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Project Title and Project ID: Practical strategies for improving swine oral fluid PCR performance SHIC #20-157 and NPB #20-171

Investigators: J Zimmerman, L Giménez-Lirola, Carrillo Ávila JA, Ambagala A, Jiang S, Anderson J

Institution Iowa State University

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INDUSTRY SUMMARY:

Reports in the literature describe the use of specific treatments to improve nucleic acid detection in oral fluids by qPCR: (1) Heat. Ranoa et al. (2020) reported that direct PCR was possible when human oral fluids spiked with SARS-CoV-2 were heated at 95°C for 30 minutes. (2) Diluent. Diluents can change the oral fluid mucin configuration and reduce aggregation (Hughes et al., 2019; Ridley et al., 2014). The assumption is that mucins are less able to bind PCR targets if they are dispersed. (3) Targeted concentration. Tian et al. (2008) reported a 2-log improvement in analytical sensitivity for norovirus detection using "targeted concentration", i.e., magnetic beads with a norovirus-compatible ligand.

Based on these reports, we evaluated the effect of these three treatments on the detection of porcine reproductive and respiratory syndrome virus (PRRSV), influenza A virus (IAV), porcine epidemic diarrhea virus (PEDV), and *Mycoplasma hyopneumoniae* (*MHP*) nucleic acids detection in oral fluid samples by qPCR: (1) Heat at 95°C for 30 minutes; (2). Dilution 1:2 with Tris Borate EDTA (TBE); and (3). Targeted concentration.

(1) Heat treatment was actually detrimental to the detection of PRRSV, IAV, PEDV, and *MHP* in oral fluid samples by qPCR. (2) Dilution with TBE showed no gain when compared to the undiluted sample. Overall, among positive samples, the standard extraction and amplification protocols produced the lowest Cqs in Treatments 1 and 2. (3) We were unable to complete the development and assessment of "targeted concentration", but preliminary results showed that capture of PRRSV-specific nucleic acids is achievable with a probe-based hybridization capture protocol. Further work is needed for full optimization.

Key Findings:

- Heating oral fluid samples was detrimental to the detection of PRRSV, IAV, PEDV or *Mycoplasma hyopneumoniae* nucleic acids by PCR.
- Dilution of oral fluid samples with Tris Borate EDTA showed no gain in detection when compared to a mock dilution with oral fluid or an undiluted sample
- Preliminary testing for probe-based targeted concentration showed potential for improved detection of PRRSV RNA, but will require further optimization.

Keywords: Oral fluid, qPCR, heat treatment, sample dilution, hybridization capture.

SCIENTIFIC ABSTRACT:

Objective

Early work attempting to improve swine oral fluid PCR performance involved "fine tuning" extraction protocols (Chittick et al., 2011). Later work focused on improving PCR performance by chemically degrading potential PCR inhibitors (guanidinium chloride, trypsin, dithiothreitol, sodium borohydride, and others) (Weiser et al., 2018). Improvements were made, but the significant breakthroughs sought were not achieved. The goal of this current research was to identify practical methods to improve the detection of PRRSV, IAV-S, and *Mycoplasma hyopneumoniae* (*MHP*) nucleic acids in swine oral fluids by evaluating sample treatments reported to achieve improved nucleic acid detection by qPCR: (1) Heat. Ranoa et al. (2020) reported that direct PCR was possible when human oral fluids spiked with SARS-CoV-2 were heated at 95°C for 30 minutes. (2) Diluent. Diluents can affect the oral fluid matrix by changing the mucin configuration and reducing aggregation (Hughes et al., 2019; Ridley et al., 2014). The assumption behind this approach is that mucins are less able to bind PCR targets if they are dispersed. (3) Targeted concentration. Tian et al. (2008) reported a 2-log improvement in sensitivity for norovirus detection using "targeted concentration", i.e., magnetic beads with a norovirus-compatible ligand.

Materials and methods

TREATMENT 1. Oral fluid samples known to contain PRRSV, IAV, PEDV or *MHP* (n = 8 each) were 2-fold serially diluted (neat, 1:2, 1:4, 1:8) using oral fluid known to be free of PRRSV, IAV, PEDV and *MHP* (n = 32 diluted samples per pathogen), and split into 4 aliquots, each of which was randomized to one of 4 protocols (see Table 1): (P1) heat (95°C × 30 m) and direct qPCR; (P2) heat, cool (25°C × 20 m) and direct qPCR; (P3) heat, cool, nucleic acid extraction, and direct qPCR; (P4, i.e., control) extraction and qPCR.

