Field evaluation of non-invasive sampling for monitoring nine swine pathogens in the Republic of Korea

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Abstract

Background: Non-invasive sampling methods, such as swine oral fluid (OF) and fecal boot (FB) sampling, have gained attention due to their animal welfare considerations, ease of collection, and cost effectiveness. However, comparative data regarding non-invasive and standard sampling methods remain limited, particularly in the Republic of Korea, where research on OF and FB sampling is sparse. Therefore, we investigated representative pig pathogens, including six porcine respiratory pathogens and three gastrointestinal pathogens, using OF and FB samples for pathogen surveillance across commercial farms in the Republic of Korea.

Methods: The results from targeted polymerase chain reaction (PCR) assays for major respiratory and gastrointestinal pathogens obtained using OF and FB samples were compared with those obtained from standard samples. Non-invasive samples included 30 OF ropes and 30 FBs collected from nine farms, and 360 standard samples (blood, fecal, oral, and nasal swabs) were also collected. PCR assays for the nine pathogens were conducted, and the results from OF, FB, and combined OF+FB were compared with those from standard samples at the pen level.

Results: OF sampling showed superior test concordance with respiratory pathogens than gastrointestinal pathogens, and FB sampling demonstrated superior detection of gastrointestinal pathogens compared with OF sampling. Additionally, the combination of OF+FB sampling improved the detection concordance for all nine target pathogens investigated.

Conclusions: We demonstrated the feasibility and potential applicability of OF and FB sampling under field conditions in Korean pig farms. Therefore, this study supports OF and FB sampling as useful tools for swine pathogen monitoring in both research and practical field applications.

Keywords:

Bio-surveillance, Swine oral fluid, Fecal boots, Non-invasive sampling, Swine health monitoring, Swine respiratory disease, Swine gastrointestinal disease

Background

40 Various surveillance and diagnostic approaches have been developed to detect infectious
41 diseases in swine. Widely used conventional sampling techniques for diagnostic tests, such as
42 blood sampling and swabbing require individual animal restraint and can cause considerable
43 stress to the animals [1, 2]. In contrast, non-invasive sampling methods, which do not require
44 trained veterinarian intervention, are increasingly being adopted for improved animal welfare.
45 These methods, including the use of cotton ropes to collect oral fluid (OF) and boot socks to
46 collect feces (fecal boot, FB), not only simplify the process but also significantly reduce stress
47 in the animals. Moreover, they offer significant economic and time-saving benefits, making
48 them a prudent choice for swine farmers and veterinarians [3-6].

49 The OF sampling method, first described in 2008 [2, 7], leverages the natural curiosity of pigs
50 toward novel and flexible objects. The procedure involves suspending a cotton rope to chew
51 on in pig pens, followed by squeezing the rope to extract OF, consisting of saliva and mucosal
52 exudate [7, 8]. In addition to the simplicity and animal friendliness of this method, as OF
53 consists of a mixture of saliva, oral mucosal transudate, and gingival crevicular fluid, it is
54 similar in composition to serum. Therefore, OF has been increasingly used in veterinary
55 research to detect pathogens, such as viruses and bacteria [7]. Similarly, the FB method,
56 introduced in 1999 [9], offers similar ease of use and non-invasiveness but primarily targets
57 gastrointestinal pathogens, whereas the OF method is typically employed for respiratory
58 pathogen surveillance [10]. Despite demonstrating high sensitivity in detecting gastrointestinal
59 pathogens, particularly *Salmonella* species [5, 10], FB sampling is not as common as OF
60 sampling.

61 Various diagnostic methods have been employed to examine OF and FB samples from pigs.
62 This includes quantitative reverse transcription polymerase chain reaction (RT-qPCR), solid-
63 phase competitive enzyme-linked immunosorbent assay (ELISA), virus or bacteria isolation,

Sanger sequencing, and double antibody sandwich ELISA [11]. Several porcine pathogens, including porcine respiratory and reproductive syndrome virus (PRRSV) [12], influenza A virus in swine (IAV-S) [13], foot-and-mouth disease virus, African swine fever virus [14], porcine circovirus 2 (PCV2) [15], and *Mycoplasma hyopneumoniae* (MHP) [16] have been detected by OF samples, which are effective for diagnosis via PCR-based methods. That is, combining OF sampling with PCR-based techniques is useful for pathogen surveillance in laboratories and some field settings [3, 4].

