# Rapid detection of *A. pleuropneumoniae* from clinical samples using recombinase polymerase amplification

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## Abstract

*Actinobacillus pleuropneumoniae* (APP) is the causative agent of porcine pleuropneumonia, resulting in high economic impact worldwide. There are currently 19 known serovars of APP, with different ones being predominant in specific geographic regions. Outbreaks of pleuropneumonia, characterized by sudden respiratory difficulties and high mortality, can occur when infected pigs are brought into naïve herds, or by those carrying different serovars. Good biosecurity measures include regular diagnostic testing for surveillance purposes. Current gold standard diagnostic techniques lack sensitivity (bacterial culture), require expensive thermocycling machinery (PCR) and are time consuming (culture and PCR). Here we describe the development of an isothermal point-of-care diagnostic test - utilizing recombinase polymerase amplification (RPA) for the detection of APP,

targeting the species-specific *apxIVA* gene. Our APP-RPA diagnostic test achieved a sensitivity of 10 copies/µL using a strain of APP serovar 8, which is the most prevalent serovar in the UK. Additionally, our APP-RPA assay achieved a clinical sensitivity and specificity of 84.3% and 100%, respectively, across 61 extracted clinical samples obtained from farms located in England and Portugal. Using a small subset (n = 14) of the lung tissue samples, we achieved a clinical sensitivity and specificity of 76.9% and 100%, respectively) using lung imprints made on FTA cards tested directly in the APP-RPA reaction. Our results demonstrate that our APP-RPA assay enables a suitable rapid and sensitive screening tool for this important veterinary pathogen.

## 1 Introduction

*A. pleuropneumoniae* (APP) is a highly contagious respiratory pathogen and the causative agent of porcine pleuropneumonia. It is one of the most frequently identified bacterial agents causing porcine respiratory infections (1), and is responsible for high economic losses to the swine industry worldwide (2). The clinical symptoms of APP infection are often indistinguishable from other bacterial and viral respiratory infections, with clinical signs including a decrease in growth rate, breathing difficulties, fever, and high mortality (1,3).

Economic losses incurred by APP infection are largely due to increased mortality rates, animals requiring a longer time to reach their finishing weight, and costs incurred by treatment and metaphylaxis (1,2). APP responds well to antibiotics, however, the spread of antimicrobial resistance genes has been extensively reported and may limit the efficacy of treatment (4–6). Therefore, there is an ongoing need for continued disease surveillance and rapid diagnosis to reduce antibiotic usage and losses incurred to the industry.

There are geographical differences in the seroprevalence of the currently identified 19 serovars of APP (7,8), with serovar 8 predominant in the UK (9). Production of certain key virulence factors vary between different serovars, which can influence the severity of disease and affect vaccine efficacy. Typically, isolates of a given serovar produce one or two of three different Apx toxins, with a fourth (ApxIV) produced by all APP isolates. Surveillance for the presence of the pathogen, as well as detection of predominant serovars, is important for biosecurity measures as well as determination of appropriate disease mitigation strategies. Historically, serotyping was performed using serological methods, with detection of specific capsule antigens determining the serovar. However, similarities between lipopolysaccharide O-antigens amongst subsets of serovars (e.g., 1/9/11, 3/6/8/15, and 4/7) can result in cross-reactions (10–12), thus a shift towards more robust molecular serotyping has occurred (13).

The current gold standard molecular diagnostic technique is the polymerase chain reaction (PCR), typically targeting the species-specific ApxIV toxin gene, *apxIVA*. However, PCR is costly and time-consuming, requiring 2-3 hours to complete with highly trained personnel and complex equipment. Numerous PCR and qPCR assays with high sensitivity and specificity have been described for the detection of APP (14–16). Isothermal amplification techniques rely on enzymes to denature DNA, facilitating primer annealing and subsequent amplification of target sequences at a single temperature. One such isothermal technique is recombinase-polymerase-amplification (RPA), which utilizes T4 bacteriophage enzymes (UvsX, UvsY and Gp32) to anneal primers to their complementary sequence within DNA (17). An advantage of RPA over other isothermal techniques is the requirement of only two primers, similar to PCR. Typically, RPA primers are longer in length than PCR primers, as increased primer length (>28 bp) has been found to increase the rate of amplification as UvsX exhibits a higher rate of ATP hydrolysis when forming filaments with longer oligonucleotides (17,18).

78 RPA amplification can be monitored in real-time with the addition of an exonuclease probe, with
79 several sensitive and specific RPA assays previously described for porcine respiratory pathogens (19–
80 22). Fluorometers are commercially available in a small form, portable device, for a fraction of the cost
81 of a complex thermocycler. Furthermore, RPA reagents are available in a lyophilized format,
82 facilitating stable transportation, making RPA the most versatile point-of-care molecular diagnostic
83 technique currently available.

84 A significant difficulty in the implementation of a simple and rapid point-of-care test is the
85 process of sample preparation. Labor intensive spin column methods (for binding, purification, and
86 elution of nucleic acids) require high-speed centrifugation, which is unsuitable for use in a field setting.
87 We have previously described an alternative sampling method, directly imprinting infected tissue on
88 FTA cards, which chemically lyse and entrap nucleic acids. We have shown the effectiveness of this
89 method, when combined with a simple water wash and our multiplex PCR, for the detection and
90 serotyping of APP (23). Further adaptation of this sample preparation for combination with RPA could
91 prove a powerful tool to screen for APP infection on-site, i.e., directly at the farm or abattoir.

