Method development for post-extraction purification of animal feed in support of the Bovine Spongiform Encephalopathy (BSE) program

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Abstract

Bovine Spongiform Encephalopathy (BSE) is a fatal neurological disease with the primary route of infection through consuming contaminated protein. This disease can be transmitted to humans, and there is no treatment or vaccine. Therefore, preventing contaminated feed from entering food supply is the best means to protect public health. Per 21 CFR 589.2000-2001 and CVM Compliance Program #7371.000, the FDA prohibits certain ruminant materials in animal feed and feed ingredients.

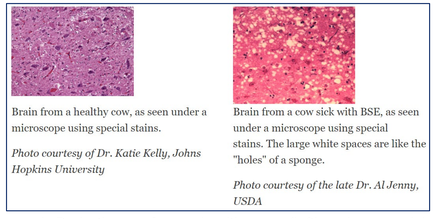
A DNA extraction method followed by a real-time PCR (qPCR) has been validated for the BSE program to identify prohibited materials in feed samples. Due to the complexity in animal feeds such as different ingredients and processing conditions, qPCR inhibitors are sometimes present in DNA extract, potentially leading to inconclusive situations that need repeats or further investigation. Simple remediation efforts such as re-run the qPCR did not correct the problem. For continuous improvement on analytical efficiency, we developed a post-extraction purification procedure to remove inhibitors for the downstream qPCR.

We evaluated a precipitation method and four commercial kits for purification of DNA extract that contained inhibitory factors. The precipitation method was not able to remove inhibitors. Among the four kits tested here, only the DNeasy® PowerClean® Pro Cleanup Kit completely removed qPCR inhibitors without affecting the detection of positive signals. Our findings revealed great potential of a post-extraction procedure for the BSE analysis. These results support a large-scale evaluation of the kit on robustness and sensitivity with more archived feed samples.

Introduction

Commonly known as the mad cow disease, Bovine Spongiform Encephalopathy (BSE) belongs to a family of fatal neurological disorders called Transmissible Spongiform Encephalopathies (TSE). This disease gradually destroys brain cells and causes tiny holes in the brain (Figure 1). The causative agent is believed to be a prion, i.e., an misfolded protein. BSE can be transmitted to human through consumption of contaminated meat from infected animals. There is no treatment or vaccine to prevent it, posing a serious threat to public health [1-3]. FDA bans high-risk materials from bovine, caprine, and ovine species in animal feed and feed ingredients for meat producing animals through 21 CFR 589.2000-2001 and CVM Compliance Program #7371.000. The validated laboratory analyses include a qPCR using feed DNA extract and an Animal Feed Microscopy (AFM). These two approaches serve as the screening and confirmatory methods in the BSE program [4].

Commercial animal feeds vary widely with regard to formulation, including different ingredients, ingredient concentrations/sources, as well as processing conditions. PCR inhibitors sometimes remain in feed DNA after the initial extraction. We observed that certain feeds and feed ingredients gave inconclusive results. Simple remediation efforts such as re-run the qPCR did not correct the problem. To improve turnaround time, conserve resources, and advance the efficiency of the analysis, we developed a post-extraction process using a commercial kit to remove qPCR inhibitors. Further purified feed DNA showed greater compatibility with the downstream qPCR, which was indicated by cycle threshold (Ct) values of the internal amplification control (IAC).



Brain from a healthy cow, as seen under a microscope using special stains.

Photo courtesy of Dr. Katie Kelly, Johns Hopkins University

Brain from a cow sick with BSE, as seen under a microscope using special stains. The large white spaces are like the "holes" of a sponge.

Photo courtesy of the late Dr. Al Jenny, USDA

Figure 1. Brain tissues from a healthy cow (left) and a cow sick with BSE (right) under microscope.
https://www.fda.gov/animal-veterinary/animal-health-literacy/all-about-bse-mad-cow-disease

Disclaimer

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Materials and Methods

Spiked references included bovine, caprine, and ovine genomic DNA (Zyagen catalog numbers GB-110F, GG-150, and GS-190F, respectively) with concentrations described in LIB 4666 [5], and standard meat and bone meals (MBM) from these 3 species provided by Dr. Myers at Center for Veterinary Medicine. Zyagen DNA also served as qPCR positive controls following SOP-000611 [6]. The extraction of feed DNA and downstream qPCR were performed following Laboratory Information Bulletins (LIB) 4486 and 4657 [7-8], respectively, to detect the presence of bovine, caprine, and ovine DNA. The internal amplification control (IAC) targets the *gfp* gene [8] with its primers and fragment synthesized by the Integrated DNA Technologies, Inc. The ethanol DNA precipitation with sodium acetate followed an online protocol [9]. The four commercial purification kits were Qiaquick® PCR Purification Kit, DNeasy® PowerClean® Pro Kit, GeneJET™ Genomic DNA Purification Kit, and SpinPrep™ PCR Clean-up Kit with detailed information in Table 2. DNA purification procedures using these kits followed each manufacturer's instruction. The study workflow and major steps using the most satisfactory kit are outlined in Figures 2 and 3. Per LIB 4657 [8], qPCR inhibition is indicated if the Ct value of a sample's IAC shows a significant delay compared to that of the positive control, i.e., for IAC, $Ct_{sample} - Ct_{positive\ control}$ (δCt) = 3.3 cycles. As $\log_2 10 \approx 3.3$, δCt≥3.3 means that amplification is suppressed by at least ten times.