Furthermore, compared with blood sampling or oral swabbing, which require individual restraint of animals, OF- and FB-based testing allows for simultaneous sampling from multiple animals. This method enables relatively high sampling throughput and provides valuable health data for entire swine herds. Monitoring herd-level biological data can aid in preventing the spread of pathogens by asymptomatic carriers [2]. In clinical swine practice, pooling samples has become increasingly common, with results often interpreted as representative of the age group from which the samples were collected. In this way, monitoring for pathogens through sample pooling effectively reduces veterinary diagnostic costs and facilitates testing large numbers of animals for pathogens [1, 2].

Despite the proven benefits of OF and FB sampling, research on their applicability and practicality in field conditions specific to the Republic of Korea remains limited, particularly in the context of comprehensive pathogen surveillance [6, 17-20]. Therefore, this study addresses this gap to enhance biosecurity measures in swine herds in the Republic of Korea. Pig pathogens representative of the most commonly tested pathogens in the Republic of Korea were investigated, including six porcine respiratory pathogens and three gastrointestinal pathogens [PRRSV, IAV-S, PCV2, MHP, *Actinobacillus pleuropneumoniae* (APP), *Glaesserella parasuis* (GSP), porcine epidemic diarrhea virus (PEDV), *Lawsonia*

88 *intracellularis* (LI), and *Brachyspira hyodysenteriae* (BH)], using OF and FB samples across
89 commercial Korean farms. The results were compared with those obtained through standard
90 sampling methods (blood and nasal, oral, and fecal swabs), and a practical monitoring program
91 was proposed for future pathogen detection on farms.

92

93 **Methods**

94 The study was conducted in compliance with the Institutional Animal Care and Use Committee
95 (IACUC) of Konkuk University, Republic of Korea, ensuring the ethical use of animals in
96 research, and all swine owners entered into a written agreement with Konkuk University's
97 IACUC to permit ethical review of the study (under project number KU24100), and all samples
98 were collected by trained veterinarians.

99 Routine blood sampling and other sample collections approved by the IACUC were performed
100 on the animals under normal farm conditions. The animals were not euthanized as part of the
101 study. After the completion of the study, the animals were processed according to the farm's
102 standard slaughter procedures, which align with local agricultural and ethical standards. No
103 additional anesthetic or euthanasia methods were applied, as the animals followed the normal
104 lifecycle for commercial livestock.

105 **Targeted pathogen selection**

106 Targeted pathogens for this study were selected based on data from the Korea Animal Health
107 Integrated System and the Swine Health Information Center's Swine Disease Matrix
108 (https://www.swinehealth.org/swine-disease-matrix/), as well as the opinions of professional
109 veterinarians. Six porcine respiratory pathogens and three gastrointestinal pathogens were

110 identified as significant concerns for swine health. The selected respiratory pathogens were
111 PPRSV (PRRSV-1, PRRSV-2), IAV-S, PCV2, and MHP, which cause porcine enzootic
112 pneumonia; APP, which causes porcine pleuropneumonia; and GSP, which causes Glässer's
113 disease. The selected gastrointestinal pathogens were PEDV, which causes PED; LI, which
114 causes porcine proliferative enteropathy; and BH, which causes swine dysentery.

115 **Sample collection**

116 From July 2024 to November 2024, non-invasive samples (OF and FB) and standard samples
117 (blood, fecal swab, nasal and oral swab) were collected from nine commercial pig farms in the
118 Republic of Korea. To minimize selection bias, three pigs were randomly selected from each
119 pen for standard sampling regardless of clinical signs. The pigs within each pen were of similar
120 age (growing-finishing stage, 6 to 18 weeks old) to reflect the typical herd composition in
121 commercial swine farms (Supplementary Table 1). A total of 420 samples were obtained from
122 nine farms in the Republic of Korea, and they were divided into 60 non-invasive samples (30
123 OF and 30 FB samples) and 360 standard samples (90 blood, 90 fecal swabs, 90 nasal swabs,
124 and 90 oral swabs). The detailed collection information for each sample type are described
125 below and in Supplementary Table 1.

126 To prevent cross-contamination, disposable gloves were changed between the handling of each
127 rope and between each pen during FB sampling. Additionally, all equipment and collection
128 materials were disposable or sterilized prior to use. All samples were transported to the
129 laboratory on ice packs, and the average transfer time was approximately 4 h (maximum: 6 h).
130 Upon arrival at the laboratory, samples were immediately preprocessed according to their
131 specific characteristics and temporarily stored at 4 °C until DNA and RNA extraction. All
132 extractions were completed within 24 hours of sample collection.

