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RNA Isolation from Cell Free – Saliva of Water Buffalo

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A B S T R A C T

Non-invasive methods are easy, simple, and effortless ways of collecting resources for the detection of biomarkers in water buffaloes. Among non-invasive fluid resources, saliva is a rich source of biomarkers, unlike milk and urine samples with no time restriction to obtain a sample. Saliva is a real-time indicator of the pathophysiology of animal health status, it has a source of many biomarkers including RNA and miRNAs, Studying RNA biology is less complex as compared to other "omics" study, but the isolation of salivary extracellular RNA is difficult in water buffaloes saliva due to high alkaline pH (pH ≥ 9), feed material and microbial contamination in the oral cavity. In addition, available kits prepared for humans were also not suitable in animal studies. To address these issues, 32 different protocols which already existed were tested. Finally, a protocol has been developed first time in buffalo for isolating total RNA from cell-free saliva has A260/280 is 1.89 ± 0.24 and concentrations 25.69 ± 7.49 ng/µL. The present study, however, unfortunately, could not isolate a very high-quality RNA to meet the general canonical principles of RNA quality and quantity for NGS transcriptomics and miRnomics.

Introduction

The water buffalo saliva is a clear, basic pH of more than 9.0, and a complex biological fluid formed by the secretions of salivary glands (parotid, sublingual and sub-mandibular). Saliva is a more attractive material used for biomarker discovery for various diseases and physiological conditions, as it is easy to collect and non-invasive, unlike blood collection. In addition, this fluid is available every time unlike other body fluids, such as milk, cervical vaginal fluids, which are available during selective physiological conditions. Therefore, saliva is a great resource for the identification of biomarkers. It contains a variety of metabolites like enzymes, antibodies, antimicrobial

constituents, growth factors, and total RNAs. Ladi *et al.,* 2014 suggested saliva as an alternative to blood sampling and they compared it with the serum of healthy women and breast cancer patients and observed a positive correlation between saliva and serum. Saliva is a mirror of the body because it is a plasma ultrafiltrate, this means most biomarkers found in blood are also present in saliva (Yoshizawa, *et al.,* 2013*).* A method developed by Pandit *et al.,* 2013 by using QIAzol lysis reagent yielded 0.89- 7.1 mg/ ml of "complete saliva" for clinical studies. Onteru *et al.,* 2015 first time attempted to isolate cellular RNA from "cell-free saliva" of water buffaloes, but could not achieve the desired quantity and quality of RNA, and they did a direct salivary transcriptomic analysis (DSTA) in Buffalo saliva, find out significant expression was recorded, Heat shock protein 70 (HSP70) and Toll-like receptor 4 (TLR4) at estrus than diestrus in buffaloes. Mostly cellular RNA of the saliva derived from the buccal mucosa, cell-free RNA originates from a wide variety of sources within the body, so that supernatant saliva would be better than the whole saliva for clinical screening (Li Y. *et al.,* 2004*).* Sullivan *et al.,* 2020 used "RNA later" for clinical samples; improve the yield from 4912ng/µl to 15,473 ng and RNA Integrity Number (RIN) from 4.5 to 7.

Based on the available literature on salivary RNA isolation different authors tried to obtain a NGS quality, but its difficulty in obtain good concentration from cell free-saliva to study the systemic patho-physiology. It is further becoming difficulty in Water buffaloes to isolate RNA from unstimulated cell-free saliva, due to high alkaline pH (pH ≥ 9), feed material, and microbial contamination in the oral cavity. In addition, available kits prepared for humans were also not suitable for animal studies. To address these issues, 32 different protocols which already existed were tested to get good quality and quantity of RNA, but no favorable results could be obtained. Hence we attempted one more method of isolation of RNA developed by Pandit *et al.,* 2013 with few modifications got satisfactory results to study the physiology of reproduction in water buffaloes.

Materials and Methods

Collection of the Saliva

The collection of the saliva samples from eight buffaloes by following the procedure established in our laboratory earlier (Ravinder *et al.,* 2016). The animals were maintained in calm stress-free environmental conditions were observed every day and the saliva samples were also collected daily before feeding early in the morning. The unstimulated saliva accumulated at the lower lip was collected by using 3 ml Pasteur pipettes (Tarson) and immediately transferred into autoclaved 1.5 - 2 ml RNAse and DNAse free microcentrifuge tubes by deflating the Pasteur pipettes gently (Ravinder *et al.,* 2016). The collected saliva samples were brought immediately to the lab and centrifuged at 3000 g for 5 minutes to remove the cells, debris, and any feed materials. The clear cell-free supernatant was removed carefully and transferred to 2 ml nuclease-free microcentrifuge tubes by using a 1 ml sterile pipette. This 'cell-free saliva' was used for the standardization of RNA isolation either immediately or after storage at -80ºC. The collection of saliva samples were under the approval of the NDRI Institutional Animal Ethics Committee (Approval No. 41-IAEC-18-2). Total RNA quantity and quality can be checked by Nanoquant.

