# Suitability of undenatured ethanol for DNA and RNA preservation in pig oral fluid and fecal samples used for PCR-based pathogen detection

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## Summary

Nucleic acid integrity in pig oral fluid and fecal samples is important for polymerase chain reaction-based pathogen detection and appropriate preservation during shipping is required. A final concentration of 70% undenatured ethanol was sufficient to maintain DNA and RNA quality for up to 7 days.

**Keywords:** swine, oral fluids, feces, ethanol

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**Resumen - Eficacia del etanol sin desnaturalizar para la conservación de ADN y ARN en muestras fecales y de fluidos orales de cerdos utilizados para la detección de patógenos basada en la PCR**

La integridad de los ácidos nucleicos en el fluido oral y las muestras fecales de cerdos es importante para la detección de patógenos basada en la reacción en cadena de la polimerasa, y se requiere una conservación adecuada durante el envío. Una concentración final de 70% de etanol sin desnaturalizar fue suficiente para mantener la calidad del ADN y el ARN hasta por 7 días.

**Résumé - Pertinence de l'éthanol non dénaturé pour la préservation de l'ADN et de l'ARN dans les échantillons de salive et de selles de porc utilisés pour la détection d'agents pathogènes par PCR**

L'intégrité de l'acide nucléique dans les échantillons de salive et de selles de porc est importante pour la détection des agents pathogènes par réaction d'amplification en chaîne par la polymérase et une conservation appropriée pendant le transport est requise. Une concentration finale de 70% d'éthanol non dénaturé était suffisante pour maintenir la qualité de l'ADN et de l'ARN jusqu'à 7 jours.

Respiratory and enteric diseases in pigs remain major health concerns for pork producers.[1] One of the evolving strategies for monitoring and surveillance of pig respiratory and enteric diseases are oral fluid (rope) sampling as well as pooled fecal (sock) sampling, methods proven to be suitable for detecting multiple pathogens of concern.[2,3] Resulting sample types are complex matrices, which means that their mixed composition of water, proteins and enzymes, microorganisms, host cell components, and other environmental additives such as soil[2,4] pose a major challenge for preserving the integrity (eg, molecular weight and size) of target pathogen analytes in the DNA and RNA compartment prior to nucleic acid extraction and downstream molecular analysis. Enzymatic-driven nucleic acid degradation, dilution of target pathogen analytes by continued pathogen overgrowth, or overwhelming presence of nontarget species exacerbate inaccurate and nonsensitive pathogen diagnostics in both sample types. To overcome these challenges, various storage and preservation methods have been tested.[5,6]

Undenatured ethanol is one of the most common, least toxic, and least expensive preservation methods used for animal tissues.[7] Ethanol easily replaces water molecules in biological tissues and cells and leads to major alterations of cellular and membranous proteins by disrupting hydrophobic bonds within the tertiary structure. This inactivates nucleic acid-degrading enzymes, such as DNases, when used in concentrations of 95% to 99%.[8] Undenatured ethanol does not contain other chemicals, such as methanol, which are often added during the denaturing process making the substance unsuitable for different end uses. Furthermore, this preservation method complies with biosecurity import regulations of many countries. The objective of this study was to test a final concentration of 70% undenatured ethanol as a method to preserve the integrity of total nucleic acids in pig oral fluid and fecal samples held for at least 7 days at ambient temperature before use in downstream polymerase chain reaction (PCR)-based molecular applications.

## Animal care and use

Oral fluid and fecal samples were collected by a veterinarian between April and May 2022 from farms with previously

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detected *Streptococcus suis* and rotavirus infections. Samples were sent to Genics Laboratories for diagnostic purposes and transferred to the study with the permission of the farm owners and veterinarian. No specific animal ethics approval was required.