92 In this study, we describe an RPA assay, initially targeting the *apxIVA* gene for detection of all
93 APP (APP-RPA), regardless of serovar, with validation of the assay using 61 extracted clinical
94 samples, 17 lung homogenates and a small subset (n=14) of lung imprints made on FTA cards.

95 **2 Materials and Methods**

96 **2.1 RPA primer design**

97 The APP species-specific *apxIVA* gene was used to design APP-RPA primers and probe. The
98 *apxIVA* gene from serovar 8 strain MIDG2331 (GenBank: LN908249.1, nt: 1,131,104-1,136,935) was
99 subjected to a BLAST 2.0 search against the available genome assemblies of APP (taxid ID: 715)
100 available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The resulting consensus
101 sequence when mapped to the MIDG2331 genome matched an alternative gene annotated *hemolysin*
102 *A* (nt: 1,124,268-1,128,977 ), but has subsequently been reported as a partial duplication of the *apxIVA*
103 gene, denoted *apxIVS* (24). Originally we designed an RPA assay against the same target region of
104 *apxIVA* that is amplified in the qPCR assay described by Tobias *et al*. (16). However, this was
105 subsequently found to result in several alternative priming sites due to being located within a repetitive
106 region of the *apxIVA* sequence. Therefore, a 49 bp probe was designed within a conserved region
107 located at the 3' end of the *apxIVA* sequence (nt: 1,124,315-1,124,454), in addition to 10 forward and
108 10 reverse 35 bp primers, designed in the flanking region of the probe in accordance with the
109 manufacturer's instructions (TwistDx, Maidenhead, UK). In silico analysis of primer dimerization was
110 assessed using Thermo Fisher multiple primer analyzer tool (Thermo Fisher UK, Loughborough, UK).

111 To achieve optimal performance, primer combinations were experimentally screened in duplicate
112 against 200 copies/µL of the serovar 8 reference strain 405 (25). The primer pairs displaying the highest
113 sensitivity are shown in Table 1, covering a 140 bp region of the *apxIVA* gene (Supplementary Figure
114 1).

115

116 **2.2 RPA reaction conditions**

117 RPA reactions were performed using the TwistAmp Liquid Basic kit (TwistDx, Cat
118 #TALQBAS01), according to the manufacturer's recommendations, with final concentrations of 0.6

119 μM of each primer (Thermo Fisher UK), 0.12 μM probe (LGC Biosearch Technologies, Risskov,
120 Denmark, Cat #RPA-BF-2), 2 U/μL *Escherichia coli* Exonuclease III (NEB, Hitchin, UK, Cat
121 #M0206L), 14 mM MgAc, and 1 μL of DNA template, in a total of 5 μL/reaction for limit of detection
122 and specificity experiments. For extracted clinical samples and FTA card amplifications, 25 μL
123 reactions were used, since their use yielded more consistent results over 5 μL reactions. Amplification
124 was visualized with a Bio-Rad CFX Connect Real-Time PCR detection system (Bio-Rad, Cressier,
125 Switzerland, Cat #1855201), running for 20 minutes at 37°C, with a pause at 4 minutes where the
126 reactions were manually agitated (by inversion 10-times), a step which is favorable for reaction kinetics
127 (26). Fluorescence data acquisition was taken once every thirty seconds (i.e., once per cycle) for a total
128 of 40 cycles (20 minutes).

129

130 **2.3 RPA FTA card reaction conditions**

131 RPA reagent concentrations remained as above, however reaction volumes were increased to
132 25 μL to account for the FTA card disc. Samples on FTA classic cards (Whatman plc, Little Chalfont,
133 Buckinghamshire, UK, Cat #WB120205) were prepared by pressing the card surface against lung
134 tissue as previously described (23). FTA cards were allowed to dry fully prior to being processed, 3
135 mm discs were removed from the inoculated card with a sterile biopsy punch, which was rinsed in
136 ethanol and deionized water between uses to prevent cross-contamination. The 3 mm discs were
137 washed twice for 5 minutes in deionized water and added directly to the 25 μL RPA reaction for
138 amplification.

139

140 **2.4 RPA lung homogenate reaction conditions**

141 RPA reagent concentrations remained as above. 50 mg of lung tissue was excised with a sterile
142 scalpel, placed in a 2 mL lysing matrix A microcentrifuge tube (MP Biomedicals, Cat #116910050-
143 CF) along with 400 μL of distilled water. The sample was then homogenized using a FastPrep-25 5G
144 (MP Biomedicals, Cat #116005500) set at 6.0 m/s for 60 seconds. The microcentrifuge tube was left
145 to settle at room temperature for 5 minutes prior to the supernatant being removed and used in
146 subsequent 25 μL APP-RPA reactions.

