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Purification methods to reduce interference by dextran sodium sulfate with quantification of gene expression in intestinal tissue samples from a piglet model of colitis

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Abstract

Dextran sodium sulfate (DSS) is commonly used to induce intestinal (i.e., colonic) inflammation in a variety of animal models. However, DSS is known to cause interference when using quantitative-real time polymerase chain reaction (qRT-PCR) methods, thereby invalidating accurate and precise measurement of tissue gene expression. Therefore, the goal of this study was to determine whether different mRNA purification methods would reduce DSS-interference. Colonic tissue samples were collected at postnatal days (PND) 27 or 28 from pigs that had not been administered DSS (Control), and two independent groups of pigs that received 1.25 g of DSS/kg of BW/d (**DSS-1** and **DSS-2**) from PND 14 to 18. Tissue samples collected were subsequently stratified into three purification methods (i.e., 9 total treatment × method combinations), including: 1) no purification, 2) purification with lithium chloride (LiCl), or 3) purification using spin column filtration. All data were analyzed using a one-way ANOVA in the Mixed procedure of SAS. The average RNA concentrations across all treatments were between 1,300 and 1,800 µg/µL for all three in vivo groups. Although there were statistical differences among purification methods, the 260/280 and 260/230 ratios fell between acceptable limits of 2.0 to 2.1 and 2.0 to 2.2, respectively, for all treatment groups. This confirms the RNA quality was adequate and not influenced by purification method in addition to suggesting the absence of phenol, salts, and carbohydrate contamination. For pigs in the Control group that did not receive DSS, qRT-PCR Ct values of four cytokines were achieved, though these values were not altered by purification method. For pigs that had undergone DSS dosing, those tissues subjected to either no purification or purification using LiCl did not generate applicable Ct values. However, when tissues derive from DSS-treated pigs underwent spin column purification, half of the samples from DSS-1 and DSS-2 groups generated appropriate Ct estimates. Therefore, spin column purification appeared to be more effective than LiCl purification, but no method was 100% effective, so caution should be exercised when interpreting gene expression results from studies where animals are exposed to DSS-induced colitis.

Lay Summary

Dextran sodium sulfate (DSS) is a chemical used to experimentally induce colonic inflammation in animal models. However, DSS causes chemical inhibition of processes involved with quantitative real-time polymerization chain reaction, thereby inhibiting the measurement of gene expression in tissues. In this study, differing methods of RNA purification were applied to remove DSS inhibition. Because no purification methods were 100% effective in alleviating this interference, caution should be exercised when interpreting gene expression results from studies where animals are exposed to DSS-induced colitis.

Key words: dextran sodium sulfate, gene expression, inhibition, interference, qRT-PCR

Abbreviations: cDNA, complementary deoxyribonucleic acid;Ct, cycle threshold; DSS, dextran sodium sulfate; IFN, interferon; IL, interleukin; LiCl, lithium chloride; mRNA, messenger ribonucleic acid; PND, postnatal day; TBCD, gamma-cyclodextrin encapsulated tributyrin; TNF, tumor necrosis factor; qRT-PCR, quantitative real-time polymerase chain reaction; RQN, ribonucleic acid quality number

Introduction

Dextran sodium sulfate (**DSS**) is utilized for the experimental induction of localized intestinal inflammation in animal models (Kerr et al., 2012; Viennois et al., 2013; Juritsch and Moreau, 2019). Specifically, it is a widely used model to understand human inflammatory bowel diseases, such as ulcerative colitis (Kerr et al., 2012; Viennois et al., 2013; Krych et al., 2018; Juritsch and Moreau, 2019). The exact mechanism by which DSS induces colonic inflammation is not known, but the clinical and histological outcomes associated with DSS are similar to those observed in patients experiencing ulcerative colitis (Viennois et al., 2013; Krych et al., 2018; Juritsch and Moreau, 2019). In an animal research context, DSS-induced colitis allows researchers to examine supplements and therapeutics that can decrease and ameliorate colonic inflammation, along with understanding immune responses and gene expression of pro- and anti-inflammatory cytokines (Kerr et al., 2012; Juritsch and Moreau, 2019).