One of the biggest bottlenecks in the current research work is to get NGS quality RNA from saliva samples. Several protocols have been tried to isolate good-quality RNA from saliva samples. Unfortunately, we didn't get

NGS-quality RNA for sequencing. The isolation of RNA from cell-free saliva was very difficult due to the high alkalinity of the buffalo saliva and high amounts of plant phenolic components. Nearly 32 protocols were tried to isolate the high-quality RNA for performing Next Generation Sequencing. The details of these protocols are presented in Table 1.

All these protocols did not result in good quality RNA for transcriptome analysis

Results and Discussion

RNA isolation

Isolation of Total RNA from cell-free buffalo saliva for NGS

The results of the 32 methods of RNA isolation were presented in the Table No.1. It is well known that a good quality total RNA is required for Next-generation sequencing (NGS) to explore a comprehensive profile of mRNA and miRNA in buffalo cell-free saliva. The present study, however, unfortunately, could not isolate a very high-quality RNA to meet the general canonical principles of RNA quality and quantity for NGS transcriptomics and miRnomics. Finally, a protocol established by Pandit *et al.,* 2013 for human saliva was utilized with a few modifications for NGS. The detailed protocol used is presented below.

Modified method (Pandit *et al.,* 2013)

A total of 250 µl of cell-free saliva was taken into a 1.5 ml nuclease-free microcentrifuge tube and 500 µl Trizol reagent (Ambion life technologies, cat # 15596018) and 20 µl of 0.2 M acidic potassium acetate were added serially. The content was mixed gently by inverting the tubes for few seconds and incubated for 10 minutes at room temperature. 200 µl of chloroform was added to each sample and mixed the contents vigorously for few seconds and incubated the tubes for 5 minutes at room temperature. Then the tubes were centrifuged at 12000 rpm for 10 minutes at 4ºC, and the supernatant from each tube was transferred to the separate 1.5 ml microcentrifuge tubes. 200 µl of the chloroform was then again added and repeated the previous step once again. To the obtained supernatant, 1/10 volume of 8 M Lithium chloride and 2.3 volumes of absolute ethanol were added and the tubes were kept for incubation at -20°C for 20 minutes. The incubated tubes were centrifuged at 12000 rpm for 10 minutes at 4ºC, and the solution was removed carefully by decanting without disturbing the pellet by using absorbent paper towels.

The pellet was washed thrice with 600 µl of 75% ethanol by centrifugation at 7500 g for 5 minutes. The supernatant was discarded and the RNA pellet was air-dried and the pellet was dissolved in 15 µl nuclease-free water. The solution containing RNA pellet was heated on a heat block at 62°C for 10 seconds, and the brief vortex was done immediately to dissolve the pellet completely in a solution.

Then the quality and quantity were measured by Nanodrop spectrophotometer. The good quality samples were stored at -80°C for future use. The results were presented in the Table No.2.

The sample used in Pandit *et al.,* 2013 was complete saliva of human, where as we tried with cell-free saliva of Water buffaloes. With a few modifications yielded a reasonable average concentration (25.69 ± 7.49 ng/µl) and quality (1.89 ± 0.24) total RNA to proceed for NGS (the values were of an average ±SE of 6 saliva samples, 3 each from the estrus and diestrus stages).

Table.1 The results of the different methods of RNA isolation from cell free saliva of water buffalo.