147 **2.5 Extraction and quantification of genomic DNA**

148 APP reference strains of serovars 1−19 (i.e., strains 4074, S1536, S1421, M62, L20, Femø,
149 WF83, 405, CVJ13261, D13039, 56153, 1096, N273, 3906, HS143, A-85/14, 16287-1, 7311555, and
150 7213384-1, respectively) and two APP clinical isolates, with transposons interrupting or flanking their
151 *apxIVA* genes, one designated as capsule type K3:O7 and one serovar 15 (MIDG2206 and MIDG 3936,
152 respectively) were used in this study. APP isolates were grown overnight on Bacto Brain Heart Infusion
153 broth (BD, Berkshire, England, UK, Cat #2237500) containing 1.5% agar (Sigma-Aldrich, Gillingham,
154 Dorset, UK, Cat #W201201) and supplemented with 0.01% nicotinamide adenine dinucleotide (MP
155 Biomedicals, Eschwege, Germany, Cat #100319). Genomic DNA (gDNA) was extracted from
156 bacterial cells harvested from the plate cultures. Briefly, half a 10 μL loop of bacterial colonies were
157 resuspended in 200 μL of PBS and DNA extraction was performed using a FastDNA spin kit (MP
158 Biomedicals, Cat #116540600-CF) according to the manufacturer's instructions. All extracted gDNA
159 was quantified using a Nanodrop-1000 spectrophotometer (Thermo Fisher UK, discontinued) and run
160 on a 1.5% w/v agarose gel to ensure genomic integrity.

161

162 **2.6 DNA extraction from clinical tissues**

163 A total of 61 clinical lung samples, were obtained from farms in England (n=36) and Portugal
164 (n=25) with a history of APP infection. Lung samples were obtained during necropsies from pigs
165 displaying clinical signs of infection, suspected to be APP. For the extraction of lung tissue,
166 approximately 50 mg of defrosted tissue were collected with a sterile disposable scalpel and
167 homogenized in a lysing matrix A 2-mL microcentrifuge tube with 360 µL ATL buffer (Qiagen Ltd.,
168 Manchester, UK, Cat #19076) using a FastPrep-25 5G set at 6.0 m/s for 60 seconds. Subsequent DNA
169 extraction was performed with a QIAmp DNA Mini Kit (Qiagen Ltd., Cat #51304) and eluted using
170 200 µL buffer AE (Qiagen Ltd., Cat #19077).

171 Five oral fluid samples were obtained from farms in England. Each oral fluid sample was
172 extracted from a cotton rope, with each rope used to sample 25 animals at a time. Each rope was
173 individually extracted using a MagMAX Express-96 Particle Processor (Thermo Fisher UK, Cat
174 #4400074), as previously described (27).

175

176 **2.7 Analytical sensitivity and specificity**

177 The APP-RPA assay performance was assessed using gDNA from APP serovar 8 strain 405
178 diluted to give between $2x10^5$ to 0.5 copies/µL in 10-fold serial dilutions made in TE buffer. Further
179 dilutions of gDNA in TE buffer were made in smaller intervals between detection limits. Limit of
180 detection experiments using serovar 8 strain 405 were run using two separate dilutions of template,
181 with 12 replicates of each condition tested alongside no template controls. The APP-RPA assay
182 sensitivity was additionally assessed using gDNA from the reference strains of all currently known (n
183 = 19) APP serovars, each at 200 copies/µL.

184 The APP-RPA assay specificity was assessed by amplifying duplicate APP-RPA reactions with
185 10 ng of gDNA obtained from eight other bacterial species (n=27), including bacteria commonly found
186 to occupy the same niche as APP, as well as other members of the *Pasteurellaceae* family, as previously
187 described (7). All species tested for specificity are shown in Supplementary Table 1.

188

189 **2.8 APP quantification of clinical samples**

190 Designation of the 61 extracted clinical samples as APP-positive or APP-negative was
191 determined by qPCR targeting the APP species-specific *apxIVA* gene designed and validated by Tobias
192 et al. (16). qPCR reactions containing 0.5 µM of each primer (Thermo Fisher Scientific), 0.3 µM
193 Taqman probe (Sigma) and 1x GoTaq probe qPCR master mix (Promega, UK) for a total reaction
194 volume of 5 µL. Reactions were run in a Bio-Rad CFX-1000 thermocycler with the following cycling
195 conditions: 2 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with
196 data acquisition taken at each cycle.

197 A qPCR standard curve was obtained from Ct values obtained from 10-fold serial dilutions of
198 serovar 8, strain 405, amplified by qPCR in triplicate across three separate dilutions. The standard
199 curve (semi-log linear regression line) was calculated using GraphPad Prism version 9.2.0 for Mac
200 (GraphPad Software, San Diego, California USA). Relative copy numbers of APP present in clinical

201 samples were estimated by extrapolation from the standard curve using two Ct values obtained from
202 each clinical sample, within a 95% confidence interval.

203 The clinical performance of the APP-RPA assay was assessed using the extracted clinical
204 samples, each tested in duplicate. Samples were scored as APP-RPA positive if both replicates
205 overcame the threshold fluorescence level, whilst samples were scored as APP-RPA-negative if one or
206 both replicates failed to reach the threshold fluorescence level.