To understand the inflammatory and immune responses associated with colitis, quantitative real-time polymerase chain reaction (**qRT-PCR**) is routinely utilized to measure the mRNA expression of cytokines (Oldak et al., 2018). This

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measurement of mRNA expression occurs by the utilization of a fluorescent molecule that tracks the amplification of complementary deoxyribonucleic acid (**cDNA**) during each cycle (Bustin and Mueller, 2005). When fluorescence levels of the sample exceed background levels, a key outcome of the qRT-PCR method is obtained, commonly referred to as a cycle threshold (**Ct**; Bustin and Mueller, 2005; Wong and Medrano, 2005; Kralik and Ricchi, 2017). As such, a lower Ct value is interpreted as a greater quantity of target mRNA in the sample, because more starting material translates into fewer amplification cycles when considering the exponential phase of gene amplification (Wong and Medrano, 2005; Kralik and Ricchi, 2017).

Substances that interfere with the biological machinery involved in qRT-PCR may inadvertently bias gene expression results. One known interfering factor of the qRT-PCR assay is DSS, which can be directly detected in tissues after administration in live-animal studies (Kerr et al., 2012; Viennois et al., 2013; Juritsch and Moreau, 2019). During qRT-PCR, DSS causes the inhibition of enzymatic reactions integral to the PCR process, including reverse transcriptase and polymerase enzymes (Krych et al., 2018; Oldak et al., 2018). When DSS is administered to animals in water at concentrations of 5% or higher, the presence of DSS is known to disrupt the qRT-PCR assay, therefore significantly increasing the number of amplification cycles before a Ct value is achieved or even causing an undetermined Ct value (Kerr et al., 2012; Viennois et al., 2013; Krych et al., 2018; Oldak et al., 2018). While research is scant on quantitatively detecting DSS in animal tissues after administration, cDNA polymerization interference due to DSS has been noted in literature since the 1970's (Hitzeman et al., 1978). As such, there exists a need to remove DSS from tissue samples prior to use of the qRT-PCR assay to analyze cytokine gene expression in models employing DSS-induced colitis (Kerr et al., 2012). Previously, two methods have been primarily utilized to remove DSS interference in exposed tissue samples: 1) purification using lithium chloride as a binding agent to isolate DSS, and 2) purification using spin column filtration (Juritsch and Moreau, 2019). Our objective in this research was to compare lithium chloride and spin column purification methods to mitigate the tissue-level interference of qRT-PCR assays in young pigs exposed to DSS-induced colitis.

Materials And Methods

Tissue sampling and processing

Animal husbandry was previously reported by Sommer et al. (2022) with tissue samples collected on postnatal days (**PND**) 27 or 28 from pigs that had not been administered DSS (**Control**) and two independent groups of pigs had been administered DSS (1.25 g/kg BW/d, ([**DSS-1, DSS-2**]) daily from PND 14 to 18. DSS-1 and DSS-2 correspond to treatments DSS and DSS+TBCD, respectively from Sommer et al. (2022). Tissues were flash frozen in liquid nitrogen then stored at −80 °C until analyzed. Between 50 and 100 mg of tissue was placed in microcentrifuge safe lock-top tubes with one 5 mm stainless steel bead per tube. Afterwards, 1 mL of Trizol solution (Invitrogen, Carlsbad, CA) was added to each tube, and the tissue was then homogenized (TissueLyser II, Qiagen, Valencia, CA) for 2 min at 30 Hz, with the tube blocks rotated 180° at 1 min, and finally centrifuged. Subsequently, 0.2 mL of chloroform was added to each tube, which was then homogenized for 30 s at 30 Hz with the tube blocks flipped after 15 s. Finally, the tubes were then centrifuged at 14,000 × *g* for 15 min at 4 °C, at which point the clear upper aqueous layer was removed and placed into a new tube for subsequent purification steps. Due to variance in volume between sample tubes, increments of 200 µL were utilized until the upper aqueous layer was completely removed; the total extracted volume was recorded for each tube.

