites Krebs II cells

From the practical point of view, express diagnostics has to take a minimum time. Therefore, quick preparaion of crude extracts that contain total RNA and its characterization steps were examined.

Crude ascites RNA was prepared using ATCA as described in *Methods*. For comparison, control preparation of total RNA was isolated from the supernatant obtained after cell nuclei precipitation (*see Methods*) by the standard method using SDS-phenol/chloroform procedure. Properties of both RNA preparations were compared by UV-spectrophotometry and agarose gel electrophoresis (see Fig. 6 and 7).

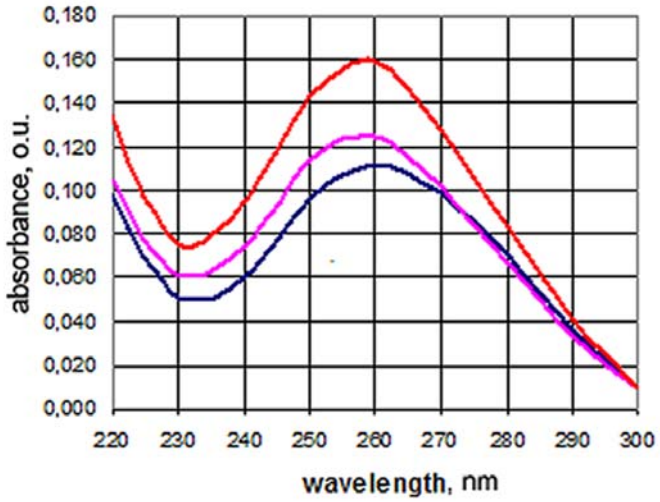

Fig 6: UV-absorption spectra of the crude RNA preparations extracted from Krebs II ascites tumor cells with: red - LDB (~1.8 M ATCA) from cells infected with Mengo virus; dark blue - extracted by SDS-phenol procedure and pink – with LDB (~1.8 M ATCA) from uninfected Krebs II cells. Note -absorbance values are not normalized to the quantity of the cells.

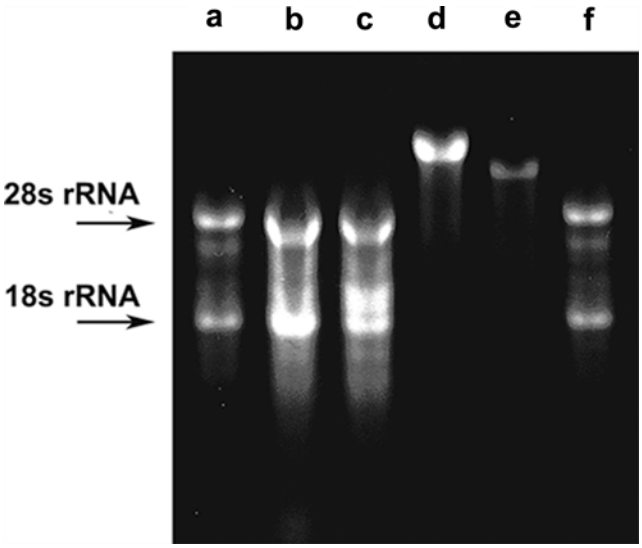

Fig7: Comparision of electrophoretic characteristics of the crude ascites cell RNA preparations obtained with LDB (~1.8 M ATCA) and by SDS-phenol extraction in 1% agarose gel. Staining was done with ethidium bromide. (**a** and **f**) total RNA isolated by SDS-phenol-method from uninfected Krebs II cells; (**b** and **c**) total RNA isolated by ATCA technique from uninfected and Mengo virus-infected Krebs II cells, respectively; (**d**) Mengo virus RNA and (**e**) TMV RNA, both virus RNAs were isolated by SDS-phenol method.

3.7 Extraction and analysis of total RNA from *Escherichia coli*

Crude preparations of total RNA were obtained from the cleared cell lysate of *E. coli* (*see* Methods). Fig. 8 shows the results of electrophoretic analysis of the RNA preparations.

