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Comparative analysis of rodent tissue preservation methods and nucleic acid extraction techniques for virus screening purposes

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A B S T R A C T

The polymerase chain reaction (PCR) has become an essential method for the detection of viruses in tissue specimens. However, it is well known that the presence of PCR inhibitors in tissue samples may cause false-negative results. Hence the identification of PCR inhibitors and evaluation and optimization of nucleic acid extraction and preservation methods is of prime concern in virus discovery programs dealing with animal tissues. Accordingly, to monitor and remove inhibitors we have performed comparative analyses of two commonly used tissue storage methods and five RNA purification techniques using a variety of animal tissues, containing quantified levels of added MS2 bacteriophages as the indicator of inhibition.

The results showed (i) no significant difference between the two methods of sample preservation, viz. direct storage at −80 °C or 4 °C in RNAlater, (ii) lung rodent tissues contained lower levels of inhibitor than liver, kidney and spleen, (iii) RNA extraction using the EZ1 + PK RNA kit was the most effective procedure for removal of RT-PCR inhibitors.

1. Introduction

The polymerase chain reaction (PCR) is a powerful tool for nucleic acid detection because of its high sensitivity (Mullis and Faloona, 1987). In addition to its use as an essential tool for molecular biological research, it is used routinely and widely for diagnostic purposes in human and veterinary medicine and for the detection of microorganisms in quality management systems such as food production and water treatment (Chung et al., 1996; Fitzner et al., 2000; Mignotte et al., 1999; Robertson and Leek, 2006; Schlindwein et al., 2009). Initially, PCR tests were notorious for the production of false-positive results high levels of sequencing errors, problems that have decreased significantly with the use of improved protocols, high fidelity enzymes and the shift toward real-time (rt) assays using labeled probes (Rys and Persing, 1993; Schafer et al., 2000). However, in the diagnostic environment, field samples continue to raise technical problems in particular, false-negative results are widely observed due to the presence of PCR inhibitors in clinical and field samples (Ninove et al., 2011). Indeed, many compounds can act as inhibitors of PCR; the most common are hemoglobin, fat, glycogen, cell constituents, Ca^{2+}, high concentrations of nucleic acids and/or DNA binding proteins, myoglobin, immunoglobulin G, complex polysaccharides, urea or poorly preserved tissues (Al-Soud et al., 2000; Belec et al., 1998; Drosten et al., 2002; Khan et al., 1991; Monteiro et al., 1997; Queipo-Ortuno et al., 2008; Rossen et al., 1992; Tichopad et al., 2004; Wilson, 1997). All of these factors may impact both qualitatively and quantitatively on the results of diagnostic tests. The importance of such inhibitors in human studies was recently emphasized in a large-scale study, showing that they were involved much more frequently than commonly believed (Ninove et al., 2011). Since virus detection tests in animal tissue increasingly use PCR or RT-PCR, we have addressed the problem of PCR inhibitors in specific animal tissues. To this end, the presence of inhibitors was monitored comparatively following two methods of tissue preservation (−80 °C and RNAlater reagent) prior to RNA extraction and five RNA extraction and purification procedures on tissue samples containing known concentrations of

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added Enterobacteria phage MS2 (MS2), to indicate the presence of PCR inhibitors).

2. Materials and methods

2.1. Study design

The experimental model used in this study was the house mouse (*Mus musculus domesticus*). The mice were live-trapped using Sherman traps baited with sardine paste in the Montpellier area of southern France (Espiguette, Mauguio, Grammont). The captured mice were maintained in pairs at the laboratory breeding facilities (quarantine room) of the University of Montpellier II (CECEMA – authorization n° C34-172-23) under standard conditions (12 h light/12 h dark, 21 °C). One litter of five progeny from each of two breeding pairs (6 males, 4 females) were selected for the experiment and euthanized with CO_2 at 7 weeks of age.

The trapping and experimental procedures performed in this study complied with the legislation currently enforced in France (authorization n° C34-269 for JBD).