133 Oral fluid: A cotton rope with a thickness of 12 mm and a length of 150 cm was tied to a metal
134 bar on the side of the pen at pig shoulder height, ensuring that the rope did not touch the floor,
135 and left in the pen for the pigs to chew for approximately 30–40 min. OF samples were
136 collected by placing the chewed ropes into an individual plastic zipper bag. In the laboratory,
137 the ropes were manually squeezed into 50 mL conical tubes (SPL Life Science, the Republic
138 of Korea). The samples were then left at 4 °C for 30 min, and 5 ml of the supernatant was
139 carefully transferred and stored again at 4 °C until further analysis.

140 Fecal boot: FB samples were collected from pen floors using the boot-sock method (10). Two
141 layers of disposable polypropylene shoe covers were worn over the veterinarian's boots. During
142 standard sampling, veterinarians walked within the pen, and the outer layer collected fecal
143 matter and other environmental debris from the surface. The inner layer was used to avoid
144 contamination from boots, whereas the outer layer was used for collection and testing. After
145 sampling, the socks were placed in an individual plastic zipper bag. In the laboratory, 10 mL
146 of PBS (Welgene Inc., the Republic of Korea) was added to the plastic zipper bag, and the
147 contents were manually squeezed into 15 mL conical tubes (SPL Life Science, the Republic of
148 Korea). The samples were then left at 4 °C for 30 min, and 5 ml of the supernatant was carefully
149 transferred and stored again at 4 °C until further analysis.

150 Blood: Blood samples were randomly collected from three pigs per pen via jugular
151 venipuncture. Pigs were appropriately restrained, and blood was drawn using a sterile needle
152 and syringe, then transferred into tripotassium EDTA (Becton Dickinson, NJ, USA) as an
153 anticoagulant.

154 Fecal, nasal, and oral swabs: Swab samples were collected from the same pigs used for blood
155 sampling using DNA/RNA-free sterile swabs (Fisher Scientific, NH, USA). Each swab was

156 gently inserted into the rectum, nasal cavity, or oral cavity and rotated for sample collection.
157 In the laboratory, they were diluted in 3 mL PBS (Welgene Inc., the Republic of Korea).

158 **DNA and RNA extraction**

159 Genomic DNA and total RNA were manually extracted using the AccuPrep® Genomic DNA
160 Extraction Kit (Bioneer, the Republic of Korea) and the GeneAll® Hybrid-R™ kit (GeneAll,
161 the Republic of Korea), respectively, according to the manufacturers' instructions. Both DNA
162 and RNA were extracted from 250 μL of input and eluted in 50 μL of elution buffer.

163 **Targeted PCR**

164 RT-qPCR was performed for PRRSV [21], IAV-S [22], and PEDV [23]; qPCR was performed
165 for PCV2 [24], MHP [25], LI [26], and BH [27]; and conventional PCR was performed for
166 APP [28] and GSP [29] (Supplementary Table 2).

167 The RT-qPCR reaction mixture totaled 20 μL and included 10 μL of GoTaq® Probe qPCR
168 Master Mix with dUTP (2×) (Promega, Madison, USA), 0.4 μL of RT Enzyme (50x) (Promega,
169 Madison, USA), 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 1 μL of 10 μM
170 probe, 5 μL of RNA template, and the remaining volume of nuclease-free water. RT-qPCR was
171 performed using the CFX Opus Real-Time PCR Systems (Bio-Rad Laboratories, CA, USA)
172 under the following conditions: 45 °C for 15 min, 95 °C for 2 min, and 45 cycles of 95 °C for
173 15 s and 60 °C for 1 min. Each run included positive and negative controls.

174 Similarly, the qPCR reaction mixture totaled 20 μL and included 10 μL of GoTaq® Probe
175 qPCR Master Mix with dUTP (2×), 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse
176 primer, 1 μL of 10 μM probe, 5 μL of DNA template, and the remaining volume of nuclease-

177 free water. These reactions were also performed using the CFX Opus Real-Time PCR Systems
178 under the same cycling conditions as the RT-qPCR, except for the omission of the 45 °C step.

179 Bio-Rad CFX Maestro 1.1 software (Bio-Rad Laboratories, Hercules, CA, USA) was used to
180 analyze the RT-qPCR and qPCR results. Cycle threshold values were generated with a
181 threshold of 0.1 for the target gene and 10.00% of the maximum amplitude of the sigmoidal
182 amplification curves for positive controls.