207

208 **3 Results**

209 **3.1 APP-RPA sensitivity**

210 Twenty APP-RPA primer combinations were screened using gDNA from the serovar 8
211 reference strain 405 at 200 copies per µL reaction (data not shown), and the best performing primer
212 pair was taken forward to assess the sensitivity of the APP-RPA assay. The chosen primer pair, APP-
213 RPA_F and APP-RPA_R, amplified a 140 bp sequence that was well conserved, as assessed by BLAST
214 searches against all available APP genomes, with 100% pairwise identity and 97.9% identical sites
215 across 100% sequence coverage. The partial duplication seen in some APP isolates (24) results in an
216 APP-RPA alternative priming site in these isolates (Supplementary Figure 1).

217 The APP-RPA assay sensitivity was assessed using gDNA from the serovar 8 reference strain
218 405, as this serovar is the most common cause of clinical pleuropneumonia in England and Wales (9).
219 The APP-RPA assay achieved a sensitivity of 10 gDNA copies per µL reaction (Figure 1A), with all
220 positive amplification occurring in under 10 minutes (Figure 1B).

221 Two APP clinical isolates which had previously undergone whole genome sequencing (WGS)
222 (unpublished data) showing they contain transposases, within or adjacent to their *apxIVA* genes were
223 also used (Supplementary Figure 1). MIDG2206 originated from Denmark and designated as having a
224 K2:O7 capsule type, contains a hypothetical protein (WP_017357847) between *apxIVA* and *lacZ*, with
225 the sequence immediately 3' to the hypothetical protein being identical to the 3' end of *apxIVA*
226 resulting to an alternative priming site for the APP-RPA assay. MIDG3936, a serovar 15 isolate,
227 contains a fragmented *apxIVA* with three ORFs, with the longest being 3867 bp. Additionally, this
228 isolate contains a partial duplication of *apxIVA*, designated as *apxIVS'*, with a transposon flanking the
229 duplication (WP_005599960). These isolates were verified to be detected with APP-RPA. Both
230 MIDG2206 and MIDG3936 displayed a similar onset time at 200 copies/µL as their reference strain
231 serovars, serovar 2 and serovar 15, respectively (data not shown). Therefore, isolates with the
232 WP_017357847 hypothetical protein between the *apxIVA* and *lacZ* genes, or a fragmentation pattern
233 of *apxIVA* similar to that seen in MIDG3936, are unlikely to interfere with their positive APP
234 identification using RPA-APP.

235

236 **3.2 APP-RPA specificity**

237 The sequence of the APP-RPA amplicon (140 bp) was used in a BLASTn interrogation of the
238 NCBI nucleotide database (excluding APP), with no significant similarity found for any species,
239 indicating that only APP should cause amplification to occur. Additionally, we experimentally tested
240 gDNA from 27 isolates, including 8 different species found in the same host niche as APP, as well as

241 related members of the *Pasteurellaceae* family. None of these bacterial species caused detectable
242 amplification with the APP-RPA primer set (Supplementary Table 1). Taken together, these results
243 suggest that our APP-RPA assay is highly specific, detecting only the APP species-specific gene,
244 *apxIVA*.

245 **3.3 Clinical sensitivity**

246 The 61 clinical samples with suspected APP infections were obtained from farms throughout
247 Europe (UK: n = 36, Portugal: n=25). Extracted gDNA from these clinical samples were characterized
248 in *apxIVA*-qPCR to determine APP infection status, along with the relative bacterial load. For this,
249 qPCR standards were performed using gDNA from serovar 8 strain 405, with the assay proving highly
250 sensitive, being able to consistently detect 10 gDNA copies per µL reaction (Figure 2A). Of the 61
251 clinical samples, 51 were positive for APP by qPCR and 10 were negative, with samples giving a
252 relative quantification value below the limit of detection considered negative. The relative
253 quantification of clinical samples showed a range of copy numbers were present, from 11 to $10^6$ copies,
254 (Figure 2B).

255 In order to assess our APP-RPA performance, the APP-qPCR characterization was compared
256 to the results obtained with the same clinical samples tested in our APP-RPA assay. Only samples
257 displaying positive amplification in both replicates were considered positive. Samples showing a low
258 burden (<200 copies) by qPCR performed poorly in APP-RPA, with only 10 of the 18 samples
259 amplifying, giving a sensitivity of 55.6%. Of these low burden APP-positive samples, APP-RPA
260 detected all four samples from oral fluid, and 6 out of 14 extracted from lung tissue. All the low burden
261 samples missed by APP-RPA were quantified by qPCR as having a bacterial load lower than 55 copies
262 (equating to less than 2.2 copies per µL in the RPA reaction). For samples characterized as having a
263 medium bacterial burden (201-2000 copies) by qPCR, APP-RPA detected all 15 of the samples,
264 including one oral fluid sample, equating to a sensitivity of 100%. The one oral fluid sample in the
265 medium bacterial load group contained 260 copies, determined by qPCR. Of the high burden samples
266 (>2001 copies), our APP-RPA detected all 18 of the samples, therefore, the high burden samples gave
267 a sensitivity of 100%. All samples in this burden category were derived from lung tissue. Overall (i.e.,
268 across all burden levels), our APP-RPA assay achieved a sensitivity of 84.3%, with 100% specificity
269 (Table 2).