2.2. Tissue preservation

Immediately after euthanasia, liver, spleen, lungs and kidneys of each rodent were removed. Individual liver and spleen were divided into two equal parts of approximately 100 mg. They were each placed in 1.7-ml tubes: one was directly stored at −80 °C, while the other was stored in RNAlater RNA Stabilization Reagent (RRSR, Qiagen, Hilden, Germany) at 4 °C. The lungs and kidneys of each animal (weighing approximately 100–120 mg each) were placed in 1.7-ml tubes, and then stored either at −80 °C or in RRSR at 4 °C.

2.3. Sample processing

For analysis, stored samples were thawed at room temperature and ground mechanically in the presence of a 3-mm tungsten carbide bead using a Mixer Mill MM300 (Qiagen, Hilden, Germany) at 30 cycles/s frequency for 5 min with 600 μl of Minimal Essential Medium (MEM) supplemented with 3% Fetal Bovine Serum (FBS), 1% glutamine, 1% Penicillin, 1% Streptomycin and 3% Kanamycin. Homogenized tissues were then centrifuged at 13,000 × *g* for 5 min. For each sample, five aliquots of 100 μl of the supernatant fluid were prepared and spiked with 10 μl of Enterobacteria phage MS2 (MS2). This is the type species of the genus *Levivirus* within the *Leviviridae* family. Virions are non-enveloped, spherical particles with icosahedral symmetry. They contain a single-stranded positive-sense genomic RNA molecule that is 3569 nucleotides long. MS2 was selected for this study since it has been used previously as an internal control for the detection of RNA viruses (Ninove et al., 2011). MS2 was obtained from the American Type Culture Collection (ATCC ref 15597-B1). Primers and probes targeting MS2 phage (MS2F CTCTGAGAGCGGCTCTATTGGT, MS2R GTTCCCTACAACGAGCCTAAATTC, MS2-probe VIC-TCAGACACGCGGTCCGCTAT AACGA-TAMRA) were designed from published genomic sequences. The MS2 suspension was diluted to provide a Cycle threshold (Ct) value of 30 for the PCR assay. Five other aliquots of 100 μl of MEM spiked with 10 μl of MS2 were prepared in order to serve as control samples.

2.4. Nucleic acid purification

Five aliquots of each sample were used for viral RNA purification using five different techniques: (i) the EZ1 virus mini kit v 2.0 on the BioRobot EZ1XL (both from QIAGEN, Hilden, Germany), (ii) the TRIzol reagent (Invitrogen, Karlsruhe, Germany), (iii) the RNAnow™ ready-to-use reagent (Biogentex, Seabrook, TX, USA), (iv) the NucleoSpin 96 virus nucleic acid extraction kit (Macherey Nagel, Düren, Germany) and (v) Digestion with 20 μl of 20 mg/ml proteinase K (Qiagen, Hilden, Germany) for 1 h at 56 °C, associated only with the EZ1 virus mini kit. MS2 control samples were included in the nucleic acid extraction procedures of the five methods listed above. All procedures were performed according to the manufacturer's recommendations, and the final elution volume was 60 μl.

2.5. Reverse transcription and detection of MS2

Reverse transcription (RT) was performed using the TaqMan® Reverse Transcription Reagents (Life Technologies SAS, Saint Aubin, France) following the manufacturer's protocol. The program cycle consisted of 25 °C for 10 min, 48 °C for 90 min, 95 °C for 3 min and 18 °C for 20 s. Detection of MS2 was performed as previously described (Ninove et al., 2011).

2.6. Methods of analysis

The presence of residual inhibitors in each sample was estimated from the difference between the expected and obtained Ct values (shift) of the target sample as compared to that observed with the control (Ninove et al., 2011).

Overall 400 results were available for the analysis. Results were grouped into series and the analysis performed according to the following three defined criteria: the method of sample preservation (2 series with $n = 200$/series); the technique of RNA extraction (5 series with $n = 80$/series); the tissue organ (4 series with $n = 100$/series).

For each series of samples, the average Ct value (Ct_m) and its associated standard deviation (SD) were calculated as described previously (Ninove et al., 2011). The analysis was performed using two independent algorithms that correspond to different stringencies, namely 1SD and 2SD, the latter being the least stringent. Briefly, in the more stringent algorithm, the result was classified as "correct detection of MS2" (CDM) when the individual Ct value of a sample was equal to or lower than [Ct_m of its series + 1SD]; in the second algorithm, 2SD was used as the criterion.