183 For the PCR, the reaction mixture totaled 20 μL and included 10 μL of GoTaq® Probe qPCR
184 Master Mix with dUTP (2×), 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 5
185 μL of DNA template, and 3 μL of nuclease-free water. All primers were used at a concentration
186 of 10 μM. PCRs were performed using the T100 Thermal Cycler (Bio-Rad Laboratories, CA,
187 USA) under the following conditions: 95 °C for 2 min, and 45 cycles of 95 °C for 15 s and 60 °C
188 for 1 min. After the PCR was completed, the PCR product was electrophoresed on a 2.00%
189 agarose gel in 1× TAE buffer (Biosesang, Republic of Korea) and visualized by Midori-Green
190 staining (Nippon Genetics, Japan) at 100 V for 20 min. The DNA separated on the gel was
191 recorded using a GelDoc Go System (Bio-Rad Laboratories, Hercules, CA, USA).

192 **Analytical sensitivity**

193 To determine the analytical sensitivity, limit of detection (LoD) tests were performed for all
194 target pathogens. The sensitivity results of RT-qPCR for PRRSV, IAV-S, and PEDV; qPCR
195 for PCV2, MHP, LI, and BH; and PCR for APP and GSP (Supplementary Table 2) were
196 evaluated using 10-fold serially diluted synthetic plasmids (Bionics, Republic of Korea) for
197 each target pathogen, ranging from 5×10^{-1} to 5×10^{7} copies/L as templates. For detection, the
198 Ct value was considered, and the lowest concentration of synthetic plasmids that could be
199 reproducibly detected was regarded as the presumed LoD.

200 The LoD was established at 50 copies/μL for PRRSV-1, PRRSV-2, IAV-S, PCV2, APP, GSP,
201 LI, and BH and 5 copies/μL for MHP and PEDV. At the LoD concentrations, the Ct values
202 were approximately 34–39. Consequently, we set the Ct value at 40 as the cut-off indicating
203 positivity at Ct ≤ 40 and negativity at Ct > 40. This means Ct and Tm values were not detected
204 in negative controls.

205 **Statistical analysis**

206 The primary aim of this study was to evaluate the detection concordance between non-invasive
207 (OF and FB) and standard sampling methods (blood, nasal swab, oral swab, and fecal swab)
208 for pathogen detection in pigs at the pen level. Pen-level positivity was determined based on
209 PCR results from standard samples collected from three randomly selected pigs per pen. A pen
210 was classified as positive when at least one of the 12 standard samples (blood, nasal swab, oral
211 swab, and fecal swab from three pigs) tested positive for a pathogen. Samples were regarded
212 positive if the qPCR and RT-qPCR results showed a Ct value of less than 40 or if the target
213 size (bp) was observed by electrophoresis.

214 The detection concordance between standard and non-invasive sampling methods was assessed
215 using Cohen's kappa coefficient (κ), which adjusts for agreement expected by chance [30].
216 Additionally, a positive rate was defined as the proportion of samples that tested positive for a
217 specific pathogen out of the total number of samples of each type, regardless of pen-level
218 matching. Test agreement was defined as the proportion of pens for which both the standard
219 and non-invasive sampling methods yielded identical PCR results (either both positive or both
220 negative). Sensitivity and specificity were defined respectively as the proportions of true
221 positives and true negatives obtained by non-invasive sampling, using standard sampling as
222 the reference.

223 All statistical analyses were conducted using R (version 4.2.2), with the epiR package (version
224 2.0.54) used to calculate the test agreement, sensitivity, specificity, and Cohen's kappa with
225 95% confidence intervals (CIs).

226

227 **Results**

228 In this study, detection concordance of non-invasive sampling methods (OF, FB, and combined
229 OF+FB) was evaluated at the pen-level by comparison with standard sampling methods (Tables
230 1- 4). As an initial step, we summarized the raw positivity rates of the nine pathogens across
231 different sample types (Table 1), which served as a reference for the subsequent concordance
232 analyses.

233 Overall, the combined OF+FB sampling demonstrated improved detection concordance
234 compared to individual OF or FB sampling and exhibited higher test agreement, sensitivity,
235 and κ values for most pathogens. Specifically, the combined OF+FB sampling showed the
236 highest diagnostic accuracy for detecting respiratory pathogens such as IAV-S (test agreement:
237 86.7%; sensitivity: 92.9%; κ = 0.73) and MHP (test agreement: 86.7%; sensitivity: 90.5%; κ =
238 0.68). Moreover, combined OF+FB sampling showed higher accuracy for PRRSV (test
239 agreement: 78.3%; sensitivity: 84.0%; κ = 0.57) compared with OF sampling alone (test
240 agreement: 76.7%; sensitivity: 63.3%; κ = 0.53).