Previous research suggests there are two methods that may consistently mitigate the interference of DSS contamination in animal tissues (Kerr et al., 2012; Viennois et al., 2013; Oldak et al., 2018; Juritsch and Moreau, 2019). In our study, three tissue purification methods were independently applied to biological replicates within each pig to extract, purify, and quantitate mRNA: 1) no purification, 2) purification with lithium chloride (**LiCl**), and 3) purification via filtration using silica-based spin columns (Part 100002334, Thermo Fisher Scientific Inc., Waltham, MA). For tissues that did not undergo any purification, the extracted upper aqueous layer from each sample was simply used for subsequent qRT-PCR steps as described below.

LiCl purification and RNA precipitation began by adding 0.1 volume of 8 M LiCl and 2 volumes of 100% ethanol proportional to the aqueous layer collected during extraction. Samples were then incubated for 2 h at −20 °C and subsequently, samples were centrifuged at 14,000 × *g* for 30 min at 4 °C. The supernatant was then decanted, and the pellet was resuspended in 200 µL of RNase-free water. This process was then repeated once more for a total of two LiCl purification cycles for each sample. Once resuspended, 0.1 volumes of sodium acetate and 2 volumes of 100% ethanol were added, and then samples were incubated for 30 min at −20 °C to allow for pellet precipitation. Samples were then centrifuged at 14,000 × *g* for 10 min at 4 °C, and the supernatant was once again decanted. The remaining pellet was then washed twice with 70% ethanol, resuspended in 20 µL of RNase-free water, and the final product stored at −80 °C.

A second clean-up method was applied with the use of spin columns as part of a purification kit (Thermo Fisher Scientific Inc.). Once extracted from the tissue, the upper aqueous layer was combined with an equal volume of 70% ethanol and quickly vortexed. Next, 700 µL of the resulting sample was pipetted into a spin column and centrifuged at 12,000 × *g* for 15 s at 4 °C. The flow-through was discarded, and the spin column was re-inserted into the collection tube. This process was repeated until all of the sample has been processed through the spin tube. Once repeated, 700 µL of the supplied wash buffer was added to the spin column and centrifuged at 12,000 × *g* for 15 s at 4 °C. The flow-through was then discarded and the spin column was re-inserted into the collection tube. After this, 500 µL of the supplied wash buffer was added to the spin column, and again centrifuged at 12,000 × *g* for 15 s at 4 °C with the flow-through discarded and spin column re-inserted into the tube. The steps with the wash buffer were repeated once more and then the samples were centrifuged at 12,000 × *g* for 1 min at 4 °C to dry the membrane of the spin column. The tube was then discarded, and the spin column was placed in a new tube with 30 µL of RNase-free water added and then incubated for 1 min. Lastly, the samples were centrifuged at 12,000 × *g* for 2 min at 4 °C, with the resulting samples being stored at −80 °C.

Extracted RNA resulting from the three purification treatments was quantified with a spectrophotometer (NanoDrop