For the preservation methods, RNA extraction techniques and organ tissue samples, pairwise comparisons between series of CDMs were performed using the Mann–Whitney nonparametric test. The alpha probability threshold of significance used was 0.05. All statistical analyses were performed using the IBM SPSS® statistic 20 software (http://www-01.ibm.com/software/fr/analytics/spss/statistics20/).

3. Results

In all samples, the methods for detecting the presence of inhibitors were as described above (Table 1). The specific method for storage of animal tissue (−80 °C or RNAlater) did not significantly influence the role of inhibitors regardless of the type of tissue being analyzed (Fig. 1).

The performance of the EZ1 virus kit + proteinase K (PK), the EZ1 virus kit alone and the RNAnow reagent were significantly better in the presence of inhibitors than the NucleoSpin virus kit ($p = 0.003$/EZ1, $p = 0.0001$/EZ1 + PK, $p = 0.001$/RNAnow) and the TRIzol reagent ($p = 0.001$/EZ1, $p = 0.0001$/EZ1 + PK, $p = 0.0001$/RNAnow). When compared to the EZ1 virus mini kit alone and the RNAnow extraction, the EZ1 + PK was significantly the least affected by the presence of inhibitors ($p = 0.003$/EZ1, $p = 0.01$/RNAnow). The EZ1 virus kit and the RNAnow reagent were not significantly different in the presence of inhibitors ($p = 0.804$) (Fig. 1).

Irrespective of the method used for RNA purification, lungs and kidneys were the organs in which PCR was the least hindered by

Table 1
Ct_m and quantity of CDM according to sample treatment methods.

Organ	Storage		Extraction technique	Total	CDM (1 SD)	CDM (2 SD)	Ct_m
Lungs	-80°C	1	EZ1	10	8	9	36.89
		2	NucleoSpin	10	7	7	37.92
		3	RNAnow	10	**9**	9	36.42
		4	TRIzol	10	6	7	37.28
		5	EZ1 + Proteinase K	10	**9**	**10**	**33.72**
	RNAlater	6	EZ1	10	8	9	34.02
		7	NucleoSpin	10	6	8	35.51
		8	RNAnow	10	8	9	**33.43**
		9	TRIzol	10	6	7	36.26
		10	EZ1 + Proteinase K	10	**10**	**10**	**33.68**
Kidneys	-80°C	11	EZ1	10	8	**10**	34.96
		12	NucleoSpin	10	5	7	38.68
		13	RNAnow	10	8	**10**	34.37
		14	TRIzol	10	5	5	38.61
		15	EZ1 + Proteinase K	10	**9**	**10**	**33.92**
	RNAlater	16	EZ1	10	**8**	**10**	34.46
		17	NucleoSpin	10	5	6	38.34
		18	RNAnow	10	**8**	**10**	**33.87**
		19	TRIzol	10	5	6	37.7
		20	EZ1 + Proteinase K	10	**9**	**10**	**33.98**
Liver	-80°C	21	EZ1	10	6	6	**36.65**
		22	NucleoSpin	10	3	4	38.92
		23	RNAnow	10	6	7	**36.45**
		24	TRIzol	10	3	4	38.28
		25	EZ1 + Proteinase K	10	**9**	**10**	**36.63**
	RNAlater	26	EZ1	10	**8**	8	**36.44**
		27	NucleoSpin	10	3	4	38.09
		28	RNAnow	10	**8**	9	**36.3**
		29	TRIzol	10	3	4	38.81
		30	EZ1 + Proteinase K	10	**8**	**10**	**36.62**
Spleen	-80°C	31	EZ1	10	7	8	37.25
		32	NucleoSpin	10	6	7	38.57
		33	RNAnow	10	8	9	36.66
		34	TRIzol	10	4	5	36.67
		35	EZ1 + Proteinase K	10	**9**	**10**	**34.47**
	RNAlater	36	EZ1	10	6	7	35.26
		37	NucleoSpin	10	6	7	38.25
		38	RNAnow	10	7	7	35.79
		39	TRIzol	10	4	5	38.45
		40	EZ1 + Proteinase K	10	**9**	**10**	**34.1**

Green represents experiments with CDM ≥ 8.
Light gray represents experiments with CDM < 8 and >4.
Red represents experiments with CDM ≤ 4.
Bold characters represent experiments which enabled the best values of CDM or Ct_m, within a group of samples to be derived from a single organ (and stored similarly).

the presence of inhibitors (Fig. 1). However, the NucleoSpin virus kit and the TRIzol reagent were the least effective methods, with CDMs ranging from 5 to 7 [1SD] or 5 to 8 [2SD]. The best performance was obtained with the EZ1 mini kit + PK, the EZ1mini kit alone and the RNAnow reagent, all being above or equal to 8 [1SD] or 9 [2SD] (Table 1).