TREATMENT 2. Oral fluid samples containing PRRSV (n = 9), IAV (n = 10), PEDV (n = 10) or *MHP* (n = 10) were split into 3 aliquots: (D1) diluted 1:2 with TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA); (D2) diluted 1:2 with oral fluid free of PRRSV, IAV, PEDV and MHP; (D3) undiluted ("neat"). Samples were randomly ordered and then tested.

TREATMENT 3. A pool of PRRSV-specific biotin-tagged probes targeting sequences of different PRRSV-specific genes were attached to streptavidin-conjugated magnetic beads. A PRRSV modified live vaccine (MLV) was rehydrated and ten-fold diluted with molecular grade water, and dilutions 10^4 and 10^5 were processed in duplicates by a probe-based hybridization capture protocol, and compared to the standard protocol (extraction and amplification).

Results and discussion

TREATMENT 1. P4 (control) produced 32/32 positives for PRRSV, IAV, PEDV, and 31/32 for *MHP*. Cumulatively, P1, P2, and P3 produced 1/96 positive for PRRSV, 5/96 for IAV, 15/96 for PEDV, and 47/96 for *MHP*. Among positives, P4 produced the lowest Cqs, i.e., produced the strongest positive results.

TREATMENT 2: Overall, D1 and D2 did not improve detection, with D3 producing the lowest Cqs.

Overall, the results clearly showed that the methods described in the literature for heat and dilution treatments not only did not improve the process but were actually detrimental to the detection of PRRSV, IAV, PEDV, and *MHP* nucleic acids in oral fluid samples by qPCR. To understand these unanticipated results, we re-examined the original reports. Most notably, we found that the work often did not include comparisons with standard methods. That is, quantitative comparisons, e.g., standard method vs the proposed alternative, was typically lacking. Thus, quantitative measures of the gains or losses in performance achieved by alternative methods was not provided.

TREATMENT 3. We were unable to complete the work on targeted concentration within the allotted time. However, preliminary results showed that capture of PRRSV-specific nucleic acids is achievable with the probe-based hybridization capture protocol we developed. Further optimization is needed but the probe-based hybridization capture protocol could result in development of specific target capture methodology that would improve qPCR detection.

INTRODUCTION

We have worked on optimization of swine oral fluid PCR performance for some time. Early work involved "fine tuning" extraction protocols (Chittick et al., 2011). Later work focused on improving PCR performance by chemically degrading potential PCR inhibitors (guanidinium chloride, trypsin, dithiothreitol, sodium borohydride, and others) (Weiser et al., 2018). Improvements were made, but the significant breakthroughs we sought were not achieved.

Publications in the refereed literature reported that the use of specific sample treatments achieved improved nucleic acid detection by qPCR:

1. Heat treatment (95°C for 30 minutes) Ranoa et al. (2020) reported that direct PCR was possible, i.e., no RNA extraction was necessary, when human oral fluids spiked with SARS-CoV-2 were heated at 95°C for 30 minutes. Lower temperatures and/or short incubation times did not achieve the same results.

2. Diluent Diluents can affect the oral fluid matrix by changing the mucin configuration and reducing aggregation (Hughes et al., 2019; Ridley et al., 2014). The assumption behind this approach is that mucins are less able to bind PCR targets if they are dispersed.

3. Targeted concentration Tian et al. (2008) reported a 2-log improvement in sensitivity for norovirus detection using "targeted concentration", i.e., magnetic beads with a norovirus-compatible ligand. Others have corroborated this work (Strubbia et al., 2019; Suresch et al., 2019).

More efficient detection of nucleic acids in swine oral fluid specimens would be highly desirable. Therefore, the purpose of this study was to evaluate the effect of these sample treatments on the detection of PRRSV, IAV, PEDV and *MHP* nucleic acids in swine oral fluids.

OBJECTIVE

The overall objective of this research was to identify practical methods to improve the detection of PRRSV, IAV-S, and *Mycoplasma hyopneumoniae* nucleic acids in swine oral fluids. To achieve this objective, we tested three approaches for improved and/or direct PCR based on treatments (TRT) described in the literature: (1) heat treatment (95°C for 30 minutes), (2) diluent, and (3) targeted concentration.

