Accepted author version posted online: 3 August 2018

SHORT COMMUNICATION

Alternative RNA Extraction Method in *Vibrio vulnificus* Infected Brown-Marbled Grouper, *Epinephelus fuscoguttatus*

Fahmeeda Mohamad Jazamuddin, Wan Mohd Aizat, Hoe-Han Goh, Chen-Fei Low and Syarul Nataqain Baharum*****

Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

*Corresponding author: nataqain@ukm.edu.my

Abstrak. Vibriosis adalah penyakit akuatik berleluasa yang disebabkan oleh spesies *Vibrio* dan telah menyebabkan kerugian yang besar terhadap spesis akuatik terutamanya kerapu harimau, *Epinephelus fuscoguttatus*. Kerumitan mekanisme molekul yang berkaitan dengan pertahanan imun ikan dapat dikaji melalui pendekatan seperti analisis transkriptomik. Kualiti dan kuantiti jumlah RNA yang tinggi adalah penting dalam ketepatan penjujukan RNA dan analisis pengekspresan gen. Kualiti RNA yang rendah akan menjejaskan analisis dan mengakibatkan kerugian masa dan wang sekiranya data perlu dianalisa semula. Oleh itu, kaedah pengekstrakan RNA yang andal dan cekap adalah langkap pertama dan paling penting untuk mendapatkan RNA jumlah yang berkualiti tinggi terutamanya bagi kajian pengekspresan gen. Terdapat banyak aspek yang perlu diambil kira apabila memilih kaedah pengekstrakan seperti keberkesanan protokol tersebut, tempoh pendedahan kimia, tempoh masa yang diambil untuk pengekstrakan dan bilangan pemindahan sampel. Protokol pengekstrakan RNA yang baik juga perlu dapat menghasilkan jumlah dan ketulenan RNA yang tinggi dan bebas daripada perencat enzim seperti nuklease (RNase), fenol, alkohol, sisa bahan kimia, protein, dan genomik DNA bagi memastikan keutuhan RNA yang diekstrak sentiasa dalam keadaan baik. Dalam kajian ini, *TransZol*™ Up Plus menghasilkan sampel RNA yang bersih dan tulen daripada tisu insang kawalan sahaja tetapi bukan daripada tisu insang yang dijangkiti dan juga tisu seluruh badan ikan. Kaedah konvensional CTAB (conventional hexadecyltrimethylammonium bromide) yang diubah suai kemudiannya digunakan sebagai kaedah alternatif untuk mengasingkan RNA dari insang dan tisu badan *E. fuscoguttatus* yang dijangkiti *Vibrio*. Kaedah CTAB telah menghasilkan jalur RNA yang utuh pada elektroforesis gel dengan nilai RIN yang lebih tinggi (>6.5), maka kaedah ini juga sesuai dalam pengekstrakan RNA jumlah yang berkualiti tinggi daripada sampel *E. fuscoguttatus.* Oleh itu kaedah ini berpotensi untuk digunapakai dalam pengestrakan RNA daripada spesis ikan terutamanya yang telah dijangkiti penyakit.

Kata kunci: CTAB, Insang, Pengekstrakan RNA, *TransZol*™ Up, Tisu Badan

Abstract. Vibriosis is a prevalent aquatic disease caused by *Vibrio* species and has led to massive loss of brown-marbled grouper, *Epinephelus fuscoguttatus*. The complexity of molecular mechanisms associated with immune defence can be studied through transcriptomics analysis. High quality and quantity of total RNAs are crucial for the veracity of RNA sequencing and gene expression analysis. A low quality RNA will compromise downstream analysis, resulting in loss of time and revenue to re-acquire the data again. Thus, a reliable and an efficient RNA isolation method is the first and most important step to obtain high quality RNA for gene expression studies. There are many aspects need to be considered when deciding an extraction method, such as the cost-effectiveness of the protocol, the duration of chemical exposure, the duration required for a complete extraction and the number of sample-transferring. A good RNA extraction protocol must be able to produce high yield and purity of

RNA free from enzyme inhibitors, such as nucleases (RNase), phenols, alcohols or other chemicals carryover, apart from protein and genomic DNA contamination, to maintain isolated RNA integrity in storage condition. In this study, *TransZol*™ Up produced clean and pure RNA samples from control gills only but not from the infected gill and whole-body tissues. Modified conventional CTAB (conventional hexadecyltrimethylammonium bromide) method was then used as an alternative method to isolate RNA from gill and whole-body tissues of *Vibrio*-infected *E. fuscoguttatus*. Modified CTAB method produced intact RNA on gel electrophoresis with higher RIN number (>6.5) for infected gill and whole-body tissues, suggesting that this method could also be used to isolate high quality RNA from fish samples. Therefore, this method is potentially suitable to be used to extract RNA from other fish species especially those that have been infected.