## Materials and methods

Oral fluids were collected using 3 cotton ropes hung in the pig pen for 30 minutes. The chewed-on ropes were drained into one tube, mixed, and divided into 2 aliquots. One aliquot was immediately preserved with ethanol as described herein. Three fecal droppings were collected from the pen floor and pooled into one container. After shipping to the laboratory, both sample types were further aliquoted, and feces were diluted with > 99.5% undenatured ethanol using a 1:2.5 ratio to obtain a final concentration of approximately 70%. Ethanol-preserved samples were stored at ambient temperature whereas undiluted samples were stored at 4°C and -80°C (n = 5-6 aliquots per group and time point). The extraction of total nucleic acids (TNA = DNA + RNA) was carried out on day 1, 4, and 7 of storage using the MagMAX CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific) following the manufacturer's instruction with some modifications (see Supplementary Material). Concentration of DNA was determined using a Qubit 4 fluorometer and Qubit dsDNA high sensitivity assay (Thermo Fisher Scientific). Additionally, DNA quality was estimated by spectrophotometric analysis at 260 and 280 nm using a ClarioSTAR microplate reader (BMG LABTECH).

In a follow-up study, 405 oral fluids and 405 pooled fecal samples were collected from three different Australian pig farms over a period of three months using the previously described sampling methods. Due to varying transit times during shipping, individual samples were assigned to different storage durations ranging from 1 to 6 days.

Extracted TNA from oral fluid and fecal samples were tested on the high-throughput PCR-based Pork MultiPath respiratory (PMP1) and enteric (PMP2) panels (Genics Pty Ltd) which were inclusive of a reverse transcription and PCR step. The PMP1 panel contained assay targets for *Actinobacillus pleuropneumoniae* serotypes 1, 5, 7, and 15, *S suis*, *S suis* serotypes 2 and 3, *Pasteurella multocida*, *Glaesserella parasuis*, *Mycoplasma hyorhinis*, *Mycoplasma hyopneumoniae*, and porcine circovirus 2 (PCV-2). The PMP2 panel contained assay targets for *Lawsonia intracellularis*, *Brachyspira pilosicoli, Brachyspira hyodysenteriae*, *Salmonella enterica, S enterica* serovar Typhimurium, *Escherichia coli* virulence genes (F4, F5, F6, F18, F41, LT1, ST1, ST2, STX2e, and eaeA), Porcine rotavirus A, B, and C, and PCV-2. Both panels included two assays that targeted the housekeeping gene beta-2-microglobulin (B2M), which serve as an internal control for detection of pig genomic DNA (gDNA) and messenger RNA (mRNA). Each assay also included a synthetic positive control, an extraction control, and a no-template control. The presence of target genes was determined by copy number per reaction.

All assays of both PMP panels were previously assessed for analytical specificity (ASp; inclusive and exclusive), analytical sensitivity (ASe) or limit of detection (LOD), and dynamic range or limit of quantitation (LOQ) of each assay (Tables 1 and 2). Data analysis was performed using the MedCalc Statistical Software version 20.111 (MedCalc Software Ltd). Statistical significance was tested using a two-way analysis of variance with Bonferroni corrected pairwise comparisons (dependent variable was concentration; independent variables were time and storage conditions). All data was presented as the mean (SD) and $P < .05$ was deemed statistically significant.

**Table 1:** Summary of LOD and upper and lower LOQ for each respiratory pathogen assay

| Assay* | Upper LOQ†, No. copies | Lower LOQ†, No. copies | LOD† |
|---|---|---|---|
| *Actinobacillus pleuropneumoniae* serovar 1 | 1800 | 10 | 2 |
| *A pleuropneumoniae* serovar 5 | 1800 | 5 | 3 |
| *A pleuropneumoniae* serovar 7 | 2000 | 1 | 2 |
| *A pleuropneumoniae* serovar 15 | 1800 | 10 | 2 |
| *Streptococcus suis* | 2000 | 10 | 4 |
| *S suis* serotype 2 | 1800 | 25 | 2 |
| *S suis* serotype 3 | 1000 | 1 | 13 |
| *Glaesserella parasuis* | 1800 | 0.1 | 6 |
| *Pasteurella multocida* | 2000 | 2.5 | 2 |
| *Mycoplasma hyorhinis* | 1800 | 10 | 3 |
| *Mycoplasma hyopneumoniae* | 1800 | 5 | 4 |
| Porcine circovirus 2 | 1800 | 25 | 3 |

* Extracted total nucleic acids from orthogonal samples were tested on the high-throughput PCR-based Pork MultiPath respiratory panel (Genics Pty Ltd).

† Above Upper LOQ assay called HIGH, between Upper and Lower LOQ assay gives the numerical value, between Lower LOQ and LOD assay called LOW, below LOD assay called negative.