ND-1000, Nano-Drop Technologies, Wilmington, DE) and automated capillary electrophoresis (5200 Fragment Analyzer System, Agilent, Santa Clara, CA), with all samples exhibiting a 260:280 absorbance ratio of 1.8 or higher and RQN score of 5.5 or higher, respectively. The extracted RNA was then reverse-transcribed into cDNA using a high-capacity kit (Thermo Fisher Scientific Inc.). Samples were then placed in a thermocycler (Bio-Rad, Hercules, CA) for 10 min at 25 °C, 120 min at 27 °C, 5 min at 85 °C, and then cooled to 4 °C before being stored at −20 °C until plating. The TaqMan Gene Expression Assay (Thermo Fisher Scientific Inc.) was utilized to perform qRT-PCR to quantify relative gene expression for interferon-γ (NM_213948.1; Thermo Fisher Scientific Inc.A), tumor necrosis factor-α (NM_214022.1; Thermo Fisher Scientific Inc.), interleukin-10 (NM_214041.1; Thermo Fisher Scientific Inc.), interleukin-12β (NM_214013.1; Thermo Fisher Scientific Inc.), and interleukin-6 (NM_214399.1; Thermo Fisher Scientific Inc.). Gene expression using qRT-PCR was also performed for the reference swine ribosomal protein-19 gene (XM_003131509.5; Thermo Fisher Scientific Inc.). Sample cDNA was amplified utilizing TaqMan (4304437; Thermo Fisher Scientific Inc.) oligonucleotide probes that contained a 3′ nonflorescent quencher dye and 5′ fluorescent reported dye. A QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems, Foster City, CA) was utilized to determine fluorescence (Oelschlager et al., 2019) with a total of 40 cycles. Normalization of gene expression was done through parallel amplification of ribosomal protein-19 for each sample. The comparative Ct method (Schmittgen and Livak, 2008) was utilized to calculate relative gene expression and results were expressed as a fold-change relative to Control pigs that were not administered DSS.

Statistical analysis

Data were subjected to an analysis of variance (**ANOVA**) using the MIXED procedure of SAS (version 9.4; SAS Institute, Cary, NC). A one-way ANOVA was used to determine whether the overall model was significant, and in those instances, means separation was conducted assuming an alpha level of 0.05. Results are presented as least squares means with their respective standard errors of the mean. Detectable Ct values were processed using the FREQ procedure of SAS (version 9.4; SAS Institute) to produce *F*-test comparisons. Detectable Ct values were separated into binary datasets (Ct value detectable vs. not detectable) prior to statistical analysis. After creating the binary datasets per type of tissue sample, a frequency analysis was applied to the data due to the data lacking a normal distribution of errors and percent of pigs expressing Ct scores.

Results and Discussion

Gene expression

In animal models, DSS is an efficient and cost-effective approach to induce intestinal inflammation, such as colitis (Kerr et al., 2012). After colitis induction, qRT-PCR may be utilized to understand immune and inflammatory responses (Oldake et al., 2018). However, since the 1970's, the scientific literature has noted that DSS may directly interfere with cDNA polymerization (Hitzeman et al., 1978). Importantly, such DSS-induced interference not only affects enzymes required for cDNA polymerization, but also reverse transcription of

Table 1. Effect of different purification methods on concentration, quality, and contamination of RNA extracted from distal colon tissues in young pigs[1]

In vivo Group	Purification method			SEM	*P*-value
	No purification	LiCl	Spin column		
Control					
N	8	8	8		
Concentration, μg/μL	1,934	1,800	1,646	88.3	0.09
260/280 ratio[2]	1.95[a]	2.04[b]	2.04[b]	0.005	<0.001
260/230 ratio[3]	1.91[a]	2.18[b]	2.21[c]	0.029	<0.001
RQN[4]	8.35[a]	5.50[b]	8.01[a]	0.659	0.011
DSS-1					
N	7	7	7		
Concentration, μg/μL	1,542	1,107	1,341	224.9	0.41
260/280 ratio[2]	1.92[a]	2.00[b]	2.05[c]	0.013	<0.001
260/230 ratio[3]	1.99	2.15	2.17	0.026	0.08
RQN[4]	7.41	5.57	7.40	0.572	0.05
DSS-2					
n	8	8	8		
Concentration, μg/μL	1,821[a]	1,366[b]	1,593[a,b]	175.5	0.048
260/280 ratio[2]	1.95[a]	2.03[b]	2.05[b]	0.006	<0.001
260/230 ratio[3]	1.91	2.18	2.21	0.029	<0.001
RQN[4]	8.16	6.54	7.75	0.515	0.09

[1]All samples (*N* = 7 to 8) were snap frozen in liquid nitrogen, then stored at −80 °C pending analysis.
[2]The 260/280 ratio indicates the purity of RNA at wavelengths of 260 and 280 nm.
[3]The 260/230 ratio indicates the absence of RNA contaminants at wavelengths of 260 and 230 nm.
[4]The RQN indicates the integrity of RNA on a scale from 1 to 10, with 1 being completely degraded and 10 being completely intact RNA.
[a-c]Means lacking a common superscript letter within a row differ (P < 0.05).