The results obtained with liver and spleen indicated a higher impact of inhibitors, most likely due to a higher content of blood and hence of heparin. The only method that provided CDM that was consistently higher or equal to 8 [1SD] and 10 [2SD] was the EZ1 virus kit + PK. The treatment of samples with PK prior to the EZ1-based extraction clearly improved the results. Each tissue sample treated with this protocol resulted in material apparently devoid of inhibitors.

4. Discussion

The detection of RNA viruses in animal tissue samples using RT-PCR is greatly hindered by the presence of inhibitors, which reduce or inhibit the efficiency of enzymatic reactions (Das et al., 2009; Dreier et al., 2005). The aim of this study was to identify suitable methods for storing tissue specimens and purifying viral RNA to render it free from inhibitors of RT-PCR.

To estimate the impact of inhibitors potentially present in tissues, 200 μL of sample tissue was spiked with a quantified amount of bacteriophage MS2. As indicated above, this RNA bacteriophage shares many features with known animal virus pathogens and is therefore commonly used in virus discovery programs that investigate tissues from wild caught animals. The use of MS2 has at

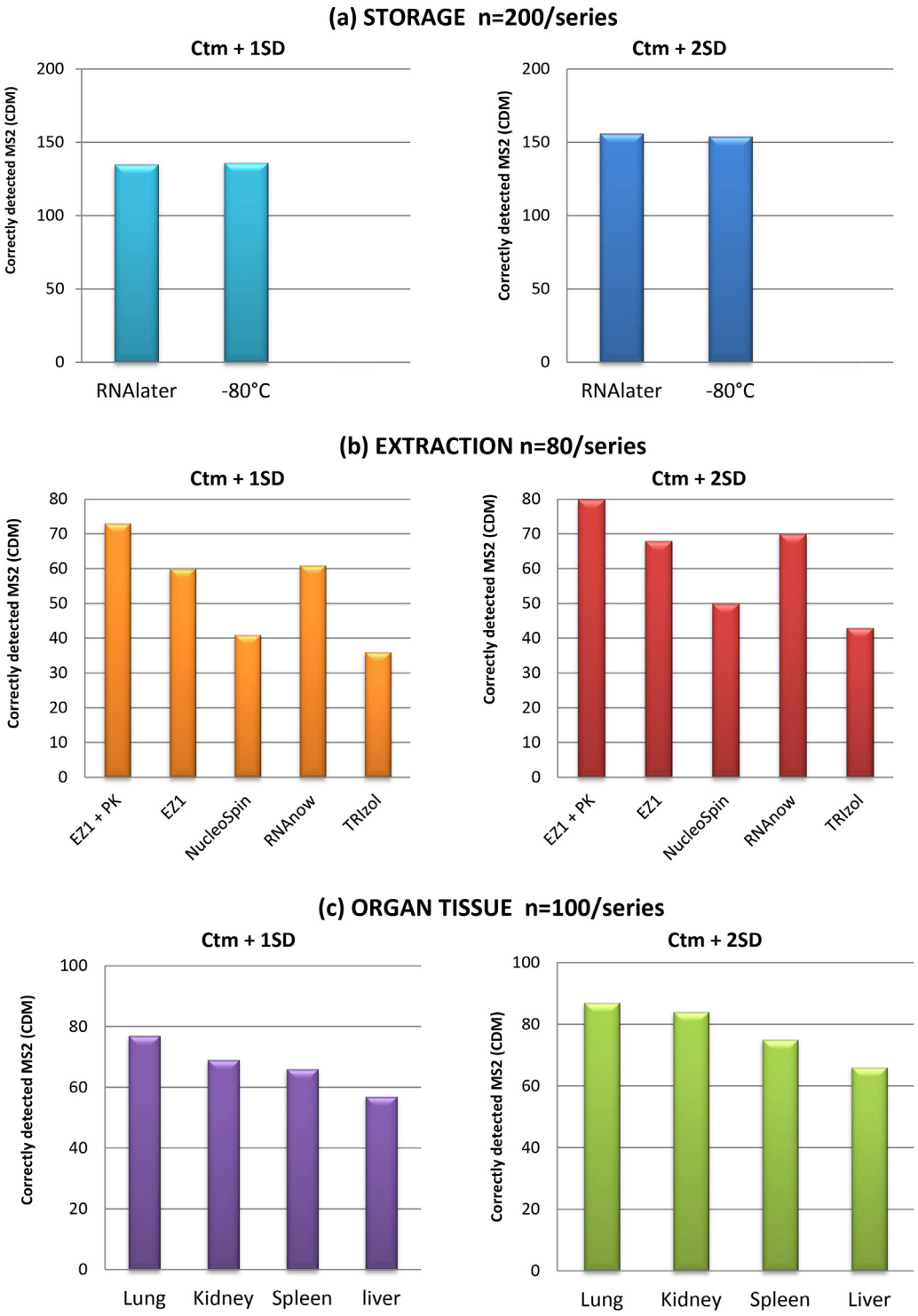

Fig. 1. (a–c). Comparison of RT-PCR efficiency among (a) preservation methods, (b) extraction techniques and (c) organ tissues.

least two advantages over pathogenic animal RNA viruses; (i) it possesses an infectious positive-stranded RNA virus genome, the stability of which probably resembles the likely viruses in the discovery program, (ii) it is non-pathogenic for humans and therefore presents no significant safety issues for laboratory personnel (Dreier et al., 2005).