MATERIALS & METHODS

Treatment 1. Heat (95˚c for 30 minutes) Oral fluid samples containing PRRSV (n = 8), IAV (n = 8), PEDV (n = 8) or *MHP* (n = 8) nucleic acids were tested using a protocol described by Ranoa et al., (2020) for the extraction-free detection of RNA by qPCR. In brief, samples were 2-fold serially diluted (neat, 1:2, 1:4, 1:8) using oral fluid known to be free of PRRSV, IAV, PEDV and *MHP* (n = 32 diluted samples per pathogen), split into 4 aliquots, each of which was randomized to one of 4 protocols (see Table 1): (P1) heat (95°C × 30 m) and direct qPCR; (P2) heat, cool (25°C × 20 m) and direct qPCR; (P3) heat, cool, nucleic acid extraction, and direct qPCR; (P4, i.e., control) extraction and qPCR. Every heat step consisted in placing samples in a dry block heater (Standard Dry Block Heater, VWR International®, Radnor, USA) and allowing them to reach 95°C for 5 min. Thereafter, samples were heated at 95°C for 30 min, and held at that temperature until either cooling, nucleic acid extraction or testing by qPCR. Each "cool step" consisted of allowing samples to cool down to 25°C in an incubator (Model 12-140E, Quincy Lab, Inc, Chicago, IL) for 20 min, and holding them at 25°C until either nucleic acid extraction or testing by qPCR.

Table 1. Protocols evaluated for TRT 1. Heat (95˚C for 30 minutes)

Protocols	Heat (95°C x 30 min)	Cool (25°C)	Extraction	PRRSV qPCR
1	Yes	No	No	Yes
2	Yes	Yes	No	Yes
3	Yes	Yes	Yes	Yes
4	No	Yes	Yes	Yes

Treatment 2. Effect of diluent oral fluid samples known to contain PRRSV (n = 9), IAV (n = 10), PEDV (n = 10), or *MHP* (n = 10) were diluted 1:2 with Tris borate EDTA (TBE), as described by Ranoa et al., (2020) for improved qPCR performance. For comparison, the same samples were also tested undiluted ("neat') and tested after dilution (1:2) with oral fluid free of PRRSV, IAV, PEDV or *MHP*. To perform the experiment, oral fluid samples were thawed overnight at 4°C and split into three aliquots. Each aliquot was prepared as follows: (D1) 1:2 dilution with TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA; Sigma-Aldrich); (D2) 1:2 dilution with oral fluid known to be free of PRRSV, IAV, PEDV and *MHP*; (D3) undiluted (neat) was tested as control. Aliquots were completely randomized and then subjected to nucleic acid extraction in consecutive random number order.

Treatment 3. Targeted concentration A probe-based hybridization capture protocol was designed to target PRRSV-specific nucleic acids. In brief, a pool of PRRSV-specific biotin-tagged probes (4 Nmole Ultramer® DNA Oligo, Integrated DNA Technologies) targeting sequences of different PRRSV-specific genes were attached to

streptavidin-conjugated magnetic beads (Dynabeads® M-270, Invitrogen™). A PRRSV modified live vaccine (MLV) was rehydrated and ten-fold diluted with molecular grade water. Subsequently, dilutions 10^4 and 10^5 were tested in duplicates by the capture protocol, and for comparison, by the standard extraction-amplification protocol. The probe-based capture of PRRSV-specific nucleic acids was performed by incubating the conjugated magnetic beads with the sample at 65°C (annealing temperature) for 30 min while gently shaking in a thermomixer (1000 rpm). The magnetic beads were recaptured using a 6-tube magnetic stand (New England Biolabs, Inc.), and washed twice in an astringent solution of saline sodium citrate. Elution was performed by resuspending in 100 µl buffer Tris EDTA (TE) (10 mM Tris-EDTA pH 7.5; Fisher Scientific) and heating at ~97°C for 5 min. The magnetic beads were captured and the supernatant tested by real-time PCR.

For all treatments, total nucleic acid extraction was performed using the RealPCR* DNA/RNA Spin Column Kit (IDEXX Laboratories, Inc, Westbrook, Maine, USA) following the instructions of the manufacturer. All qPCR reactions were performed using the Magnetic Induction Cycler qPCR (Mic qPCR Cycler, Bio Molecular Systems, Australia) as directed by the manufacturer, using commercially available PCR reagents (RealPCR* RNA Master Mix, RealPCR* DNA Master Mix, RealPCR* PCR Grade Water, RealPCR* PRRS Types 1-2 RNA Mix, RealPCR* Influenza A RNA Mix, RealPCR* PEDV RNA Mix, RealPCR* M. hyo DNA Mix; IDEXX Laboratories, Inc.)