LOD = limit of detection; LOQ = limit of quantification.

**Table 2:** Summary of LOD and upper and lower LOQ for each enteric pathogen assay

| Assay* | Upper LOQ†, No. copies | Lower LOQ†, No. copies | LOD† |
|---|---|---|---|
| *Lawsonia intracellularis* | 2000 | 10 | 3 |
| *Brachyspira pilosicoli* | 2000 | 50 | 3 |
| *B hyodysenteriae* | 1800 | 50 | 4 |
| *Salmonella enterica* | 1500 | 25 | 4 |
| *S enterica* serovar Typhimurium | 1500 | 50 | 6 |
| *Escherichia coli* F4 | 2000 | 10 | 6 |
| *E coli* F5 | 1250 | 2.5 | 3 |
| *E coli* F6 | 1800 | 0.1 | 3 |
| *E coli* F18 | 1000 | 50 | 7 |
| *E coli* F41 | 1800 | 2.5 | 5 |
| *E coli* LT | 1800 | 25 | 4 |
| *E coli* ST1 | 1000 | 50 | 10 |
| *E coli* ST2 | 1500 | 50 | 7 |
| *E coli* STX2E | 1000 | 50 | 3 |
| *E coli* EAE | 2000 | 50 | 4 |
| Porcine rotavirus A | 2000 | 250 | 6 |
| Porcine rotavirus B | 1800 | 25 | 4 |
| Porcine rotavirus C | 1000 | 10 | 4 |
| Porcine circovirus 2 | 1800 | 25 | 4 |

* Extracted total nucleic acids from orthogonal samples were tested on the high-throughput PCR-based Pork MultiPath enteric panel (Genics Pty Ltd).

† Above Upper LOQ assay called HIGH, between Upper and Lower LOQ assay gives the numerical value, between Lower LOQ and LOD assay called LOW, below LOD assay called negative.

LOD = limit of detection; LOQ = limit of quantification.

## Results

Statistical analysis revealed that both time and storage condition had a substantial impact on the yield of DNA extracted from oral fluid samples. Amongst 3 different storage methods, freezing samples at -80°C yielded the lowest oral fluid DNA ($P < .001$) across all time points (Figure 1A). Oral fluids were not stored at -80°C before day 1 because samples were shipped during the first 24 hours. In fecal samples, no difference in DNA yield was observed between the different storage methods ($P = .16$; Figure 1A). The greatest impact on DNA yield for both sample types was time of storage with a decrease in fecal samples in all storage groups after day 1 ($P < .001$; Figure 1A).

Further, the A260/280 ratio was used as a quality indicator of extracted TNA (Table 3). The mean A260/280 ratio for oral fluids stored at 4°C and preserved with ethanol across all time points was closest to the acceptable threshold of 1.8 to 2.0, while the mean ratio for oral fluids stored at -80°C substantially deviated below 1.7 (Table 3). The quality of TNA extracted from feces was not influenced by time or method of storage (Table 3).

Additionally, the effect of different storage methods on the performance of the multiplex PCR-based assay in extracted TNA from oral fluid and feces was investigated. For PMP1, this study focused on the housekeeping gene B2M_gDNA, an internal control for detection of pig gDNA, and *S suis* glutamate dehydrogenase (SS_gdh, a generic *S suis* assay), used as a proxy for the putative performance of DNA pathogen targets. The results showed that the copy number of B2M_gDNA gradually decreased in all three storage groups over time. Statistical analysis also confirmed that for both B2M_gDNA and SS_gdh, a considerably higher detection rate occurred in undenatured ethanol samples compared to other storage methods (Table 3 and Figure 1B). Even though the concentration of total dsDNA extracted from oral fluid samples was equal or even slightly higher ($P = .14$) in samples stored at 4°C compared to the 70% undenatured ethanol preserved samples, the degradation rate of DNA was much more pronounced over time in samples stored at 4°C as seen by copy number decrease of DNA targets.

Similar but less distinct effects were observed in fecal samples when running the PMP2 panel. This panel contained several DNA and RNA pathogen targets as well as two quality control assays for pig gDNA (B2M_gDNA) and mRNA (B2M_RNA). Analysis of DNA assays targeting B2M_gDNA and the *E coli* F4 antigen showed the highest detection rate in 70% undenatured ethanol for up to 7 days of preservation (Table 3 and Figure 1C).