241 Among gastrointestinal pathogens, PEDV and BH showed higher diagnostic sensitivity and
242 agreement with either the FB or combined OF+FB sampling methods. Specifically, combined
243 OF+FB sampling yielded a sensitivity of 87.5%, test agreement of 86.7%, and a κ value of 0.68
244 for PEDV, while for BH, sensitivity was 92.9%, agreement was 80.0%, and κ value was 0.61.
245 FB sampling alone also demonstrated reasonable performance for gastrointestinal pathogens,

notably for PEDV and BH, confirming its potential effectiveness in field applications. LI showed comparatively lower detection values, although FB or combined OF+FB sampling slightly improved detection performance compared to OF sampling alone.

High sensitivity was consistently observed for PCV2 across all sampling methods, reflecting the widespread vaccination across farms included in this study. However, specificity was markedly low (33.3%) and the κ values remained limited (κ = 0.35), indicating a reduced ability to differentiate vaccination-induced positivity from natural infections when employing these PCR-based sampling strategies. For GSP, 100% sensitivity was observed across all sampling methods, but specificity and κ values were not calculated due to the absence of negative samples, reflecting the organism's common occurrence in swine herds as a commensal rather than as a true pathogen.

Collectively, these findings suggest that OF sampling alone provides reliable diagnostic performance for detecting respiratory pathogens while FB sampling or a combination of OF+FB is more effective for gastrointestinal pathogens under practical farm conditions. The combined OF+FB sampling strategy notably enhanced pathogen detection concordance across all categories tested, thus demonstrating its suitability for practical implementation in swine health management.

Discussion

Blood sampling and swabbing have been the standard methods for monitoring disease levels in pig farms. However, these invasive procedures can negatively impact animal welfare and productivity by causing stress, pain, and increased risk of infection during sample collection

268 [31]. As a result, non-invasive sampling methods such as OF and FB sampling have gained
269 attention as practical alternatives to conventional techniques [1, 5, 6].

270 In this study, we evaluated the feasibility of OF and FB sampling for pathogen surveillance
271 under commercial farm conditions in Korea by comparing pathogen detection results from
272 standard and non-invasive samples using PCR. According to a previous study, pen-based OF
273 sampling devices in barns housing approximately 50 pigs were able to detect PRRSV in over
274 80% of pens when 10% of the herd was infected [6]. Based on this evidence, we minimized
275 our sampling to one OF sample per pen, with an average of 50 pigs per pen, aligning with
276 previous recommendations for effective OF sampling. Sampling protocols were implemented
277 based on established guidelines for both OF and FB methods.

278 Overall, combined OF+FB sampling demonstrated higher test agreement and sensitivity across
279 most respiratory and gastrointestinal pathogens compared to either method alone, suggesting
280 its suitability for pen-level disease surveillance. For respiratory pathogens such as PRRSV,
281 IAV-S, PCV2, and MHP, moderate to substantial agreement was observed between OF
282 sampling and standard sampling results. MHP showed the highest κ value (0.680) and
283 sensitivity (86.7%) among the respiratory pathogens. PRRSV and PCV2 also exhibited
284 acceptable diagnostic performance, with κ values of 0.570 and 0.35, and sensitivities of 70.0%
285 and 96.0%, respectively. These findings support earlier reports suggesting that OF sampling
286 may serve as an alternative to blood sampling for detecting PRRSV and PCV2 [6, 32, 33].

287 The combined use of OF and FB sampling was confirmed to be effective for the detection of
288 various pathogens. For example, a previous study evaluating multiple individual swab types
289 for PRRSV surveillance in weaning pigs reported sensitivities of 83.0% for ear-vein blood,
290 83.0% for nasal swabs, and 75.0% for oral swabs [29]. In comparison, our study demonstrated

291 similar or higher sensitivity (approximately 84.0%) and specificity for PRRSV detection using
292 OF samples.