RNA into cDNA (Juritsch and Moreau, 2019). This is unexpected as DSS is an orally supplemented compound, meaning that interference must occur directly through the absorption and retention of DSS or DSS-derived compounds by colonocytes in live animals. In the current study, pigs received DSS orally from PND 14 to18, after which time no DSS was provided until tissue sampling on PND 27 or 28. On PND 27 or 28, complete interference of reverse transcriptase and polymerization processes were still observed in pigs that received DSS, thereby completely preventing gene expression quantification and the generation of applicable Ct values. In an attempt to ameliorate this DSS-specific interference, differing methods of RNA purification were applied.

RNA concentration, purity, and presence of contaminants were checked using standardized spectrophotometric methods on all samples across all purification methods (Table 1). No purification, LiCl purification, and spin column purification all resulted in average RNA concentrations between 1,300 and 2,000 μg/μL for each in vivo group. The 260/280 ratio provides an approximation of extracted RNA quality, and this ratio should be greater than 1.8 for the sample to be considered of acceptable quality (Fleige and Pfaffl, 2006; USDA, 2015; Oelschlager et al., 2019). While we did observe differences among purification methods, the 260/280 ratio ranged between 1.9 and 2.1, indicating that RNA quality was adequately high and not negatively influenced by either purification method when compared with samples with no purification method applied. To confirm the absence of contaminants, including carbohydrates, phenols, salts, etc., the 260/230 ratio should be between 2.0 and 2.2 (Gayral et al., 2011). There were statistical differences among purification methods, such that the no purification method had a decreased 260/230 ratio (1.91 to 1.92), indicating the likely presence of contaminants compared with the other methods that were within the expected range.

Utilizing intact RNA is crucial when running qRT-PCR, as degraded RNA may lead to decreased cDNA amplification. RNA quality can be analyzed using automated capillary electrophoresis, which then provides an RNA quality number (**RQN**). The results of the electrophoresis analysis can be binned as follows: the area prior to the 18S peak, total area of the 18S and 28S peaks, and the ratio of 18S to 28S peaks

Table 2. Frequency of detectable amplification in gene expression across three purification methods in young pigs without or with DSS administration[1]

In vivo group	Purification method			*P*-value
	No purification	LiCl	Spin column	
Control[2]				
RPL-19	100.0	100.0	100.0	1.00
GAPDH	100.0	100.0	100.0	1.00
β-actin	100.0	100.0	100.0	1.00
IFN-γ	100.0	100.0	100.0	1.00
IL-6[3]	–	100.0	100.0	1.00
IL-12[3]	–	100.0	100.0	1.00
IL-10	100.0	100.0	100.0	1.00
TNF-α[3]	–	100.0	100.0	1.00
DSS-1				
RPL-19	0.00	14.3	42.9	0.041
GAPDH	28.6	14.3	57.1	0.044
β-actin	28.6	14.3	57.1	0.044
IFN-γ	100.0	14.3	42.9	0.041
IL-6[3]	–	14.3	57.1	0.012
IL-12[3]	–	14.3	57.1	0.012
IL-10	28.6	14.3	57.1	0.044
TNF-α[3]	–	0.0	57.1	0.006
DSS-2				
RPL-19	0.0	0.0	37.5	0.028
GAPDH	0.0	0.0	50.0	0.007
β-actin	0.0	0.0	50.0	0.007
IFN-γ	100.0	0.0	37.5	0.028
IL-6[3]	–	0.0	37.5	0.028
IL-12[3]	–	0.0	25.0	0.10
IL-10	0.0	0.0	50.0	0.007
TNF-α[3]	–	0.0	50.0	0.007