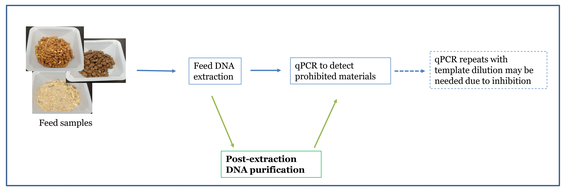

Figure 2. Study workflow. A post-extraction DNA purification step was developed for continuous improvement in analytical efficiency.

Table 1. The precipitation method couldn't remove qPCR inhibitors from DNA extract of an archived feed xxxx191.
B, bovine; C, caprine; O, ovine; G, GFP.

Sample Name	Target Name	Cт	Tm	Result
Feed DNA of xxxx191	B	Undetermined	70.8	Inhibitor indicated by IAC
	C	Undetermined	63.1	
	O	Undetermined	72.3	
	G (IAC)	Undetermined	71.7	
Ethanol precipitation	B	Undetermined	71.2	Inhibitor indicated by IAC
	C	Undetermined	63.1	
	O	Undetermined	71.9	
	G (IAC)	Undetermined	65.4	
PCR negative control	B	33.0	74.4	Controls satisfactory
	C	35.1	79.1	
	O	Undetermined	70.3	
	G (IAC)	Undetermined	62.7	
PCR positive control	B	22.0	82.0	Controls satisfactory
	C	30.8	83.1	
	O	30.0	78.8	
	G (IAC)	22.3	87.1	

Table 2. The four commercial kits evaluated in this study.

Kit name	Vendor/Catalog #	Unit price ($)	Working principle	Hands-on time	Special operation requirement
Qiaquick® PCR purification kit	Qiagen/28104	2.82	Silica Spin Filter	5 minutes	/
DNeasy® PowerClean® Pro Cleanup Kit	Qiagen/12997-50	5.40	Precipitation & Silica Spin Filter	7 min	/
GeneJET™ Gel Extraction and DNA Cleanup Micro Kit	Fisher Scientific/K0702	1.78	Silica Spin Filter	3.5 minutes	Store columns at 4°C.
SpinPrep™ PCR Clean-up Kit	Millipore/70976	3.05	Silica Spin Filter	10 minutes	Pre-warm elute buffer and assembly columns prior to experiment.

Results and Discussion

1. The precipitation method was not able to remove qPCR inhibitors from DNA of an archived test sample xxxx191 (Table 1).
2. Among the four purification kits tested in the study, the DNeasy® PowerClean® Pro Cleanup Kit (the PowerClean Kit) showed satisfactory elimination of qPCR inhibitors and the greatest sensitivity for all targets (Tables 2 and 3).
3. The PowerClean Kit showed greater efficiency and sensitivity than 1:10 dilution of the same template (Table 4).
4. DNA of an archived feed xxxx981 had been stored at -20°C for 8.5 months. The PowerClean Kit was able to remove inhibitors from this DNA extract without affecting the detection of positive signals (Table 5).

Table 3. Kit comparison using DNA of an archived feed xxxx981 that is known to be naturally positive by qPCR with diluted template and AFM. B, bovine; C, caprine; O, ovine; G, GFP. The green box highlights results achieved using DNA purified with the DNeasy® PowerClean® Pro Cleanup Kit.