293 While OF sampling has been well studied, FB sampling has received comparatively less
294 attention. However, studies utilizing pen-environment swabbing and FB sampling for detecting
295 Salmonella have shown that FB sampling can yield results comparable to OF sampling [9, 34,
296 35]. In our study, FB sampling showed higher test agreement and sensitivity than OF sampling
297 alone for gastrointestinal pathogens. Specifically, PEDV and BH achieved test agreement rates
298 of 83.3% and 76.7%, respectively, with FB samples. The combined OF+FB sampling method
299 further improved sensitivity for these pathogens, highlighting its value for gastrointestinal
300 pathogen monitoring.

301 PCV2 demonstrated high sensitivity across all sampling methods, likely due to the widespread
302 vaccination of pigs on the participating farms. However, low specificity suggests limitations in
303 distinguishing vaccinated animals from naturally infected ones using PCR. In addition, GSP
304 was detected in all samples, resulting in perfect sensitivity but precluding calculation of
305 specificity and κ values. This reflects the commensal nature of GSP rather than pathogenic
306 presence and suggests that its consistent detection is more indicative of the appropriateness of
307 OF sampling than of infection status.

308 Collectively, these findings indicate that OF sampling is particularly suitable for monitoring
309 respiratory pathogens, while FB sampling may be more effective for gastrointestinal pathogens.
310 Combined OF+FB sampling improved diagnostic coverage and represents an efficient and
311 welfare-conscious approach for pen-level pathogen surveillance in swine populations.

312 Despite these advantages, several limitations should be acknowledged. Because pathogen
313 concentrations may be lower in group samples such as OF and FB, the diagnostic sensitivity

314 of these methods may be reduced for certain pathogens compared to conventional blood or
315 swab sampling. This limitation can affect the detection of low-prevalence pathogens. Therefore,
316 additional strategies—such as increasing the sample volume or collection frequency or
317 combining non-invasive with conventional sampling—may be necessary to ensure
318 comprehensive and accurate disease surveillance.

319

320 **Conclusions**

321 This study confirmed that OF and FB sampling are practical, non-invasive alternatives to
322 conventional sampling for swine pathogen monitoring. Among the strategies evaluated, the
323 combined OF and FB sampling method consistently demonstrated improved diagnostic
324 agreement and sensitivity for both respiratory and gastrointestinal pathogens. Expanding the
325 application of these methods and optimizing sampling protocols—such as increasing sample
326 volume or collection frequency—may contribute to the development of more sustainable and
327 effective disease monitoring systems in the swine industry.

328

329 **Declarations**

330 **Author' Contributions**

331 Conceptualization Hansong Chae; Methodology Hansong Chae; Resources Hyunmin Shin,
332 Gyooha Han, Doyoung Song, Mingi Han and Huk Song; Validation Hansong Chae, Serim Kim,
333 Mingi Han and Seung-Uk Shin; Data curation Hansong Chae, Serim Kim, and Won Gyeong
334 Kim; Formal analysis Hansong Chae, Serim Kim; Investigation Seulki Kim, Seung-Uk Shin,
335 Hyunmin Shin and Gyooha Han; Writing- original draft Hansong Chae; Writing- review &

editing Hansong Chae, Serim Kim, and Huk Song. All authors read and approved of the final manuscript.

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Competing interests

The authors declare that they have no competing interests.”

Availability of data and materials

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Consent for publication

Not applicable

Ethics approval

The study was conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) of Konkuk University, the Republic of Korea, under project number KU24100, ensuring the ethical use of animals in research.

Prior publication

A portion of this work has been presented as a conference abstract at Meeting of the Microbiology Society for Korea, but the full manuscript has not been published.

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360

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469

470 **Table**

471 **Table 1. Positive percentage (%) of the nine pathogens by sample type at the pen level*.**

Sample	PRRSV	IAV-S	PCV2	MHP	APP	GSP	PEDV	LI	BH
Oral fluid	50.00	33.33	86.67	56.67	33.33	100.00	10.00	33.33	36.67
Fecal boot	40.00	23.33	36.67	43.33	16.67	63.33	23.33	26.67	36.67
Blood	56.66	39.66	63.34	10.00	0.00	0.00	23.33	0.00	16.66
Fecal swab	56.66	39.66	83.34	26.66	23.34	46.66	16.66	16.66	40.00
Nasal swab	46.67	40.00	73.33	56.67	50.00	93.33	13.33	30.00	40.00
Oral swab	30.00	43.33	76.67	50.00	23.33	100.00	13.33	40.00	50.00

472

473

474 **Table 2. Test agreement, sensitivity, specificity and kappa value of pen-level comparison between OF and standard sampling methods**