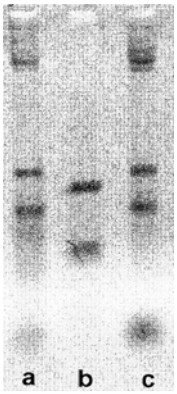

Fig 8: Extraction of RNA from the *E. coli* cleared cell lysate with (**a**) SDS-Phenol or (**c**) LDB (~3.1 M ATCA); (**b**) RNA size markers, 2300 and 1100 nucleotides. Marker RNAs were synthesized using the pGEM®Express Positive Control Template DNA producing transcripts that are 1100 and 2300 nt in length by the T7 RiboMAX™ Express Large Scale RNA Production System (Promega, USA). 2 µg samples of crude total bacterial RNA were loaded for agarose gel electrophoresis, staining was done with ethidium bromide.

4. Discussion

Viral and cellular RNAs are found to be mostly coupled with proteins. Total RNA isolation methods such as phenol-based extraction or silica gel based chromatography generally have high yields of deproteinized RNA. Currently, these methods are commonly used. Phenol and guanidine thiocyanate are powerful chaotropic agents that destabilize hydrate shells of proteins in water solutions, destroy hydrogen and hydrophobic bonds between them, dissociate most of the natural nucleoproteins and cause denaturation of proteins. Further, low-speed centrifugation separates denatured protein precipitate, leaving nucleic acids in solution. However, the toxicity of commonly used agents for deproteinization poses a grave danger, the greater quantity of isolations one has to do, the more environmental hazard it creates.

The use of less toxic chemicals for the isolation of nucleic acids have been described [13-21, 28-29]. As plants contain lot of polyphenols and polysaccharides, getting pure RNA preparations poses a challenge. Typically, total RNA extraction procedure consists of combinations of treatments of ribonucleoproteins or cells by chaotropic agents [1, 2], detergents [13, 14], proteases [15], well soluble salts of strong acids and alkali together with chloroform, polyethylene glycol [14], metal chelators[16], diluted ethanol [17], strong oxidant sodium perchlorate [14, 18], using phenol extraction and chromatography on fibrous or granular Whatman cellulose [19-21] for the last step in every RNA isolation. To isolate nucleic acids, biotech companies are commonly used magnetic particles and cartridges for chromatography with sorbents and cocktails for sorption and elution that are under patent protection.

Logemann, Schell and Willmitzer[13] compared six methods using various combinations of cetyltrimethyl ammonium bromide (CTAB), cesium chloride (CsCl), and guanidine for the extraction of RNA from potato tubers. To determine an optimal protocol for grape leaves, 15 different methods of RNA extraction were evaluated based on cost, time to complete the extraction, and quality of the RNA isolated [22].

In this paper for RNA extraction, special focus is on previously not explored, though a powerful chaotrope - ammonium trichloroacetate, virtually low toxic agent, that allows RNA isolation, which is spectorphotometerically as good as that obtained with toxic chemicals. It should be emphasized that the purified RNA was of high enough quality for molecular diagnostics by DNA probes and RT-PCR techniques.

4.1 Low Toxicity of ATCA

Trichloroacetate ion inhibits cell enzymes and dissociates nucleoproteins including plant and animal viruses. ATCA is readily soluble in water and water-organic mixtures and their constituents are less expensive than those of guanidine thiocyanate.