S.No.	Method adapted from Reference/kit	Modifications made	A260	A280	A230	Conc ng/µl	A260/230	A260/280
1	**Trizol method Chomczynski and Sacchi., 1987**	No modification done	0.39±0.10	0.28±0.07	0.58±0.08	15.60±2.71	0.67±0.21	**1.39±0.11**
2		Saliva treated with equal volume of diethyl ether then followed the Trizol	0.25±0.07	0.17±0.05	0.69±0.12	10.02±2.81	0.36±.25	**1 .48±0.04**
3		Saliva treated with 2%Triton X100 and then followed by Trizol method	0.25±0.18	0.24±0.16	0.43±0.09	11.01±7.31	0.58±0.34	**1.15±0.02**
4	**La Claire *et al.*, 1997**	No modification done	0.47±0.17	0.40±0.15	1.38±0.30	18.79±6.75	0.31±0.07	**1.19±0.05**
5	**Vanessa *et al.*, 2008**	No modification done	39.31±4.94	22.29±2.45	55.64±0.99	1572.34±197.5	0.70±0.08	**1.75±0.03**
6		No modification done	14.74±2.18	8.79+±.52	37.00±12.08	14.74±2.18	1.52±1.18	**1.71±0.10**
		Reagent						
		Method						
		obtained by						
7		the Trizol method and used it in the	26.37±2.18	18.31±1.52	95.76±12.08	806.69±2.18	0.40±1.19	**1.30±0.10**
		method of						

		Zou, *et*						
		al.,2017						
	Zou *et al.*, 2017	The supernatant of Yipping *et al.*,						
8		2017 was	0.45±0.10	0.31±0.06	1.90±0.30	17.93±3.81	0.30±0.12	**1.43±0.04**
		used as a						
		sample for						
		Trizol method						
		40 µl of 10M						
		LiCl was						
		added to the						
9		Aqueous	31.29±3.89	18.13±1.97	55.20±0.40	1251.59±155.7	0.57±0.07	**1.71±0.03**
		Phase						
		obtained by						
		the Trizol						
		method and further the method of S.No.7 was followed.						
10	**Vahed *et al.*, *2016***	No modification	0.06+0.01	0.04+0.01		2.28+0.51		**1.40+0.04**
11		Cell-Free S saliva was initially treated with diethyl Ether	0.25±0.07	0.17±0.04		10.02±2.80		**1.48±0.04**

12	**Brownlee *et al.*, 2014**	No modification	0.81±0.28	0.53±0.18		32.25±11.37		**1.51±0.**
13	**Sharma *et al.*, 2017**	No modification	1.61±0.395	1.31±0.32	3.77±0.00	64.20±15.76	0.53±0.00	**2.00v0.00**
14		The saliva was treated with Triton-x 100 and 1%SDS	1.9±0.44	1.25±0.30	3.52v0.11	77.94±17.59	0.47±0.11	**1.45±0.06**
15	**Das *et al.*, 2010**	No modification Done	0.03±0.01	0.08±0.04	0.49±0.27	1.01±0.35	0.30±0.28	**0 .46±0.14**
16	**Exosomes isolation kit**	No modification	0.02±0.00	0.01±0.00		15.50±2.8		**1.45±058**
	(Exiqon).	Done						
17	**El-Ashram *et al.*, 2016**	No modification	0.2±0.10	0.31±0.17	1.10±0.60	8.08±4.21	0.25±0.37	**1. 02±0.08**
18		The cell free saliva and lysis buffers were used in equal volumes.	0.05+0.02	0.28+.026	0.31+0.04	1.88+0.68	0.15+0.04	**1.25+0.11**
19		The supernatant from the method 18 was utilized for PVP application	0.06+0.00	0.05+0.01	3.45+0.03	2.41+0.19	0.02+0.00	**4.74+1.09**

20		S. No. 20 method was followed except the usage of Nitro cellulose paper for elution rather than Whatman No. 1 paper	0.362±0.18	0.27±0.13	0.184±0.58	14.474±7.28	1.945±0.12	**1.33±0.04**
21	**Falco *et al.*, 2008**	No modification	**39.31**±4.93	**22.29**±2.45	**55.64**±1.98	**1572.34**±197.5	**0.70**±0.07	**1.75±0.03**
22	**Huded *et al.*, 2018**	No modification	0.01+0.00	0.03+0.00	0.02+0.01	0.27+0.13	0.48+0.01	**1.32+0.12**
23	**Chan *et al.*, 2007**	The aqueous phase of **Chan *et al.*,** method was mixed with 300µl of 1.2 M NaCl	0.04±0.02	0.04±0.03	0.57±0.42	1.54±1.00	1.06±0.15	**1 .16±0.91**
24	**Vidal & Suárez, 2017**	No modification	0.05±0.02	0.28±.026	0.31±0.04	1.88±0.68	0.15±0.04	**1 .25±0.11**
25	**Hanna** and **WeiXiao 2006**	No modification	0.024±0.01	0.05±0.04	0.014±0.2	1.1±0.33.	1.63±0.27	**1.22±0.14**
26	**Rodriguez *et al.*, 2015**	No modification	1.9+0.44	1.25+0.30	3.52+0.11	77.94+17.59	0.47+0.11	**1.45+0.06**