**Figure 1:** Quantity and quality assessment of DNA and RNA extracted from pooled pig oral fluid and fecal samples stored at 4°C, -80°C, or in a final concentration of 70% undenatured ethanol (EtOH) at room temperature after 1, 4 and 7 days of storage. Due to nucleic acid instability, oral fluids were divided into EtOH-preserved and 4°C directly after sampling prior to shipping. Shipping at -80°C was not feasible, so this storage method could not be assessed on day 1. **A)** Concentration of DNA extracted from pooled pig oral fluid (left) and fecal samples (right). Molecular DNA and RNA quality was assessed using the multiplex polymerase chain reaction-based Pork MultiPath respiratory and enteric panels (Genics Pty Ltd) to quantify **B)** *Streptococcus suis* glutamate dehydrogenase gene, **C)** *Escherichia coli* F4 fimbrial antigen, and **D)** porcine rotavirus B viral protein 6. Quality of pig beta-2-microglobulin (B2M) genomic DNA in samples extracted from **E)** oral fluids and **F)** fecal samples stored in a final concentration of 70% EtOH at ambient temperature at different time periods. Quality of pig B2M mRNA in samples extracted from **G)** oral fluids and **H)** fecal samples stored in a final concentration of 70% EtOH at ambient temperature at different time periods. For each boxplot, boxes indicate the 25th percentile, median, and 75th percentile. Whiskers show the 10th and 90th percentiles. Dots (E-H) represent each sample. Asterisks indicate a significance level of $P < .05$ between storage conditions. Hashtags indicate a significance level of $P < .05$ between time points.