Key words: CTAB, Gill; RNA Extraction, *TransZol*™ Up, Whole-Body Tissues.

Vibriosis is a type of systemic disease (Chatterjee & Haldar 2012) caused by the *Vibrio* species, a Gram-negative bacteria which can cause severe illnesses to certain internal organs of the infected hosts leading to death (Chen et al. 2000; Geng et al. 2014). It often attacks grouper cultures including brown-marbled grouper (*Epinephelus fuscoguttatus*) causing the grouper production started to decrease gradually (Ilmiah et al. 2013). Information on host-bacteria interactions and the immune responses underlying *E. fuscoguttatus* and vibriosis are still largely unknown. Transcriptomic profiling analysis has been extensively applied in fish studies for the discovery of many immune-related genes and is a very useful technique in revealing the genetic response of host towards pathogen and disease infections (Ali et al. 2014; Huang et al. 2011; Qi et al. 2016; Wang et al. 2016). Thus, to achieve an overall success of gene expression analyses, a reliable and an efficient RNA isolation method is the first and most important step to obtain high quality RNA for gene expression studies (Bharudin et al. 2014). TriZol reagent, a monophasic solution of phenol and guanidinium isothiocyanate (Rio et al. 2010), is one of the most common tehcnique widely used for extracting RNA from fish samples (Ali et al. 2014; Cui et al. 2011; Lee et al. 2016; Li et al. 2016; Rajan et al. 2013). Meanwhile, conventional hexadecyltrimethylammonium bromide (CTAB) is a non-ionic detergent that is very convenient for the isolation of DNA and RNA from polysaccharides-rich organisms such as plants (Tan & Yiap 2009). Although CTAB-based method is the major nucleic acids extraction method used for plant leaf tissues (Healey et al. 2014), its usage for extracting RNA from other types of samples such as fish is yet to be explored. To our knowledge, there is currently no studies reported on extracting RNA from fish samples specifically from fish whole-body or other any organs such as gill by using CTAB method.

Fish sampling were conducted at Makmal Penyakit Ikan, Universiti Malaysia Terengganu (UMT). A total of 300 brown-marbled groupers (*Epinephelus fuscoguttatus*) fingerlings with average length of 5 cm and average weight of 2.20 g obtained from Fisheries Research Institute (FRI) Tanjung Demong, Besut in Terengganu, Malaysia were acclimatised in six glass aquariums (50 fishes/aquarium) with running aerated seawater (25 ℃, salinity ~35 parts per thousand (PPT), pH 7.9) for one week. For *V. vulnificus* experiment, 150 healthy grouper fingerlings were immersed in 20 L seawater containing 1 x 10^7 CFU mL^{-1} concentration of *Vibrio vulnificus* for 30 min; whereas for control another 150 fingerlings were immersed in clean seawater, before transferred into a new aquarium containing new seawater, and observed daily for 30 days. At day 31-post-infection, survived fish of control and infected groupers were collected at the same time point and stored separately in -80°C freezer until RNA extraction. Then, RNA were firstly extracted from gill and whole-body tissues using *TransZol*™ Up Plus RNA kit (Nanogene Solution, TransGen Biotech, Beijing, China) following the manufacturer's protocols. Total RNA extracted were then treated with DNase I (RNase-free) (Thermo Fisher Scientific Inc.) to remove DNA contamination. The second method used was modified from CTAB RNA extraction method by Kim & Hamada (2005) as well as Abdul-Rahman et al. (2017). Ground gill tissues (30 mg) and whole-body tissues (2.5 g) of the fish were transferred to tubes containing 1 mL and 25 mL of CTAB extraction buffer (0.5 mM pH 8.0, 1 M Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 8.0, 2M sodium chloride (NaCl), 2% hexadecyltrimethyl ammonium bromide (CTAB) (w/v), 2.5%