270 **3.4 FTA card clinical comparison**

271 To evaluate the use of lung smears made on FTA cards as a sample isolation method amenable
272 to rapid a diagnostic test for APP, a subset of frozen lung samples (n=13) were inoculated onto FTA
273 cards, which were then dried and washed using our previously described water-wash protocol validated
274 for use in PCR (23). In the clinical evaluation above, one of the lungs were scored as having been
275 negative for APP, three of the lungs were determined to contain low, four medium and five high
276 bacterial loads by qPCR. The overall sensitivity of the FTA-APP-RPA was 75%. However, of the three
277 samples with low bacterial loads (i.e., 96, 127 and 185 copies, as quantified by qPCR) two amplified
278 with the FTA-APP-RPA, with the lowest bacterial load (96 copies) being the sample that was missed
279 by FTA-APP-RPA. Conversely, FTA-APP-RPA failed to detect a sample displaying medium and a
280 high bacterial burden, from lung tissue that was quantified as having 572 and 82,770 copies by qPCR.

281 **3.5. Homogenized lung clinical comparison**

282 In order to achieve more rapid detection of APP, the use of crude homogenized tissue was
283 assessed. Lung samples (n=16) that had previously been subjected to genomic extraction and relative

284 quantification of APP by qPCR also underwent homogenization. Lung sections were dissected,
285 suspended in 400 µL of distilled water and homogenized. The resultant supernatant was added to APP-
286 RPA reactions. Of the 15 lung samples positive by qPCR, nine were detected with APP-RPA, a
287 sensitivity of 53.3%. However, the seven samples that failed to amplify with APP-RPA all belonged
288 to the medium and low bacterial burdens, with all eight of the high bacterial load samples amplifying
289 in APP-RPA. Eight clinical samples were paired and could be compared to one another as they were
290 run in all amplification chemistries; qPCR and APP-RPA using extracted DNA, FTA-APP and APP-
291 RPA with lung homogenate, and the results are summarized in Table 3.

## 292 4 Discussion

293 In this study, we have described a rapid, simple, sensitive, and specific diagnostic assay
294 targeting the species-specific *apxIVA* gene. Respiratory infections continue to be one of the most
295 common infectious disease burdens within the swine industry (28) and can be caused by a variety of
296 bacterial and/or viral pathogens, collectively known as the porcine respiratory disease complex (29).
297 Knowing which pathogen(s) are involved informs appropriate treatment. Acute porcine
298 pleuropneumonia, caused by APP, can present suddenly in susceptible pigs and, as it is highly
299 contagious, and can result in high morbidity and mortality. Timely diagnosis not only limits the spread
300 of disease but also antibiotic usage and economic losses. Currently, available APP diagnostic methods
301 are labor-intensive, time-consuming, and expensive. Therefore, our relatively inexpensive and rapid
302 APP-RPA assay has a huge potential in the application of point-of-care diagnostics for this important
303 respiratory pathogen.

304 A bottleneck for RPA assay design is the lack of proprietary software for designing appropriate
305 primers and probes. Therefore, the design of assays must be performed manually to achieve the most
306 efficient primer/probe combination. We initially screened 20 primers (10 forwards and 10 reverses)
307 and then selected a single combination which displayed the fastest time to positive result. Several
308 primer properties have been implicated in RPA recombination efficiency, which greatly increases the
309 reaction time. Firstly, longer primers (30-38 bp) promote more efficient recombination, although
310 shorter (e.g., 18 bp) primers (30) and those designed for PCR (31,32) have been shown to work in
311 RPA. Secondly, as for PCR, 3' guanine and cytosine anchors are beneficial. Thirdly, GC content should
312 be between 30-70%, and repetitive elements of tandem repeats should be avoided to decrease the
313 likelihood of primer dimerization events.

314 The dual-labelled RPA exonuclease probes require internal modifications, as opposed to qPCR
315 probes which are typically labelled at 3' and 5' ends. Thus, RPA probes are relatively expensive and
316 must be designed with great care. Recently an RPA based assay (33) was described for detection of the
317 APP *apxIVA* gene which utilized the ZC BioScience™ Exo kit. However, we found that the primer-
318 probe combination used in that study was not able to detect all APP serovar reference strains using the
319 Twist Dx reaction format. Specifically, serovars 9 and 11 were not detected due to a 16-base deletion
320 in the target region of the designed probe (Data not shown).

321 We verified that our APP-RPA assay detected all 19 currently known serovars of APP, which
322 is (to our knowledge) the first isothermal assay to have achieved this. Furthermore, all serovars
323 amplified rapidly (under 6 minutes at 200 copies/µL) with no significant difference in time-to-positive.
324 This suggests that our *apxIVA* region is highly conserved between all currently known APP serovars.
325 Whilst our APP-RPA assay allows rapid detection of the presence of APP (via the species-specific
326 *apxIVA* gene), determination of the relevant serovar (important for epidemiological surveillance and

327 for informing vaccination strategies) would require further testing of positive samples, currently
328 provided at centralized diagnostic laboratories.