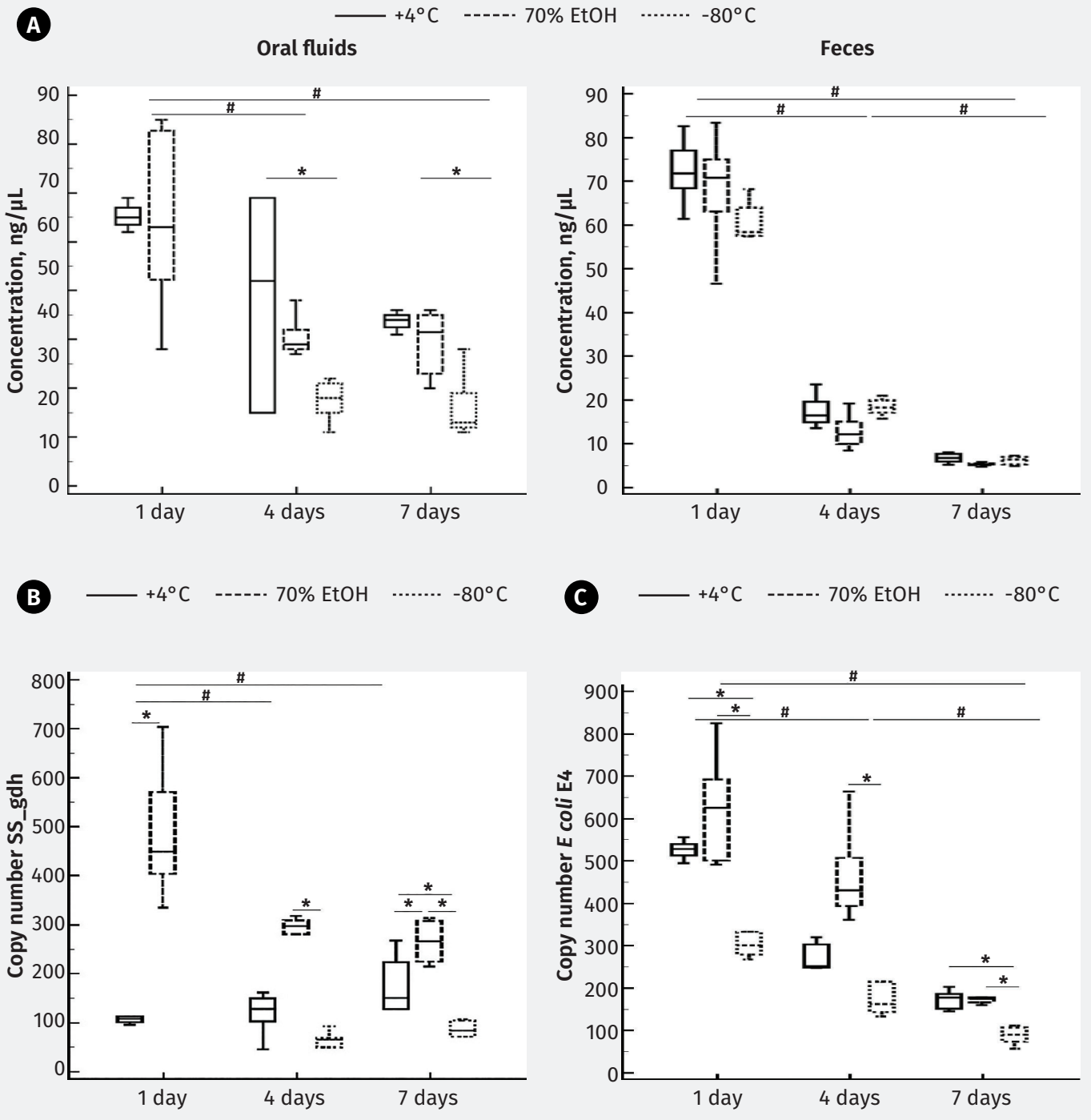

**Figure 1:** Continued

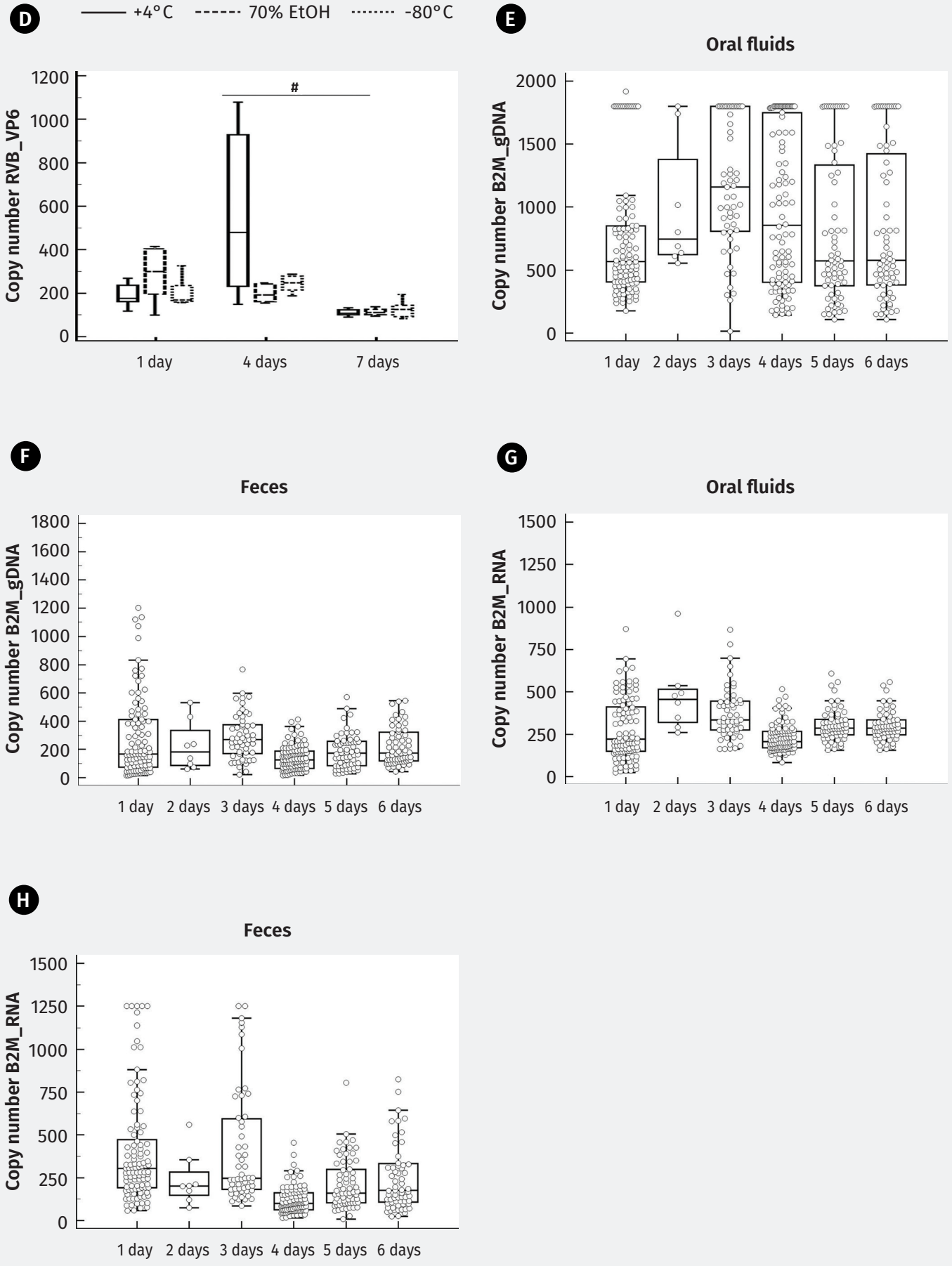

**Table 3:** Quality assessment of TNA isolated from oral fluid and fecal samples stored under different conditions

| TNA quality assessment, mean (SD) | Sample storage conditions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 day | | | 4 days | | | 7 days | | |
| | 4°C | -80°C | EtOH | 4°C | -80°C | EtOH | 4°C | -80°C | EtOH |
| **Oral fluids** | | | | | | | | | |
| A260/280* | 1.75 (0.03) | NA | 1.95 (0.02) | 1.69 (0.18) | 1.58 (0.07) | 1.93 (0.05) | 1.86 (0.08) | 1.70 (0.08) | 1.80 (0.03) |
| B2M_gDNA† | 14 (2)‡ | NA | 127 (90) | 3 (3)‡ | 3 (1)‡ | 43 (12) | 1 (1)‡ | 3 (2) | 43 (10) |
| **Feces** | | | | | | | | | |
| A260/280* | 1.93 (0.07) | 1.91 (0.01) | 1.92 (0.05) | 1.82 (0.03) | 1.90 (0.03) | 1.84 (0.02) | 1.90 (0.08) | 1.91 (0.02) | 1.86 (0.03) |
| B2M_gDNA† | 119 (30) | 98 (23) | 154 (59) | 42 (14) | 50 (14) | 55 (22) | 13 (6) | 20 (7) | 24 (12) |
| B2M_RNA† | HIGH 1359 (844) | HIGH 1502 (260) | HIGH 1728 (557) | 1151 (672)‡ | 1122 (132)‡ | 528 (194) | 849 (317)‡ | 731 (145)‡ | 318 (101) |

* Absorption at 260 and 280 nm indicates general TNA quality and purity.

† Pork MultiPath control DNA (B2M_gDNA) and RNA (B2M_RNA) assay copy number detection as reported by PMP indicates TNA integrity. All samples passed quality control assessment. HIGH score represents samples with copy number higher than limit of quantitation.

‡ Denotes means that are statistically different from EtOH means, $P < .05$. Statistical significance was tested using a two-way analysis of variance with Bonferroni corrected pairwise comparisons.

TNA = total nucleic acids; EtOH = ethanol; NA = not assessed.

The gold standard of freezing samples at -80°C was not as efficient in DNA preservation as expected. With respect to undenatured ethanol preservation on RNA in feces, PMP2 results revealed that the rotavirus B VP6 RNA target was more stable over time than the endogenous B2M_RNA control (Table 3 and Figure 1D).

In a follow-up study, pooled oral fluids and fecal samples (n = 405 each) preserved in a final concentration of 70% undenatured ethanol at ambient temperature were assessed on PMP1 and PMP2 panels at different time points after sampling (1 to 6 days). Comparison of the copy number of housekeeping genes B2M_gDNA and B2M_RNA between samples at different time points confirmed that retaining both specimen types in ethanol sufficiently preserved DNA and RNA to perform PMP analysis (Figures 1E, F, G, and H). Furthermore, screening with PMP demonstrated that detection of different bacterial and viral pathogen targets at high, medium, and low level is attainable even after 6 days of storage in a final concentration of 70% undenatured ethanol at ambient temperature (data not shown).