This approach has been validated previously with a large range of human specimens in the context of clinical microbiology laboratories dedicated to virus diagnosis in the Public Hospitals of the city of Marseille. The initial study was conducted over an 18-month period and included 8950 clinical samples (corresponding to almost 50,000 routine-PCR and RT-PCR tests) (Ninove et al., 2011). Since preliminary analyses were satisfactory, the protocols were applied routinely in diagnostic laboratories in Marseille until February 2012. A total of 94,000 samples (corresponding to 195,000 PCR and RT-PCR tests) have now been processed using this technique in order to monitor all steps of the process, from extraction to the analysis of results. This was considered to be one

of the most flexible and cost-effective approaches for such a survey, hence enabling the detection of possible false-negative results cost-effectively.

The existence of tissue-associated RT-PCR inhibitors in vertebrates has been recognized for many years. In the present studies, lung tissue proved to be less problematic than liver specimens. Spleen and kidney samples were intermediate in this respect. These results support and extend previous studies which reported that the type of tissue from which RNA was extracted had a significant effect on PCR kinetics (Tichopad et al., 2004). In a study comparing PCR inhibition in urine and genital samples, inhibition was found to be more rare in the former than in the latter (Toye et al., 1998).

Different conditions of storage of raw samples can influence the persistence of PCR inhibitors. However, this point remains ambiguous. Indeed, it has been advocated that freezing–thawing specimens may result in reduced inhibitory compounds in the sample (Sayan et al., 2009; Verkooyen et al., 1996). In contrast, another report indicated that the level of PCR inhibitors in urine and genital specimens was considerably reduced after storage at 4 °C storage as compared to storage at −70 °C (Toye et al., 1998). In the present study, no differences were identified between either storage method, both of which are commonly used in virus screening.