polyvinylpyrrolidone 40 (PVP-40) (w/v) and 2% β-mercaptoethanol), mixed gently and incubated at room temperature for 3 min and 5 min, respectively. An equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added to each sample and completely mixed by inverting the tubes gently. The solutions were centrifuged at 13, 000 rpm at 4ºC for 20 min. Supernatants formed were transferred into new tubes and the previous step was repeated once before adding 1/3 of 12 M lithium chloride. Samples were precipitated by incubation at -20ºC for 3 h. Then, solutions were centrifuged at 13,000 rpm for 90 min for whole body tissues, and 60 min for gill tissues at 4ºC. A total of 1.0 mL of 0.1% DEPC-water was added to resuspend the precipitated samples. An equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added, mixed gently, and centrifuged at 13,000 rpm for 20 min. Supernatants were transferred to new tubes and added with 1/3 of 12 M lithium chloride before kept at -20ºC for 3 h. Solutions were then centrifuged (13,000 rpm, 30 min, 4ºC) and pellets were washed twice with 70% ethanol. The RNA pellets were air-dried for 2 min and resuspended in 0.1% DEPC-water without DNase treatment. Compared to the extraction of RNA from mangosteen pericarp by Abdul-Rahman et al. (2017), the extraction of RNA from gill and whole-bod tissues differs in incubation condition and duration of the extraction. The current study's incubation time for lysing the animal tissues were 3-5 minutes at room temperature while the study conducted by Abdul-Rahman et al. (2017) incubated the sample at 65 ℃ for an hour. Besides that, Abdul-Rahman et al. (2017) took three days to extract the RNA samples whereas the current study took only half day. The quality of total RNA isolated were then assessed based on absorbance ratio of $A_{260/280}$ and $A_{260/30}$, agarose gel electrophoresis and RNA integrity number (RIN) score.

Based on **Error! Reference source not found.** and Table 2, when compared to *TransZol*™ Up, CTAB extraction method produced a comparable amount of RNA concentration for both gill (214.52 ng/µL for control and 142.67 ng/µL for infected) and whole-body (149.81 ng/µL for control and 168.03 ng/µL for infected) tissues. Besides that, based on the absorbance ratios, the A_{260}/A_{280} for total RNA extracted using CTAB method also resulted with acceptable ratio when compared to *TransZol*™ Up indicated that CTAB method also can effectively remove contamination from the total RNA extracted. CTAB protocol can be advantageous compared to *TransZol*™ Up as it has more cleaning steps during the extraction process. This is because, CTAB protocol includes liquid-liquid phase separation step that needed to be done three times during extraction process. Liquid-liquid phase separation step is the addition of chloroform with isoamyl alcohol to the extracted solution. This step is very critical because it ensures higher cleaning and removing of DNA, protein and any contaminants in the supernatant (Tan & Yiap 2009) providing the samples three times cleaner. As for *TransZol*™ Up kit, it only requires one time cleaning step, which is only the addition of chloroform for phase separation. Next, the gel electrophoresis results shown in Figure 1 are the images of 18S and 28S rRNA bands for gills and whole-body tissues after extraction by *TransZol*™ Up kit with DNase treatment and CTAB method without DNase treatment. The results suggested clear and intact 18S and 28S rRNA bands of gill (Figure 1(a)) and whole-body (Figure 1(b)) for extraction using CTAB method when compared to *TransZol*™ Up. RNA samples extracted using CTAB did not show visible genomic DNA contamination even without DNase treatment because apart from having better liquid-liquid phase separation, CTAB also has better RNA precipitation step that is by using lithium chloride (LiCl). LiCl precipitation provides a major advantage over other precipitation methods because it can efficiently precipitate RNA but does not effectively precipitate DNA, proteins, and carbohydrates making it most suitable for purification of RNA (Walker & Lorsch 2013).