Pathogen	Test agreement	Sensitivity	Specificity	Kappa
PRRSV	76.67 (64.56-85.56)	63.33 (45.51-78.13)	90.00 (74.38-96.54)	0.53 (0.33-0.74)
IAV-S	76.67 (59.17-88.21)	62.50 (38.64-81.52)	92.86 (68.53-98.73)	0.54 (0.26-0.82)
PCV2	86.67 (70.32-94.69)	92.59 (76.63-97.94)	33.33 (6.15-79.23)	0.26 (-0.26-0.78)
MHP	83.33 (66.44-92.66)	80.95 (59.60-92.33)	88.89 (56.50-98.01)	0.64 (0.36-0.92)
APP	60.00 (42.32-75.41)	44.44 (24.56-66.28)	83.33 (55.20-95.30)	0.25 (-0.04-0.54)
GSP	100.0 (88.65-100.0)	100.0 (88.65-100.0)	-*	-*

PEDV	73.33 (55.55-85.82)	30.00 (10.78-60.32)	95.00 (76.39-99.11)	0.29 (-0.04-0.36)
LI	73.33 (55.55-85.82)	58.33 (31.95-80.67)	83.33 (60.78-94.16)	0.43 (0.10-0.76)
BH	66.67 (48.78-80.77)	50.00 (29.03-70.97)	91.67 (64.61-98.51)	0.38 (0.10-0.65)

All values are presented with 95% confidence intervals.

* "-" indicates the absence of positive or negative result values, meaning no valid data were obtained.

Table 3. Test agreement, sensitivity, specificity and kappa value of pen-level comparison between FB and standard sampling methods

Pathogen	Test agreement	Sensitivity	Specificity	Kappa
PRRSV	66.67	36.67	96.67	0.33

	(54.06-77.27)	(21.87-54.49)	(83.33-99.41)	(0.14-0.52)
IAV-S	83.33 (66.44-92.66)	68.75 (44.40-85.84)	100.0 (78.47-99.99)	0.67 (0.42-0.92)
PCV2	80.00 (92.69-90.49)	85.19 (67.52-94.08)	33.33 (6.15-79.23)	0.14 (-0.28-0.57)
MHP	73.33 (55.55-85.82)	66.67 (45.37-82.81)	88.89 (56.50-98.01)	0.47 (0.18-0.76)
APP	43.33 (27.38-60.80)	16.67 (5.84-39.22)	83.33 (55.20-95.30)	0.00 (-0.23-0.23)
GSP	63.33	63.33	-*	-*

	(45.51-78.13)	(45.51-78.13)		
PEDV	83.33 (66.44-92.66)	60.00 (61.27-83.18)	95.00 (76.39-99.11)	0.59 (0.28-0.91)
LI	73.33 (55.55-85.82)	50.00 (25.38-74.62)	88.89 (67.20-96.90)	0.41 (0.08-0.74)
BH	76.67 (59.07-88.21)	61.11 (38.62-79.69)	100.0 (75.75-99.99)	0.56 (0.30-0.81)

All values are presented with 95% confidence intervals

* '-' indicates the absence of positive or negative result values, meaning no data were obtained.

482 **Table 4. Test agreement, sensitivity, specificity and kappa value of pen-level comparison between the combined OF+FB and standard**

483 **sampling methods**

Pathogen	Test agreement	Sensitivity	Specificity	Kappa
PRRSV	78.33 (66.38-86.87)	84.00 (65.34-93.60)	74.29 (57.93-85.84)	0.57 (0.36-0.77)
IAV-S	86.67 (70.32-94.47)	92.86 (68.53-98.73)	81.25 (57.00-93.41)	0.73 (0.49-0.97)
PCV2	90.00 (74.38-96.54)	92.85 (77.35-98.02)	50.00 (9.45-90.55)	0.35 (-0.22-0.92)
MHP	86.67 (70.32-94.69)	90.47 (71.08-97.35)	77.78 (45.26-93.68)	0.68 (0.40-0.97)
APP	63.33 (45.51-78.12)	81.82 (52.30-94.86)	52.63 (31.71-72.67)	0.30 (0.01-0.60)
GSP	100.0 (88.65-100.0)	100.0 (88.65-100.0)	-*	-*

PEDV	86.67 (70.32-94.69)	87.50 (92.86-97.76)	86.36 (66.67-95.25)	0.68 (0.40-0.97)
LI	73.33 (55.55-85.82)	66.67 (39.03-86.19)	77.78 (54.79-91.00)	0.44 (0.12-0.77)
BH	80.00 (62.69-90.49)	92.86 (68.53-98.72)	68.75 (44.40-85.58)	0.61 (0.33-0.88)

484 All values are presented with 95% confidence intervals

485 * '-' indicates the absence of positive or negative result values, meaning no data were obtained.