329 Although our APP-RPA gave positive amplification for all 19 serovars, the *apxIVA* gene is not
330 100% conserved between isolates, and insertions such as IS*Apl1* have been detected in the *apxIVA*
331 gene in some isolates, which could affect detection (34). When tested with isolates that have been
332 found to have transposase adjacent to or within their *apxIVA* genes, we found the isolate MIDG3936
333 with a WP_005599960 transposon flanking the *apxIVA* gene duplication (*apxIVS'*) and a fragmented
334 *apxIVA* gene, had a similar detection time to the reference serovar 2 strain. This indicates that the
335 fragmentation of the *apxIVA* gene did not interfere with detection by APP-RPA, or that *apxIVS'* rescued
336 any sensitivity that may be caused by the fragmentation of the *apxIVA* gene. The isolate MIDG2206,
337 containing a hypothetical protein (WP-017357847) between the *apxIVA* and *lacZ* genes, also resulted
338 in near-identical amplification as the reference serovar 15 strain. Therefore, this untypical arrangement
339 of the genes immediately adjacent to the *apxIVA* gene does not result in any loss in sensitivity in the
340 APP-RPA assay and is unlikely to result in any false-negative results in clinical samples. However, the
341 qPCR assay used in this study forward and reverse primers target a different region of the *apxIVA* gene
342 (GenBank: LN908249.1 nt: 1,124,391-1,124,767). Therefore, it would be difficult to ascertain the true
343 clinical sensitivity of our APP-RPA assay as an insertion in the *apxIVA* gene in the target region may
344 prevent detection in the qPCR assays. Thus, a relative clinical sensitivity of our APP-RPA assay was
345 determined against a previously validated *apxIVA* qPCR assay (16), using a collection of clinical
346 samples originating from England and Portugal. Of the 61 clinical samples from pigs with suspected
347 pleuropneumonia, 36 samples originated from farms in England, where serovar 8 is known to
348 predominate (9). Of the 36 English samples, three were APP negative, 16 had low, nine had medium,
349 and eight had high APP burden, as determined by qPCR. Comparative results with our APP-RPA assay
350 indicated an overall clinical sensitivity of 76.5% in these English isolates. The other 25 clinical samples
351 were obtained from Portuguese farms, where serovars 5 and 17 predominate (unpublished data). Of
352 these samples (all lung tissue), seven were negative, two had low, six had medium, and 10 had high
353 APP burden, as determined by qPCR. Comparatively, our APP-RPA achieved a sensitivity of 100%
354 with these Portuguese samples.

355 Overall, our APP-RPA assay achieved a sensitivity of 84.3% for the 61 clinical samples.
356 However, when relative quantification of copy number, as determined by qPCR, was considered, the
357 false-negative samples in APP-RPA were found to be largely due to samples with low copy number
358 (<200 copies), specifically samples with less than 55 gDNA copies per μL. Only 10 of the 18 low copy
359 lung samples gave positive amplification with APP-RPA. The high proportion of lung samples
360 harboring low APP burden may be indicative of sampling artefacts seen when sampling tissue with a
361 non-uniform distribution of bacteria. Alternatively, a low APP burden may indicate presence of gDNA
362 as a result of a previous APP infection. However, little is known about the rate at which the presence
363 of APP gDNA wanes during the course of APP infections.

364 Current evidence suggests that colonization of the tonsils plays a role in disease
365 transmission (35), as APP is known to be harbored in tonsillar crypts of sub-clinically-infected pigs,
366 including those which have survived acute lung infection (36,37). Asymptomatic carriage of APP is
367 thought to be responsible for the introduction of infections into naive herds, and for sporadic recurrent
368 disease presentation. However, detection of this bacterium in oral fluid samples is known to be
369 problematic (38), with some evidence of serovar differences in the ability to colonize the oral cavity
370 (39). Furthermore, antimicrobial treatment has been shown not to significantly clear carriage in the
371 tonsils (40). Therefore, there is a need to have a diagnostic tool that can detect sub-clinically, as well
372 as clinically infected swine (8). To ascertain the feasibility of detecting sub-clinically infected animals,

373 we obtained five oral fluid samples from UK farms. All five of the oral fluid samples were positive for
374 APP, with one high, and four low bacterial loads of APP - with qPCR quantification at 260, 133, 115,
375 111 and 82 copies/$\mu L$. All five oral samples were detected in APP-RPA in under 7 minutes. The rapid
376 time to positive and relatively late Ct obtained in qPCR of the oral fluid samples is unusual, as each
377 sample represented a pool of 25 animals. This inconsistency in results may be due to inhibition or
378 contaminants in the oral sample (such as other bacterial species), as RPA has been shown to be less
379 prone to common PCR inhibitors (41,42). Given the higher sensitivity of qPCR, it is encouraging that
380 we were able to detect all five of the positive oral fluid samples with our APP-RPA, which is faster
381 and more amenable to point of care use. However, the lack of overall sensitivity of our APP-RPA at
382 low bacterial loads suggests, this technique is not suitable to detect asymptomatic carrier animals.
383 Furthermore, the ability of our APP-RPA to sensitively detect medium and high copy numbers of APP
384 suggests it is more suitable for deployment as a rapid point-of-care assay, especially where APP is
385 suspected from the clinical presentation and where rapid diagnosis would have the potential to limit
386 spread through swift treatment and isolation of contact animals.