Apart from that, the quality of total RNA extracted from gill and whole-body tissues were analysed by Agilent 2100 Bioanalyzer to determine RNA Integrity Number (RIN). The corresponding electropherograms for gill and whole-body samples were shown in Figure 2. Based on the RIN score, *TransZol*™ Up produced clean and pure RNA samples from the control gill (RIN more than 7) only but not from the infected gill (**Error! Reference source not found.**) and whole-body tissues (**Error! Reference source not found.**). Extracting RNA from the infected gill and whole-body tissues could be challenging for *TransZol*™ Up due to the pathological effects caused by *V. vulnificus* infection such as hyper-production of mucus, skin discolouration, fin erosion, skin lesion, haemorrhage and reddening of the internal organs including gill. These effects might cause additional contamination to the samples.

Contamination by other organisms and excessive production of mucus are some of the challenges associated with the isolation of RNA from marine organisms (Stefanik et al. 2013). Previously, there are a few studies showing that *TransZol*^{TM} Up failed to produce high yield and quality of RNA in plant samples such as peanut seeds (Huang et al. 2012) and mangosteen pericarp (Abdul-Rahman et al. 2017). Thus, this suggested that the effectiveness of CTAB protocol showed that CTAB method can be an alternative method for extraction of RNA from diseased tissues including fish samples. In conclusion, the evaluation of two RNA extraction methods showed that *TransZol*^{TM} Up is suitable for extraction of RNA from uninfected (control) gill while CTAB method is preferred for the extraction from infected gill and whole-body tissues. High purity of total RNA, intactness of rRNA bands and higher RIN score from CTAB method was also considered to be suitable for extracting RNA from difficult tissues especially animal tissues that are greatly infected by disease.

ACKNOWLEDGEMENTS

This work was supported by Research University Grants from Universiti Kebangsaan Malaysia (UKM-GUP-2014-083 and DIP 2015-024). We acknowledged Azhani Abdul-Rahman for the technical support and Othman Mazlan for proofread.

REFERENCES

Abdul-Rahman A, Goh H -H, Loke K -K, Noor N M and Aizat W M. (2017). RNAseq analysis of mangosteen (*Garcinia mangostana* L.) fruit ripening. *Genomics Data 12*: 159–160. doi:10.1016/j.gdata.2017.05.013

Ali A, Rexroad C, Thorgaard G, Yao J and Salem M. (2014). Characterization of the rainbow trout spleen transcriptome and identification of immune-related genes. *Frontiers in Genetics 5*: 1–17. doi:10.3389/fgene.2014.00348

Bharudin I, Zaki N Z, Abu Bakar F D, Mahadi N M, Najimudin N, Illias R M and Murad A M A. (2014). Comparison of RNA extraction methods for transcript analysis from the psychroplilic yeast, *Glaciozyma antarctica*. *Malaysian Applied Biology 43*(2): 71–79.

Chatterjee S and Haldar S. (2012). Vibrio related diseases in aquaculture and development of rapid and accurate identification methods. *Journal of Marine Science Research and Development 1*: 1–7. doi:10.4172/2155-9910.S1-002

Chen F R, Liu P C and Lee K K. (2000). Lethal attribute of serine protease secreted by Vibrio alginolyticus strains in kuruma prawn Penaeus japonicus. *Zeitschrift fur Naturforschung - Section C Journal of Biosciences 55*(1–2): 94–99.

Cui M, Zhang Q Z, Yao Z J and Zhang Z H. (2011). Molecular cloning and expression analysis of heat-shock protein 70 in orange-spotted grouper Epinephelus coioides following heat shock and Vibrio alginolyticus challenge. *Journal of Fish Biology 79*: 486-501. doi:10.1111/j.1095-8649.2011.03045.x

Geng Y, Liu D, Han S, Zhou Y, Wang K Y, Huang X L, Chen D F, Peng X and Lai W M. (2014). Outbreaks of vibriosis associated with Vibrio mimicus in freshwater catfish in China. *Aquaculture 433*: 82–84. doi:10.1016/j.aquaculture.2014.05.053

Healey A, Furtado A, Cooper T and Henry R J. (2014). Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. *Plant Methods 10*(1): 21. doi:10.1186/1746-4811-10-21