As demonstrated previously, the impact of inhibitors on PCR can also be influenced by the technique of extraction. Detergents such as SDS, Triton X-100, phenol and NaOH have all been reported to have inhibitory effects on PCR (Demeke and Jenkins, 2010; Rossen et al., 1992). Ethanol and isopropanol, frequently used for elution of nucleic acids, can also inhibit PCR, when used at concentrations equal to, or higher than 1% (Peist et al., 2001). In our study, two commercial kits (EZ1 virus mini kit and NucleoSpin virus kit), two manual (RNAnow and TRIzol lysis reagents) RNA extraction techniques, as well as a technique that combines PK and EZ1 virus mini kit, were included in order to analyze their ability to reduce the amount of PCR inhibitors present in rodent tissue samples. These techniques are used routinely in virology monitoring programs in diagnostic and research laboratories. However, as far as we know, there are few data that precisely document the impact of RT-PCR inhibitors on these RNA extraction kits. The degree of inhibition was found to be lower in samples processed with the EZ1 virus mini kit or the RNAnow reagent as compared to those processed with the TRIzol reagent or the NucleoSpin virus kit. The results obtained with the EZ1 virus mini kit and the RNAnow reagent correlate with previous findings (Yera et al., 2009). The efficiency of the EZ1 virus mini kit in removing inhibitors was also demonstrated elsewhere (Montpetit et al., 2005; Peist et al., 2001). Guanidinium thiocyanate derived extraction techniques, such as RNAnow and TRIzol extraction reagents, are known to provide high yield of intact and inhibitor-free nucleic acid solutions (Chomczynski and Sacchi, 2006; Fabian et al., 2009; Lee and Lo, 2008; Li et al., 2009; Meng and Feldman, 2010; Ribaudo et al., 2001). For instance, in a study comparing four RNA extraction methods for RT-PCR detection of small round structured viruses (SRSVs) experimentally spiked in fecal specimens, it was shown that the use of guanidinium thiocyanate successfully removed PCR inhibitors from all samples, while only partial or complete inhibition was observed for the remaining methods (Hale et al., 1996). Accordingly, the results obtained with the RNAnow reagent are in line with these findings. However, in contrast, the results obtained with the TRIzol reagent contradict the aforementioned studies. This may have important consequences since TRIzol is currently one of the most widely used reagents for RNA extraction. Since the RNAnow and TRIzol reagents are both based on guanidinium thiocyanate and isopropanol precipitation, the undisputable higher efficiency of the RNAnow reagent is intriguing. The most likely explanation is that the detection of inhibitors is not implemented in research studies using human or animal tissue specimens outside of the specific diagnostic field.

The results observed with the NucleoSpin virus kit were in agreement with previously reported data: this kit did not rank highly in the comparative analysis due to its lack of efficacy to remove inhibitors (Burgener et al., 2003; Picozzi et al., 2006).

A 3 h long step of PK lysis, before the samples were processed using the EZ1 virus mini kit, consistently improved the quality of the results by decreasing the negative impact of inhibitors, regardless of the type of tissue that was processed. This is in agreement with previous reports (Guerin et al., 1995; Huijsmans et al., 2010; Kox et al., 1994; Uhrbrand et al., 2010; Wacharapluesadee et al., 2006). These data argue strongly for the systematic use of a PK-lysis step before extraction. Nevertheless, contrasting results have been reported with serum samples (Bergallo et al., 2006) and mouse tail samples (Burkhart et al., 2002). Thus, the advantage conferred by the PK lysis-based pre-treatment deserves to be further investigated using a wider range of tissues.

Finally, we are aware that the number of samples used in the present experimental study is limited. However, the results are fully corroborated by larger investigations of organs from wild-caught rodents in Europe and Africa. Indeed, data were also available for 1441 wild rodent tissue samples stored with RNA-later at 4 °C or at −80 °C, spiked with MS2 and treated with EZ1 virus mini kit or RNAnow extraction methods. All techniques also allowed the detection of MS2 RNA. Unfortunately, tissue-specific analysis could not be performed with these wild samples since various tissue organs were mixed for each rodent individual.

5. Conclusions

This study underlines the fact that inhibitors are very common in tissue samples and consequently may result in significant underestimation of the presence of RNA viruses, hence directly leading to undetected and false-negative signals. Our study also draws attention to the advantage provided by the use of an appropriate bacteriophage as an internal marker to monitor the accuracy of each step of the experimental protocol. In so doing, we demonstrate that (i) there is no significant difference between the two methods of sample preservation that were evaluated here, namely storage at 4 °C in RNA-later or at −80 °C; (ii) regardless of the technique used for RNA extraction, the lung is the tissue in which there is the lowest amount of inhibitors when compared to spleen, kidney and liver; (iii) regardless of the type of organ used for analysis, EZ1 + PK is the RNA purification method that results in the lowest lever of inhibitors.

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