387 Other isothermal techniques, such as loop-mediated isothermal amplification (LAMP) assays
388 (43,44), have been developed for the detection of APP. However, LAMP requires a higher temperature
389 to operate than RPA (60-65°C rather than 37°C), has a more complex primer design, and is not
390 currently commercially available in a lyophilized format, which is essential for use in the field in a
391 point-of-care scenario. Therefore, LAMP is more difficult to incorporate into low-resource field
392 settings. RPA has already been taken into remote settings, with some groups describing a simple 'lab
393 in a suitcase' for detection of pathogens responsible for human epidemics, including Ebola and SARS
394 CoV-2 (45,46).

395 A major challenge in point-of-care diagnostics is the extraction of samples in resource limited
396 settings, with more commercial extraction kits requiring high powered table-top centrifuges, which are
397 not mobile. The possibility to directly amplify from FTA cards with RPA would significantly simplify
398 this extraction bottleneck. Our previously described FTA-multiplex-PCR for APP detection was based
399 on imprinting the FTA card on lung tissue (visibly lesioned areas, where possible) and two 5-minute
400 water washes (23). As lung samples have been previously optimized for use of FTA cards and are the
401 most commonly collected sample type collected for APP diagnostic investigations, we did not seek to
402 validate other tissue types on FTA cards.
403 Our current small-scale validation using FTA cards in combination with the APP-RPA assay showed
404 a sensitivity and specificity of 76.9% and 100%, respectively. When compared to results using
405 extracted gDNA from paired lung samples (n = 10), all 10 were detected in extracted samples, however
406 two of these samples failed to be detected in FTA-APP-RPA. Only one of the three lung samples
407 quantified as having low bacterial load in extracted lung tissue failed to be detected in FTA-APP-RPA.
408 Surprisingly, one sample containing high and one containing medium bacterial load (82,770 and 572
409 copies, respectively) also failed to be detected. This may be due to inconsistencies in sampling, and
410 repeated sampling of different areas of the lung may increase the probability of sampling where the
411 bacteria are present. This requires further investigation but is beyond the scope of the current study.
412 However, the decrease in sensitivity when FTA cards were used in combination with RPA suggests
413 that sub-clinically infected animals with low bacterial loads will be missed with this rapid method of
414 detection. The use of a rapid processing method for lung tissue would facilitate the use of point-of-care
415 techniques on farm, we thus sought to compare the use of gDNA extracted and quantified by qPCR to
416 a crude homogenization of lung tissue would release enough bacterial gDNA to be detected by APP-
417 RPA. The use of homogenized tissues provided a higher correlation than FTA-APP-RPA with qPCR
418 copy numbers, with only the lung samples displaying the lowest copy numbers tested (below 12,000
419 copies), testing negative with RPA. The achieved APP-RPA detection limit increased from the 55

copies in extracted clinical samples to 12,000 copies in homogenized tissue. Therefore, the use of lung homogenate prioritizes the rapid preparation of samples over assay sensitivity. Overall, the use of FTA-cards or homogenized tissues in conjunction with APP-RPA are feasible, bypassing the labor-intensive and impractical commercial extraction of clinical lung samples.

Several limitations to our current study exist, firstly although *apxIVA* is widely used as an APP species marker, the instance of insertional elements within or distally to the *apxIVA* gene or partial duplicons in this locus makes assay design troublesome. We have used isolates with both transposons and small tandem duplications - which have yet to interfere with our APP-RPA diagnostic. However, it should be noted that duplications of the *apxIVA* gene result in alternative priming sites which can contain single-polynucleotide polymorphisms. Whilst our sequence analysis suggests that at least one perfect match APP-RPA binding region is present within these isolates, the assessment of how alternative priming sites may affect either the qPCR or APP-RPA assays was beyond the scope of this study. Secondly, we determined that our lower limit of detection in clinical samples equates to 55 copies/µL. In order to assess our clinical performance, we grouped our data into negative, low, medium and high bacterial loads based on qPCR relative quantification. We placed our low bacterial load group at less than 200 copies/µL for two reasons; it gave a uniform sample number in each group and the distribution of the qPCR copy numbers of samples gave a natural break across these groups. If we had chosen to classify <55 copies as ultra-low, we would have achieved 100% sensitivity and specificity across all other groups. However, this may lead to the false interpretation of our results as 8 of the 18 low copy number samples were below 55 copies, which may still retain clinical relevance to the APP status of an animal. Thirdly, due to limited availability, our number of oral fluid samples was low (5 out of the 61 clinical samples tested). Tonsil samples could have been used; however, no tonsil samples were available for analysis although we envisage that APP-RPA could be applied to such samples. Our limited data suggests that although oral samples contain a relatively low bacterial burden compared to the majority of lung samples, they may still prove a useful tool in detecting sub-clinically infected animals, although more samples are required to provide a full validation. Lastly, we describe the detection of APP in APP-RPA using alternative sampling methods, whilst our FTA cards gave inconsistent results in relation to onset time and the achieved limit of detection. We used qPCR of extracted tissue from the same lung tissue as our 'gold standard' to determine the copy number. However, the presence of bacteria may not be uniform across all the tissue areas, and the use of different sampling areas for each method may have accounted for the discrepancies seen in the ability to detect APP with APP-RPA.