Trichloroacetate (TCA) ion is an efficient chaotropic agent and takes the first position in a range of chaotropes: CCl_3COO^- >SCN^- >guanidine$^+$ >ClO_4^- >Br^->NO_3^- [23]. Thus, concentrated water solutions of TCA salts are likely to induce more profound dissociation of intermolecular complexes and/or disintegration of tertiary and secondary structures of biopolymers than common salts of thiocyanic acid. Ammonium salts of trichloroacetic acid and of most acids are readily soluble in water. Moreover, ammonium trichloroacetate is not included in the list of toxic and dangerous substances (PAN Pesticides Database) [24]. TCA - trichloroacetic acid, the main constituent of ATCA, is known as the atmospheric pollutant that is derived from chlororganic by photooxidation. Influence of TCA on Norway spruce/soil was examined in detail [25, 26]. Five months exposure of young spruce trees to TCA at concentrations unlikely to be exceeded in field conditions showed no detectable effects on growth or on visible damage. No significant enhancement of TCA in soil could be detected in the directly treated soils even during the experiment. The only product of TCA metabolism/biodegradation found in the plant/soil system was CO_2 and corresponding assimilates.

According to WHO reports (2014), evidence suggests that trichloroacetic acid is not genotoxic. In mammalian studies *in vitro*, trichloroacetic acid had no genotoxic effects [27].

Thus, employment of ATCA has safety advantages for RNA extraction in comparison with cyanides, phenol, and chloroform or SDS/salt agents, that is especially important in the case of the large-scale RNA isolation, for example, for total molecular diagnostics of infectious diseases.

Using highly potent chaotropic agent, ammonium trichloroacetate, a simple, rapid, and reliable technology for the small- and large-scale purification of total RNA from viruses, *E.coli*, animal cells and plant tissues was developed.

4.2 Isolation and purification of RNA using adsorption chromatography on cellulose and cotton wool

Recent trends of practical molecular diagnostics of virus infections by the DNA-probe and PCR techniques are leaning toward increased use of the nucleic acids sorption from crude cell extracts onto solid adsorbents like amorphous silica, bentonite, powder or granular cellulose, etc[3, 4, 28] to avoid dangerous organic solvents in the extraction cocktail.

Whatman CF-11 cellulose was used by R. Franklin [20] for isolation of the phage RNA replicative intermediate and J. Semancik[20]employed it for isolation of viroid RNA. Later Su and Comeau[29] used Whatman 3MM (Whatman, Fairfield, NJ) or No. 903 paper (Schleicher &Schuell, Keene, NH) to prepare secondary fibril-associated cellulose of high absorbing capacity (designated as SF cellulose) as a matrix to isolate a wide range of nucleic acids with high yield and quality.

4.3 Preparation of purified and crude RNA from plant viruses, *Nicotiana glutinosa, Solanum tuberosum,* ascites Krebs II cells and *E. coli*

In this work, optimization of conditions for the isolation and purification of RNA was performed on plant viruses TMV and PVX. Treatment of short duration of these plant viruses by LDB (~3.5 M ATCA) completely dissociates viral RNPs for RNAs and coat proteins. Following protein separation on cotton wool or filter paper allowed us to obtain preparations of viral RNA in quantitative yields and satisfactory UV spectral (Table 1) and electrophoretic characteristics (Fig. 2, 3) similarly to RNA obtained by classical SDS-phenol/chloroform method [1, 28].

Although *Nicotiana glutinosa* and *Solanum tuberosum* are more complicated sources of RNA, high purity of total RNA preparations with high yields were obtained after 20 min extraction of the leaf tissue with LDB (~3.0 M ATCA) followed by adsorption chromatography on the cellulose (Table 2 and Fig. 5).

Crude total RNA preparations can be generated in a shorter span and, at the same time, they are suitable for the detection of viral infection of animal cells. It should be noted that crude preparation of total RNA from cells ascites carcinoma after the nuclei precipitation was essentially free from DNA (Fig. 7) and showed UV-spectral (Fig. 6) and electrophoretic characteristics (Fig. 7) very close to those obtained by the standard SDS-phenol procedure.

Thus, obtained crude preparations of total RNA were used successfully for the early diagnosis of the HeLa cells infection with the HRV2 rhino virus and infected ascites carcinoma cells with the Mengo virus using specific DNA probes labeled by (dien)Pt-DNA [7].