486 **Supplementary Tables**

487 **S1 Table 1. Summary of the collected pig numbers, sample types, and vaccine statuses by farm.**

Farm	No. of pens	Age of pigs* (weeks)	No. of pigs per pen	No. of group samples**		No. of individual samples				Vaccine Status***
				OF	FB	Blood	Fecal swab	Nasal swab	Oral swab	
A	4	6 / 10 / 14 / 18	30 - 50	4	4	12	12	12	12	PCV2
B	4	6 / 10 / 14 / 18	30 - 50	4	4	12	12	12	12	PCV2
C	2	14 / 18	50	2	2	6	6	6	6	PCV2
D	2	14 / 18	50	2	2	6	6	6	6	PCV2
E	2	14 / 18	50	2	2	6	6	6	6	PCV2
F	4	6 / 10 / 14/ 18	60 - 80	4	4	12	12	12	12	PCV2, PRRSV-2
G	4	6 / 10 / 14/ 18	60 - 80	4	4	12	12	12	12	PCV2, PRRSV-2
H	4	6 / 10 / 14/ 18	50	4	4	12	12	12	12	PCV2
I	4	6 / 10 /14 / 18	50	4	4	12	12	12	12	PCV2
		Total		30	30	90	90	90	90	420

488 *Age of pigs in each pen is shown as 'weeks/weeks/...'; each number represents a distinct pen within the same farm.

489 **OF: oral fluid sample; FB: fecal boot sample

490 *** PCV2 vaccine was administered at 4 weeks of age on all farms, while PRRSV-2 vaccine was administered only on Farm F and G at 2
491 weeks of age

492 **S2 Table . Primer-probe sets used for targeted PCRs**

Target organism	Primer-probe	Sequence (5'→3')	Type of PCR	Size (bp)	LoD* (Copies)
PRRSV-1	Forward	CCTGCCCAYCACGTAGAAAG	RT-qPCR	187	50
	Probe	TACGCTGTGAGAAAGCCCGG			
	Reverse	AGTGCCGTTYACTGANGTY			
PRRSV-2	Forward	TGCCCACCACGTYGARAG	RT-qPCR	109	50
	Probe	AACCACGCATTYGTCGTYCGGCG			
	Reverse	GCACCARTGTGCCGTTRAC			
IAV-S	Forward	AGATGAGTCTTCTAACCGAGGTCG	RT-qPCR	99	50
	Probe	TCAGGCCCCCTCAAAGCCGA			
	Reverse-1	TGCAAAAACATCTTCAAGTYTCTG			
	Reverse-2	TGCAAAGACACTTTCCAGTCTCTG			
PCV2	Forward	CCAGGAGGGCGTTSTGACT	qPCR	99	50
	Probe	AATGGCATCTTCAACACCCGCCTCT			
	Reverse	CGYTACCGYTGGAGAAGGAA			
MHP	Forward	GTCAAAGTCAAAGTCAGCAAAC	qPCR	137	5
	Probe	ACCAGTTTCCACTTCATCGCCTCA			
	Reverse	AGCTGTTCAAATGCTTGTCC			

APP	Forward	AAGGTTGATATGTCCGCACC	PCR	960	50
	Reverse	GCCGTAGCACCGATTACG			
GSP	Forward	GTGATGAGGAAGGGTGGTGT	PCR	821	50
	Reverse	GGCTTCGTCACCCTCTGT			
PEDV	Forward	CGCAAAGACTGAACCCACTAA	RT-qPCR	196	5
	Probe	TTGCCATTRCCACGACTCCTGCTACG			
	Reverse	GCCTCTGTTGTTACTYGGAGAT			
LI	Forward	GCGCGCGTAGGTGGTTATAT	qPCR	98	50
	Probe	CACCGCTTAACGGTGGAACAGCCTT			
	Reverse	GCCACCCTCTCCGATACTCA			
BH	Forward	TGGCCTGTAACTCCTCCTAT	qPCR	157	50
	Probe	AGCTTTATCAGCAAGGACAAGAAATT			
	Reverse	CAGCACCAACTACCATAACT			

493 * LoD: Limit of detection

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