## Discussion

Preservation of biological diagnostic samples is vital before shipment. Especially when extracting nucleic acids for molecular biological analysis, temperature fluctuations and transit times can have a major impact on nucleic acid yield and quality due to nuclease activity, oxidative degradation, or both.[9] Immediate freezing or short-term storage at 4°C is regarded as best practice despite the challenges of maintaining a cold chain during shipment. For international shipments, freezing or sending samples on ice or preserved in special DNA/RNA stabilizers is often not permitted due to biosecurity import restrictions and can be very cost intensive. In an Australian context, the government's biosecurity import conditions allow the import of most animal and invertebrate samples that are preserved in 70% alcohol (ethanol for example) without requirement of an import permit. Due to the relatively low cost, nontoxicity, global availability, and proven efficiency in preservation of many sample types, undenatured ethanol is widely used and tested as an alternative storage medium for microbial community stabilization.

The presented study demonstrates that a final concentration of 70% undenatured ethanol is a suitable preservative for both pig oral fluids and fecal samples for downstream analysis with the PCR-based PMP panels when stored at ambient temperature for at least up to 7 days. In oral fluid samples, extracted DNA yield was comparable in samples preserved in 70% undenatured ethanol and stored at 4°C over 7 days, whereas storage at -80°C yielded the lowest DNA concentration presumably due to the freezing-thawing process.[10,11] Furthermore, DNA yield was greatly affected by storage time, especially in fecal samples. Degradation of DNA in feces over time has been shown in other studies and may be caused by remaining nuclease activity.[12,13]

When focusing on the impact of the different preservation methods for oral fluids and feces on the performance of the PMP panels, DNA target-based assays (B2M_gDNA, SS_gdh, *E coli*_F4)

detected the highest copy numbers in samples treated with undenatured ethanol across all time points. Even though dsDNA concentration in oral fluid samples was equal or even slightly higher in samples stored at 4°C, the rate of DNA degradation as reflected by copy number decrease of PMP DNA targets was much more pronounced over time compared to the ethanol preserved samples. With DNA input being approximately the same for all PMP1 tests conducted, these results suggest that undenatured ethanol has an immediate fixative effect on DNA and the preservation of respective target analytes. Additionally, it shows that the relatively high concentration of DNA extracted from samples stored at 4°C is likely a consequence of storage without any stabilizers allowing microbiome overgrowth which can vastly misrepresent the sample composition. Similar conclusions were reported by Marotz et al[6] where microbial communities in oral fluid and fecal samples were identified in the presence of different preservatives. The greatest changes of specific taxa were recognized in both types of samples when stored at room temperature without any stabilizers. Additionally, it was demonstrated that microbial blooming was efficiently prevented by using 95% ethanol at ambient temperature.[6] Further, the gold standard of freezing fecal samples at -80°C was not as efficient in DNA preservation as expected. A study examining the effect of storage conditions on genomic DNA in human fecal samples demonstrated that DNA degrades when samples are allowed to defrost.[14] With respect to the effect of undenatured ethanol preservation on RNA in feces, PMP2 results revealed that the rotavirus B VP6 RNA target was more stable over time than the endogenous B2M_RNA control. This distinct difference is likely a result of protective strategies of RNA viruses and their developed defense mechanisms against exonuclease degradation.[15]

This study confirms the suitability of a final concentration of 70% undenatured ethanol for the preservation of pig oral fluid and fecal samples when used at ambient temperature for up to seven days. Further studies are recommended to determine the suitability of this preservation technique on other complex sample types (eg, blood) and different applications such as next-generation sequencing.

# Implications

Under the conditions of this study:

- Multiple pathogens were detected in pig oral fluids and feces using PCR-based panels.
- Undenatured ethanol (70%) preserved nucleic acid integrity for at least 7 days.
- Both PCR-based panels can be combined with ethanol preserved samples.

# Acknowledgments

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## Conflict of interest

Gerszon, Genz, Moser, and Sellars are affiliated with Genics Pty Ltd, which provides the Pork MultiPath as a commercial service.

## Disclaimer

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