27	**EXIQON KIT** for Total RNA Isolation	No modification	0.00	0.01	0.01	0.04	0.34	**20.70**
28	**Direct Zol RNA isolation kit**	No modification	0.01	0.00	0.09	0.40	0.10	**1.67**
29	**Pandit *et al.*, 2013**	No modification	1.23±0.24	0.76±0.15	6.81±0.76	49.29±9.73	0.17±0.02	**1.61±0.02**
30		Replacement of isopropanol with Ethnol	0.44±0.10	0.32±0.10	2.27±0.051	17.79±1.2	0.21±0.04	**0.44±0.10**
31		Combination of Isopropanol with Ethnol ratio	0.19±0.06	0.11±0.08	2.82±1.29	7.41±2.51	0.11±0.04	**0.19±0.06**
32		1. 20ul of 0.2 M KOAc was added to 250 ul of saliva **2.** To this aquous phase 1/10 volume of 8M LiCl was added	**0.852±0.22**	**0.401±0.171**	**4.64±1.77**	**25.68±9.02**	**0.143±0.02**	**0.852±0.22**

Table.2 Results of the modified method (Pandit *et al.*, 2013) presented

Physiological stage	Animal No	A260	A280	A230	A260/280	A260/230	Conc. ng/µl
Estrus	7203	0.455	0.377	5.68	1.21	0.08	18.20
	7204	0.212	0.089	1.41	2.39	0.15	8.47
	7214	0.598	0.348	3.3	1.72	0.18	23.93
	Average	**0.422**	**0.271**	**3.463**	**1.773**	**0.137**	**16.867**
	SD	0.195	0.159	2.140	0.592	0.051	7.816
	SE	0.113	0.092	1.235	0.342	0.030	4.512
Diestrus	7203	1.3	0.921	9.28	1.41	0.14	51.99
	7204	0.198	0.07	1.41	1.81	0.14	7.9
	7214	1.0911	0.604	6.81	1.81	0.16	43.65
	Average	**0.86**	**0.53**	**5.83**	**2.01**	**0.15**	**34.51**
	SD	0.59	0.43	4.02	0.72	0.01	23.42
	SE	0.34	0.25	2.32	0.42	0.01	13.52

Fig.1

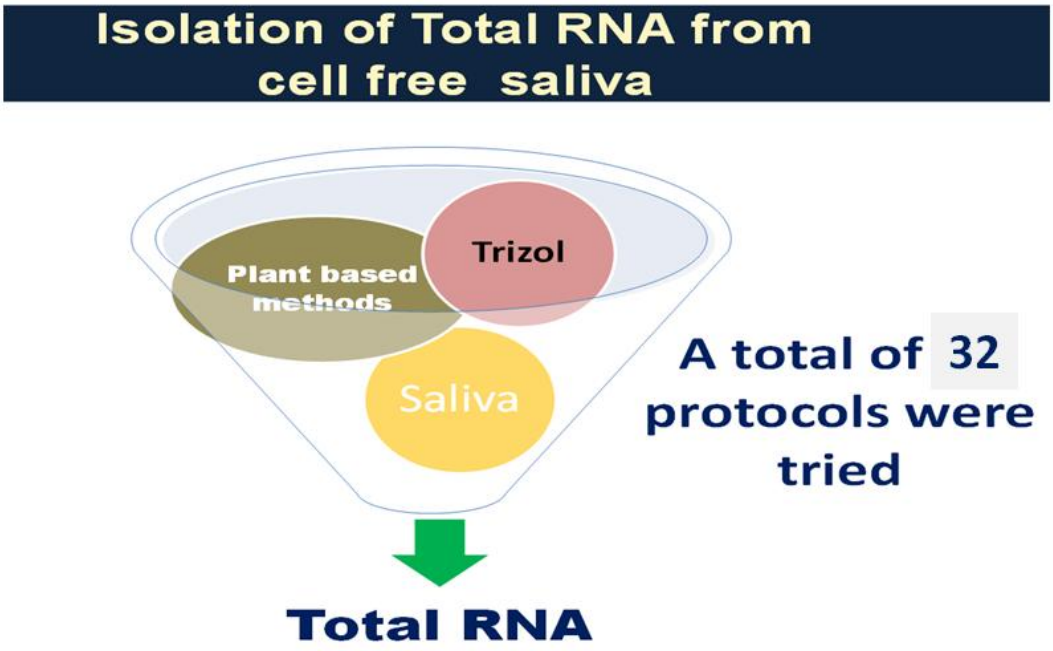
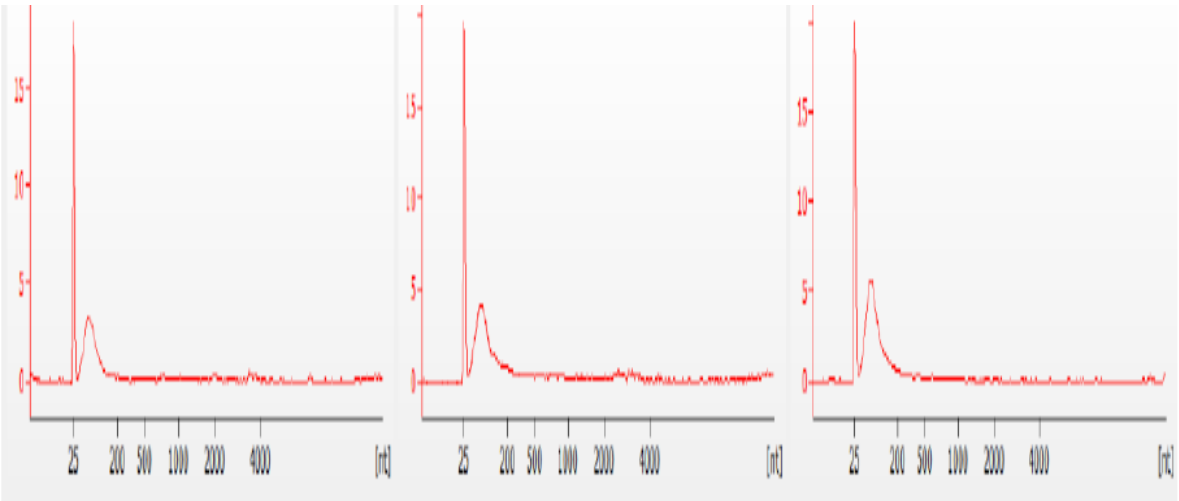