Sample Name	Target Name	Cт	Tm	Positive / negative call	Result
xxxx981 DNA without kit purification	B	Undetermined	63.3	NA	Inhibitor indicated by IAC
	C	Undetermined	63.0	NA	
	O	Undetermined	63.3	NA	
	G (IAC)	Undetermined	63.2	NA	
Purification #1: Qiaquick® PCR Purification Kit	B	Undetermined	63.3	NA	Inhibitor indicated by IAC
	C	42.5	78.2	NA	
	O	Undetermined	63.3	NA	
	G (IAC)	Undetermined	68.6	NA	
Purification #2: DNeasy® PowerClean® Pro Cleanup Kit	B	29.2	82.0	pos	B, C, and O positive with controls satisfactory
	C	29.7	82.9	pos	
	O	28.1	78.8	pos	
	G (IAC)	18.1	87.1	pos	
Purification #3: GeneJET Gel Extraction and DNA Cleanup Micro Kit	B	Undetermined	63.2	NA	Inhibitor indicated by IAC
	C	Undetermined	63.0	NA	
	O	Undetermined	63.5	NA	
	G (IAC)	Undetermined	63.3	NA	
Purification #4: SpinPrep™ PCR Clean-up Kit	B	34.4	82.0	pos	B, C, and O positive with controls satisfactory
	C	30.9	82.7	pos	
	O	46.0	78.8	pos	
	G (IAC)	19.0	86.8	pos	
PCR negative control	B	47.1	64.0	neg	Controls satisfactory
	C	30.9	79.0	neg	
	O	Undetermined	69.1	neg	
	G (IAC)	45.0	75.0	neg	
PCR positive control	B	20.3	82.2	pos	Controls satisfactory
	C	24.2	83.2	pos	
	O	24.5	78.7	pos	
	G (IAC)	18.2	86.9	pos	

Table 4. Purification with the DNeasy® PowerClean® Pro Cleanup Kit (the PowerClean Kit) showed greater purification efficiency and detection sensitivity than 1:10 dilution of the same DNA extract, based on IAC Ct and target Ct values, respectively. B, bovine; C, caprine; O, ovine; G, GFP. Sp, reference spiked.

Sample Name	Target Name	Cт	Tm	positive / negative call	Result
PCR negative control	B	33.0	74.7	neg	Bovine Ct was earlier using kit purified template than dilution
xxxx384sp, 1:10 DNA dilution	B	35.2	82.2	pos	
xxxx384sp, the PowerClean Kit	B	30.9	82.0	pos	
PCR positive control	B	25.2	82.2	pos	
PCR negative control	C	34.9	79.4	neg	Caprine Ct was earlier using kit purified template than dilution
xxxx384sp, 1:10 DNA dilution	C	30.9	83.6	pos	
xxxx384sp, the PowerClean Kit	C	27.9	83.4	pos	
PCR positive control	C	26.3	83.6	pos	
PCR negative control	O	43.0	71.6	neg	Ovine Ct was earlier using kit purified template than dilution
xxxx384sp, 1:10 DNA dilution	O	29.8	78.9	pos	
xxxx384sp, the PowerClean Kit	O	27.5	78.5	pos	
PCR positive control	O	24.6	78.9	pos	
PCR negative control	G (IAC)	Undetermined	61.8	neg	kit showed greater efficiency of inhibitor elimination
xxxx384sp, 1:10 DNA dilution	G (IAC)	19.0	87.1	pos	
xxxx384sp, the PowerClean Kit	G (IAC)	16.9	86.7	pos	
PCR positive control	G (IAC)	16.0	87.2	pos	

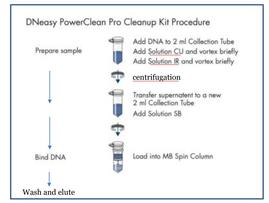

Figure 3. Major purification steps using the DNeasy® PowerClean® Pro Cleanup Kit.

Table 5. The DNeasy® PowerClean® Pro Cleanup Kit was able to purify an inhibitory DNA that had been stored at -20 °C for 8.5 months, as highlighted in the green box. Without kit purification, the qPCR couldn't produce conclusive result.

Sample Name	Target Name	Cr	Tm	Positive / negative	Result
Reference spiked xxxx981, DNA frozen for 8.5 months	B	Undetermined	63.3	NA	Inhibitor indicated by IAC
	C	Undetermined	63.0	NA	
	O	Undetermined	63.0	NA	
	G (IAC)	Undetermined	63.3	NA	
Above DNA purified by the DNeasy® PowerClean® Pro Cleanup Kit	B	29.2	82.1	pos	Spiked sample detected positive as expected
	C	26.6	83.4	pos	
	O	26.1	78.9	pos	
	G (IAC)	17.3	87.2	pos	
PCR negative control	B	Undetermined	63.3	neg	Controls satisfactory
	C	31.8	79.4	neg	
	O	Undetermined	70.4	neg	
	G (IAC)	41.7	76.3	neg	
PCR positive control	B	22.9	82.3	pos	Controls satisfactory
	C	28.2	83.2	pos	
	O	25.7	78.9	pos	
	G (IAC)	17.2	87.2	pos	

Conclusion

The DNeasy® PowerClean® Pro Cleanup Kit showed great potential for post-extraction purification of feed DNA in the BSE analysis. Our results support a large-scale evaluation of the PowerClean Kit in future study. Implementation of this new procedure will support FDA's public health mission by preventing the spread of BSE.

Future directions

1. To identify more inhibitory samples for evaluation of robustness
2. To assess detection sensitivity for all three target species by testing positive archived samples and reference-spiked matrices

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