[1]Values presented as the percentage of pigs within each method that produced detectable Ct values during gene expression analysis. All samples (*N* = 7 to 8) were snap frozen in liquid nitrogen, then stored at −80 °C until analysis was ran. Abbreviations: GAPDH, glyceraldenyde-3-phosphate dehydrogenaseRPL19, ribosomal protein L19.
[2]Statistical analysis did not produce a *P*-value as all samples had detectable Ct values, therefore a *P*-value of 1 is shown.
[3]Gene expression was not analyzed for the no purification treatment.

(Wong and Pang, 2013; Escobar and Hunt, 2017). These values are then utilized to establish an RQN for each sample, which ranges from 1 to 10, with completely degraded RNA being scored 1 and intact RNA being scored as a 10 (Escobar and Hunt, 2017). Across the three purification methods in this study, LiCl purification elicited a decreased RQN compared with spin column purification and no purification in the control samples. Though not significant, there was a numerical decrease of RQN within the LiCl purification in the DSS-1 and DSS-2 samples compared with spin column and no purification. No purification and spin column purification across all sample types had an RQN between 7.40 and 8.35, confirming that the RNA derived from these techniques was still intact and of good quality.

Though spectrometry and electrophoresis values indicated that RNA concentrations and quality were within acceptable ranges, these outcomes are independent of gene expression techniques that include use of reverse transcriptase and polymerase enzymes. As in vivo control pigs did not receive DSS, qRT-PCR was able to quantify gene expression of all endogenous (i.e., housekeeper) genes in tissues collected from all eight pigs, and this was not influenced by sample purification method (Table 2). For pigs that were administered DSS, we had limited success in achieving a detectable Ct value when no purification or LiCl purification methods were applied, with roughly 14% to 30% of samples yielding a Ct value, thus leading to the conclusion that polymerization did not work. However, spin column purification was successful in 37.5% to 57.1% of the samples from DSS-1 and DSS-2 groups, thereby allowing reverse transcriptase and polymerization to occur and the generation of applicable Ct values.

DSS can interfere with integral processes involved in qRT-PCR, specifically reverse transcriptase and polymerization (Krych et al., 2018; Oldak et al., 2018). This interference can occur after administration has ceased and tends to occur when DSS is supplemented in water at a concentration of 5% (i.e. weight-to-volume basis) or greater. Previous evidence suggests that DSS interference increases Ct values to the point where no amplification may occur before the final qRT-PCR cycle, thereby indicating no expression of a particular gene (Kerr et al., 2012; Viennois et al., 2013; Krych et al., 2018; Oldak et al., 2018). While various purification methods such as LiCl and spin column have been utilized (Juritsch and Moreau, 2019), neither method was completely effective in our study. However, spin column purification did appear to be more effective than LiCl, with at least half of the samples exhibiting Ct values within an expected range. Due to insufficient purification, more research needs to be done to identify more effective methods of purification. Without consistent and complete removal or mitigation of interference by DSS and its potential derivatives in animal tissues, results from the qRT-PCR assay cannot be trusted. Thus, if DSS is used in an experimental setting, DSS-induced interference must be considered as this has direct implications when interpreting gene expression outcomes. Thus, further research is warranted to identify a more efficient and accurate purification method for mitigating the interference from DSS in animal tissues.

In conclusion, there was some success in purification using spin column filtration, however, the methods employed were not robust enough to work consistently in all samples. Finally, it should be noted that the ability to perform relative gene expression is an indirect indication that DSS interference had been lessened or eliminated, but quantification of DSS and DSS-derived compounds is needed to provide more direct evidence that this is the source of interference during qRT-PCR assays.

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Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

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