Huang Y, Huang X, Yan Y, Cai J, Ouyang Z, Cui H, Wang P and Qin Q. (2011). Transcriptome analysis of orange-spotted grouper (Epinephelus coioides) spleen in response to Singapore grouper iridovirus. *BMC Genomics 12*(1): 556. doi:10.1186/1471-2164-12-556

Ilmiah, Jusoff K, Sukenda, Widanarni and Rustam. (2013). The role of probiotic bacteria on controlling vibriosis in tiger grouper fry (Epinephelus foscoguttatus). *World Journal of Fish and Marine Science 5*(6): 622–627. doi:10.5829/idosi.wjfms.2013.05.06.1103

Kim S H and Hamada T. (2005). Rapid and reliable method of extracting DNA and RNA from sweetpotato, *Ipomoea batatas* (L). *Lam. Biotechnology Letters 27*(23–24): 1841–1845. doi:10.1007/s10529-005-3891-2

Lee X, Yi Y, Weng S, Zeng J, Zhang H, He J and Dong C. (2016). Transcriptomic analysis of koi (Cyprinus carpio) spleen tissue upon cyprinid herpesvirus 3 (CyHV3) infection using next generation sequencing. *Fish & Shellfish Immunology 49*: 213-224. doi:10.1016/j.fsi.2015.12.007

Li P, Wang L, Kwang J, Yue G-H and Wong S-M. (2016). Transcriptome analysis of genes responding to NNV infection in Asian seabass epithelial cells. *Fish & Shellfish Immunology 54*: 342-352. doi:10.1016/j.fsi.2016.04.029

Qi Z, Wu P, Zhang Q, Wei Y, Wang Z, Qiu M, Shao R, Li Y and Gao Q. (2016). Transcriptome analysis of soiny mullet (Liza haematocheila) spleen in response to Streptococcus dysgalactiae. *Fish & Shellfish Immunology 49*: 194–204. doi:10.1016/j.fsi.2015.12.029

Rajan B, Lokesh J, Kiron V and Brinchmann M F. (2013). Differentially expressed proteins in the skin mucus of Atlantic cod (Gadus morhua) upon natural infection with Vibrio anguillarum. *BMC Veterinary Research 9*(103): 1-11. doi:10.1186/1746-6148-9-103

Rio D C, Ares M, Hannon G J and Nilsen T W. (2010). Purification of RNA using TRIzol (TRI Reagent). *Cold Spring Harbor Protocols 5*(6): 2010–2013. doi:10.1101/pdb.prot5439

Tan S C and Yiap B C. (2009). DNA, RNA, and protein extraction: The past and the present. *Journal of Biomedicine and Biotechnology 2009*: 1-10. doi:10.1155/2009/574398

Walker S E and Lorsch J. (2013). RNA purification - precipitation methods. *Methods in Enzymology 530*: 337-343 . doi:10.1016/B978-0-12-420037-1.00019-1

Wang Z, Wang B, Chen G, Jian J, Lu Y, Xu Y and Wu Z. (2016). Transcriptome analysis of the pearl oyster (Pinctada fucata) hemocytes in response to Vibrio alginolyticus infection. *Gene 575*(2): 421–428. doi:10.1016/j.gene.2015.09.014

Table 1. Quantitative analysis for RNA of control and infected gills tissue extracted using *TransZol*™ kit and CTAB method.

Method	Treatment	Samples	RMA Concentration (ng/µL)	Mean RNA Concentration (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}	RIN
TransZol™ Up Plus RNA kit	Control	1	137.82	259.30	2.10	1.32	9.5
		2	145.79		2.10	1.44	7.6
		3	494.3		2.08	2.13	7
	Infected	1	689.6	615.75	2.14	1.56	7
		2	937.89		2.03	1.19	4
		3	219.75		2.03	2.77	5.1
CTAB method	Control	1	89.67	214.52	1.95	1.30	6.9
		2	86.76		1.92	1.26	6.1
		3	467.12		1.65	1.00	3.8
	Infected	1	88.9	142.67	1.84	1.49	6.8
		2	150.17		1.97	1.54	7.3
		3	189.12		1.97	1.32	6.6

Table 2. Quantitative analysis for RNA of control and infected whole-body tissue extracted using *TransZol*™ kit and CTAB method.