For *E. coli*, our goal was to obtain quantitative extraction of total bacterial RNA. As seen in Fig. 8, a small quantity of DNA is extracted with total bacterial RNA by the ATCA simplified procedure. Low-speed centrifugation does not remove chromosomal DNA by sedimentation from the bacterial cleared lysate. Thereby, preliminary treatment of the *E.coli* lysate with DNase or an additional purification of the crude RNA specimen may need to be performed.

4.4 Preparation of total RNA samples for large in a number express-diagnostic analysis

Molecular diagnosis is the most sensitive and informative step to diagnose a sample. Detection of an infection is a special case of the definition of the target antigen or a target nucleotide sequence.

Practical diagnostics is carried out usually for rapid analysis of dozens, even hundreds of samples. To quickly obtain multiple samples of a plant sap for the primary screening, manual juicer with removable sterilizable shells (Fig. 4) has been worked out. To purify RNA from a number of samples simultaneously, the manifold device, in which cellulose chromatography is performed under reduced atmospheric pressure of the water jet pump (Fig. 1), can be used.

Large-scale analysis of viral infections practically needs modification of known protocols of diagnostics to develop useful technology as safe, so less time-consuming, less expensive and simpler.

In medicine, DNA chip technology is used often for identifying a number of pathogens. This approach has been used for laboratory analysis of viral (PVA, PVM, PLRV, PVS, PVX, PVY, and viroid) potato infections on a single low density chip on the basis collection of potato from seed stocks in Lorch Potato Institute of the Russian agricultural academy. Here, plasmids with cDNAs of potato viruses and viroid were deposited and fixed on the cellulose nitrate membrane. The isolated RNA preparations of the potato samples were labeled with (dien)platinum. After hybridization, heteroduplex RNA/cDNA hybrids were determined by dot-ELISA with the antibody against (dien)Pt-DNA [10]. However, the chip technology is not cheap.

For practical mass diagnosis in agriculture, it is economically feasible to use a two-step approach. In the first step, it is advantageous to use the fastest, high-performance and affordable molecular diagnostics method. Here the most suitable method is the lateral flow immunoassay on a test strip. Its sensitivity is sufficient to cull out the infected material [11, 30]. The most expensive component of immunoassay diagnostic kit here is the primary antibodies to the pathogen.

In the second stage, remaining accessions may be tested by using more powerful laboratory techniques. However, if in the first case it is necessary to have antibodies against the pathogen, in the second one, there is a need to know the sequence of 300 nucleotides at least of the pathogen genome. Modern molecular diagnostics of infected organisms needs only zepto-attomoles of the virus (viroid) RNAs for the infection detection. Step-by-step combining of several levels of the signal multiplication, i.e. RT-PCR + DNA (with bound hapten) probing of the PCR product + dot-ELISA with chemiluminescent substrate results in detection the only RNA molecule in the specimen [5].

At this stage, it is not critical so the integrity of all the pathogen RNA molecules (DNA), as the absence in the sample the reverse transcriptase and DNA polymerase inhibitors.

For annual plants, this two-step selection approach for the seed control shall guarantee the high harvest in the field under appropriate climatic conditions.

5. Conclusions

The methodology described in this article uses a simple and ecologically safe reagent for RNA isolation. This technology might be of particular importance commercially when rapid isolation of RNA from a number of samples is to be performed. viz. for mass molecular diagnostics at force majeure (epiphytotic, epizootics, pandemics).

We believe our findings on the biosafety method of the RNA isolation, sorption RNA on a common filter paper or cotton wool, ability of the developed technique for the express

screening of many samples simultaneously will generate interest among researchers involved not only in molecular diagnostics of infection and biotechnology of RNA, but in chemistry and biochemistry of nucleic acids and nucleoproteins as well.

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Compliance with ethical standards

The animal protocols used in this work were evaluated and approved by the institutional animal ethics committee (Protocol 2010_36). They are in accordance with the Federation of Laboratory Animal Science Associations (FELASA) guidelines.

Conflict of interest

The authors declare that they have no competing interests.

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