Fig.2 Sterile Pasteur Pipettes 3 ml quantity

Fig.3 Collection of saliva from lower lip & Floor of the mouth

Fig.4 Small RNA quality



This concentration was equivalent to 385.35 ng in a 15µl final volume of nuclease-free water used for RNA isolation, which was done from 250µl of cell-free saliva. With this average concentration of salivary RNA, we proceeded for NGS for transcriptomics, because the amount of RNA obtained in the present study appeared to be nearly 15 fold higher than the salivary RNA obtained from human saliva (54.2±20.1ng per 560µl of saliva) used for microarray studies (Li *et al.,* 2004), and 20 fold higher than human salivary RNA (50-80 ng/ml) used for RNA sequencing (Majem *et al.,* 2017). Although the RNA quantity and quality obtained from buffalo saliva appeared to be higher, these values may be overestimated by the presence of plant phenolic compounds, which are usually in large quantities in ruminant saliva due to their diets, and they would enhance the absorbance values at 260 nm.

Because of cell free saliva, traces of ribosomal RNA and large fragmental RNA were not included in the RNA sample; this was evidenced by gel electrophoresis images. The treatment of $1/10^{th}$ volume 8M lithium chloride to the aqueous phase may also be responsible for enrichment of small RNA in the form of pellet and separation of larger fragmental RNA collected into the supernatant (Prasanta *et al.,*. 2013), (Figure 1). The concentration and quality of small RNA were also not bad because A260/280 ratios more than 1.77 and the concentration were 24.90 ng/µl in water buffalo saliva, where as the NGS quality of RNA concentration in human saliva is 70.8 ng/ml (Wong *et al.,* 2018).

It is well known that a good quality Total RNA is required for Next-generation sequencing (NGS) to explore a comprehensive profile of mRNA and miRNA in buffalo saliva. The present study, however, unfortunately, could not isolate a very high-quality RNA to meet the general canonical principles of RNA quality and quantity for NGS transcriptomics and miRnomics despite the usage of 32 different protocols. Finally, the protocol adapted from Pandit *et al.,* 2013 with a few modifications yielded a reasonable average concentration (25.69 ± 7.49 ng/µl) and quality (1.89 ± 0.24) Total RNA to proceed for NGS. Although the RNA quantity and quality obtained from buffalo saliva appeared to be higher, these values may be overestimated by the presence of plant phenolic compounds, which are usually in large quantities in ruminant saliva due to their diets, and they would enhance the absorbance values at 260 nm. Therefore there is a need to establish a refined procedure to obtain enhanced quantity and quality of RNA from cell-free saliva**.**

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