Method	Treatment	Samples	RNA Concentration (ng/µL)	Mean RNA Concentration (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}	RIN
TransZol™ Up Plus RNA kit	Control	1	76.52	83.01	2.12	0.35	5.4
		2	82.97		2.02	0.65	6.8
		3	89.54		2.09	0.43	4.5
	Infected	1	52.77	64.71	1.99	0.60	N/A
		2	69.58		2.02	0.54	10
		3	71.78		2.16	0.77	5.5
CTAB method	Control	1	112.56	149.81	2.02	1.89	7
		2	201.89		2.07	2.28	7.4
		3	134.98		2.04	2.06	7.2
	Infected	1	74.66	168.03	2.14	2.70	8.1
		2	237.41		2.07	2.18	6.8
		3	192.02		2.08	2.21	7.4

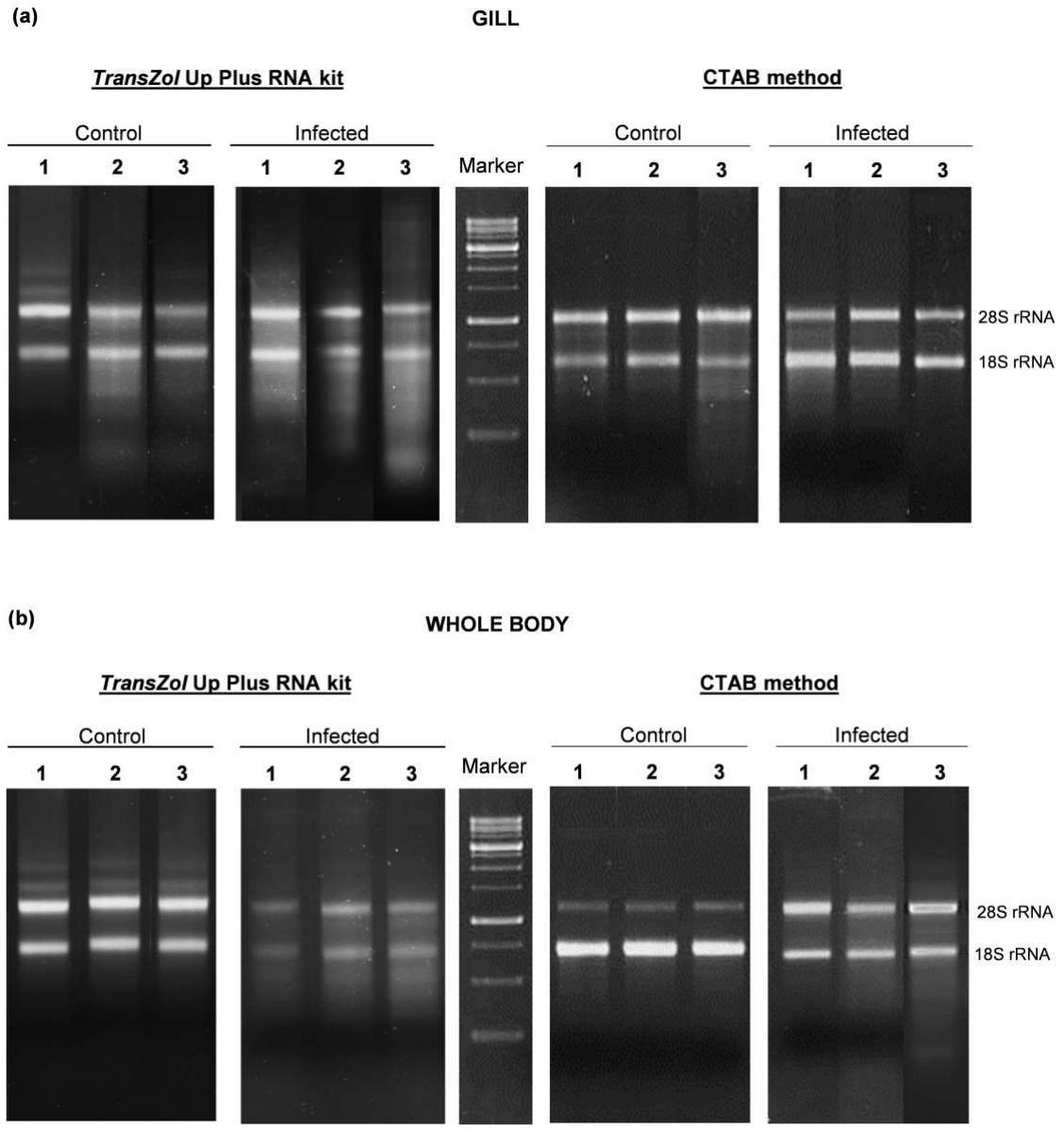

Figure 1. Agarose gel electrophoresis for RNA of control and infected (a) gill and (b) whole-body tissues extracted using TransZolTM Up Plus RNA kit and CTAB method.
161x174mm (600 x 600 DPI)

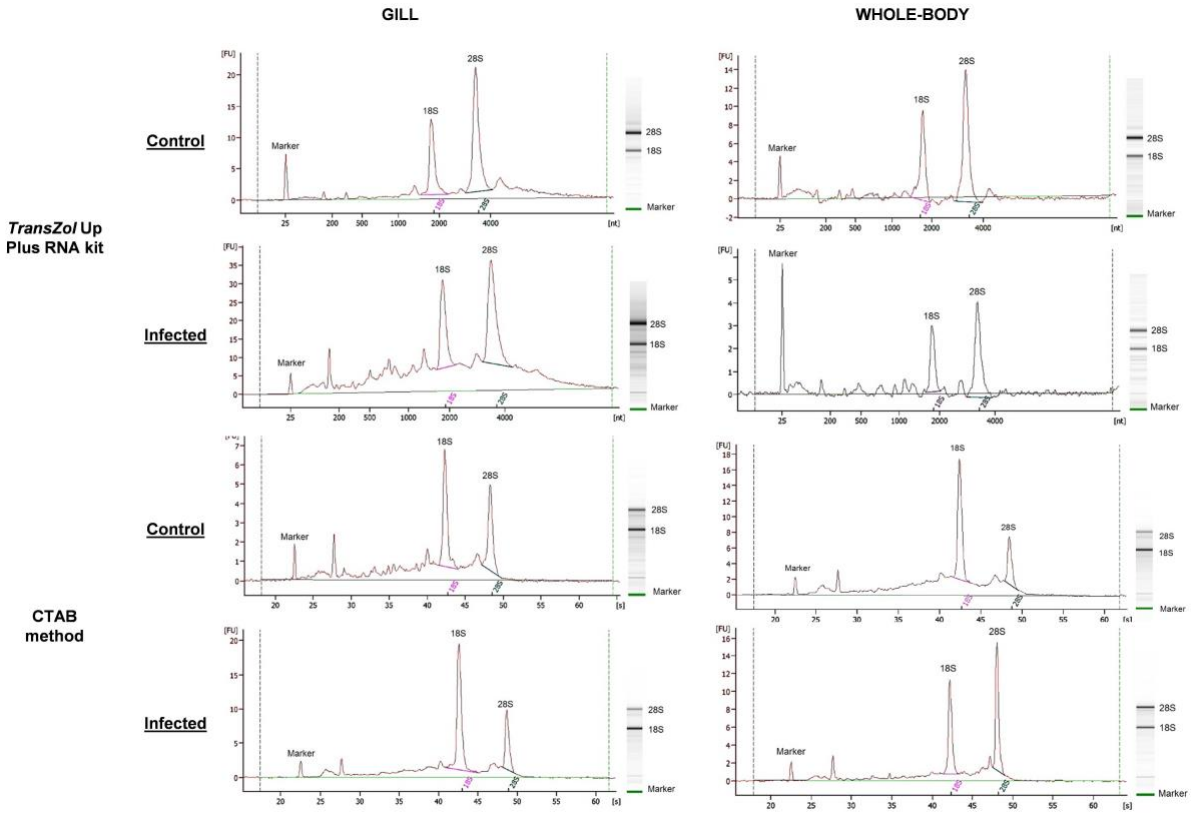

Figure 2. Electropherogram summary of Agilent 2100 Bioanalyzer for RNA samples of control and infected gills and whole-body tissues using TransZolTM Up Plus RNA kit and CTAB method.
140 x 96 mm (600 x 600 DPI)