Results were analyzed using the Mic qPCR Cycler Software 2.10.4 (Bio Molecular Systems) and reported as quantification cycles (Cq). Samples with Cq values ≤40 were considered positive. A positive and a negative extraction control and a positive and a negative amplification control were included in each plate.

RESULTS

TRT 1. Heat (95°C for 30 min) A summary of the results is provided in Table 2. Overall, it can be seen that heat treatment was deleterious to detection of PRRSV, IAV, and PEDV RNA, as well as *MHP* DNA in oral fluid samples. The best results were produced using the extraction and amplification protocols (Protocol 4) provided by the manufacturer.

Table 2. No. positives among 8 samples per dilution (mean Cq for positive samples)

Pathogen	Protocol	Dilutions			
		"Neat"	1:2	1:4	1:8
PRRSV	1	0	0	0	0
	2	0	0	0	1 (36.5)
	3	0	0	0	0
	4	8 (29.4)	8 (30.7)	8 (31.4)	8 (31.9)
IAV	1	3 (35.3)	2 (36.5)	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	8 (25.2)	8 (25.6)	8 (26.6)	8 (27.3)
PEDV	1	2 (33.6)	2 (35.7)	0	0
	2	1 (28.5)	2 (34.2)	1 (38.9)	2 (39.2)
	3	1 (33.3)	2 (38.2)	1 (37.8)	1 (39.1)
	4	8 (26.5)	8 (26.7)	8 (27.3)	8 (28.1)
IAV	1	4 (35.3)	2 (35.2)	3 (35.9)	2 (36.1)
	2	3 (36.1)	2 (36.0)	2 (35.7)	1 (36.4)
	3	7 (34.4)	7 (35.1)	7 (35.2)	7 (37.4)
	4	8 (34.7)	8 (35.1)	8 (32.1)	7 (36.7)

TRT 2. Diluent A summary of results for diluent treatment is provided in Table 3. Sample dilution with TBE (or oral fluid) showed no improvement when compared to testing the undiluted (neat) sample.

Table 3. Mean Cq response per dilution by pathogen

Pathogen	Capture		
	D1 - TBE	D2 – oral fluid	D3 – "Neat"
PRRSV	36.5	34.7	33.4
IAV	29.8	30.0	28.9
PEDV	25.5	26.0	25.5
MHP	33.0	33.5	33.9

TRT 3. Targeted concentration Preliminary results for the probe-based hybridization capture protocol are provided in Table 4. Overall, the results show that we were able to design probes and development a procedure that resulted in the capture of the target. However, achieving the long-term goal of a process that exceeds the analytical sensitivity of current extraction-amplification procedures will require further optimization.

Table 4. Preliminary results for targeted concentration (target: PRRSV MLV)

Run	Protocol	Dilution 1 x 10^4		Dilution 1 x 10^5	
		Rep[a] 1	Rep 2	Rep 1	Rep 2
1	Capture	32.4	31.9	35.3	35.5
	Extraction-amplification	NT[b]	NT	NT	NT
2	Capture	34.0	34.1	37.1	-
	Extraction-amplification	30.8	30.4	34.1	34.9

[a]Rep: replicate. [b]NT: not tested

DISCUSSION

Three different procedures described in the literature to improve nucleic acid detection by qPCR were evaluated for the detection of PRRSV, IAV, PEDV and *MHP* in oral fluid samples. Unexpectedly, the results obtained for the methods described in the literature for heat and dilution treatments were actually detrimental to the detection of PRRSV, IAV, PEDV, and *MHP* nucleic acids in oral fluid samples by qPCR. Further examination showed that these reports usually did not include comparisons with standard methods. That is, no quantitative measures of the gain or loss in performance achieved by alternative methods was provided. In this study, the inclusion of comparisons showed that optimum results were obtained using standard extraction and amplification methods.

Time constraints limited the completion of the targeted concentration work but Initial results for targeted concentration showed that PRRSV-specific nucleic acids capture is achievable using a probe-based hybridization capture protocol, but further research will be required to optimize the procedure. Full exploration of the probe-based hybridization capture protocol could result in development of specific target capture methodology that would improve qPCR detection.

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