In conclusion, we have developed an APP-RPA assay that is rapid and specific for detecting all 19 known serovars of APP. Furthermore, the APP-RPA displays good sensitivity using either extracted gDNA, homogenized samples, or imprinted on FTA-cards. Our data suggests that an RPA-based field deployable diagnostic test for APP enabling the rapid detection and screening of this highly economically important pathogen is feasible.

## 5 Figures

**Figure 1. APP-RPA performance.**

(A) Regression line of APP-RPA limit of detection across 12 replicates using two different template

463 dilutions of serovar 8 reference strain 405. Equating gDNA input to time to positive (TTP) (B)
464 Fluorescence data of the amplification of APP-RPA, calculated from the average relative fluorescence
465 units (RFU) obtained from 12 replicates of APP serovar 8. (C) TTP of all known serovars of APP
466 reference strains amplified in APP-RPA at 200 copies/µL.

467 **Figure 2. APP-qPCR performance.**
468 (A) Regression line from limit of detection of APP-qPCR performed with gDNA from serovar 8
469 reference strain, 405. (B) Relative quantification of APP copy burden for 61 clinical samples with
470 respective APP-RPA time to positive (TTP).

471

472 **6 Tables**

473 **Table 1. RPA primers and probes used in this study.**
474 Optimized primers for the detection of the APP specific *apxIVA* gene.

| Name | Sequence 5'-3' | Amplicon size (bp) |
|---|---|---|
| APP-RPA_F | GCGACACAAGAGATATCTCTCCTCCGTGCTTCTGA | 140 bp |
| APP_RPA_R | GTATTCACACCAAGATCATAAAATAGAAAATATTC | |
| APP-RPA_Prb | AAACGTTGGTGAGCACTCAGGTGGAGAAGA[T(FAM)[dspacer]G[T(BHq-1)]TGAGTCGATGGCCGG | N/A |

475

476 **Table 2. Performance of APP-RPA in 61 clinical samples.**
477 61 clinical samples (56 lung samples and 5 oral fluid samples) were obtained from various farms, 36
478 in England and 25 in Portugal. Clinical samples were characterized in qPCR and relative quantification
479 was performed based on Ct values. Subsequently, samples were run in APP-RPA to assess the clinical
480 performance of the assay.

| | **qPCR** | | | **APP-RPA** | |
|---|---|---|---|---|---|
| **Bacterial Load (Relative quantification)** | **Upper qPCR limit (Ct)** | **Lower qPCR limit (Ct)** | **Total number (n)** | **Sensitivity (%)** | **Specificity (%)** |
| **High >2001 copies** | N/A | 27.28 | 18 | 100 | N/A |

| | | | | | |
|---|---|---|---|---|---|
| **Medium**<br>**201-2000 copies** | 27.27 | 31.01 | 15 | 100 | N/A |
| **Low**<br>**<200 copies** | 31.00 | 35.64 | 18 | 55.6 | N/A |
| **Negative** | 35.63 | N/A | 10 | N/A | 100 |
| **Total** | N/A | 35.64 | 61 | 84.3 | 100 |

**Table 3. Performance of different extraction methods in APP-RPA.**
Eight clinical lung samples were extracted for gDNA, homogenized and smears made on FTA cards. The FTA cards were processed by 2 5-minute washes in deionized water. The 3 mm FTA card discs were subsequently added directly into APP-RPA reactions for detection. Time to positive (TTP) in minutes of APP-RPA detection compared across the three (Extracted gDNA, lung homogenate and FTA card) sample preparation methods.

| Bacterial burden | qPCR copies | APP-RPA TTP (mins) | | |
|---|---|---|---|---|
| | | Extracted | FTA | Homogenate |
| High | 82770 | 2.63 | Negative | 2.86 |
| | 24300 | 3.02 | 1.54 | 3.73 |
| | 21284 | 2.78 | 6.81 | 3.39 |
| | 11169 | 3.51 | 4.33 | 4.51 |
| Medium | 572 | 5.22 | Negative | Negative |
| | 246 | 6.15 | 2.97 | Negative |
| Low | 185 | 6.27 | 5.69 | Negative |
| Negative | 0 | Negative | Negative | Negative |

## 7 Author Contributions

Study concept, all authors; O.W.S. and M.S.F. designed and optimized RPA assay conditions. O.W.S. and Y.L. designed and performed experiments including data analysis; O.W.S., J.T.B., Y.L., and P.R.L. all contributed to writing the manuscript; J.H.G., A.W.T., F.C., P.M., E.V. and P.P. provided clinical

493 samples; M.S.F., J.H.G., A.W.T., F.C., P.M., E.V., P.P, and J.R.M were all involved in proof-reading
494 the manuscript; P.L. and P.G. secured funding.

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498 **9 Conflict of Interest**

499 Authors T.N., P.M., E.V. and P.P. are employed by Ceva Animal Health Ltd. The remaining authors
500 declare that the research was conducted in the absence of any commercial or financial relationships
501 that could be construed as a potential conflict of interest.

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# Figure 1

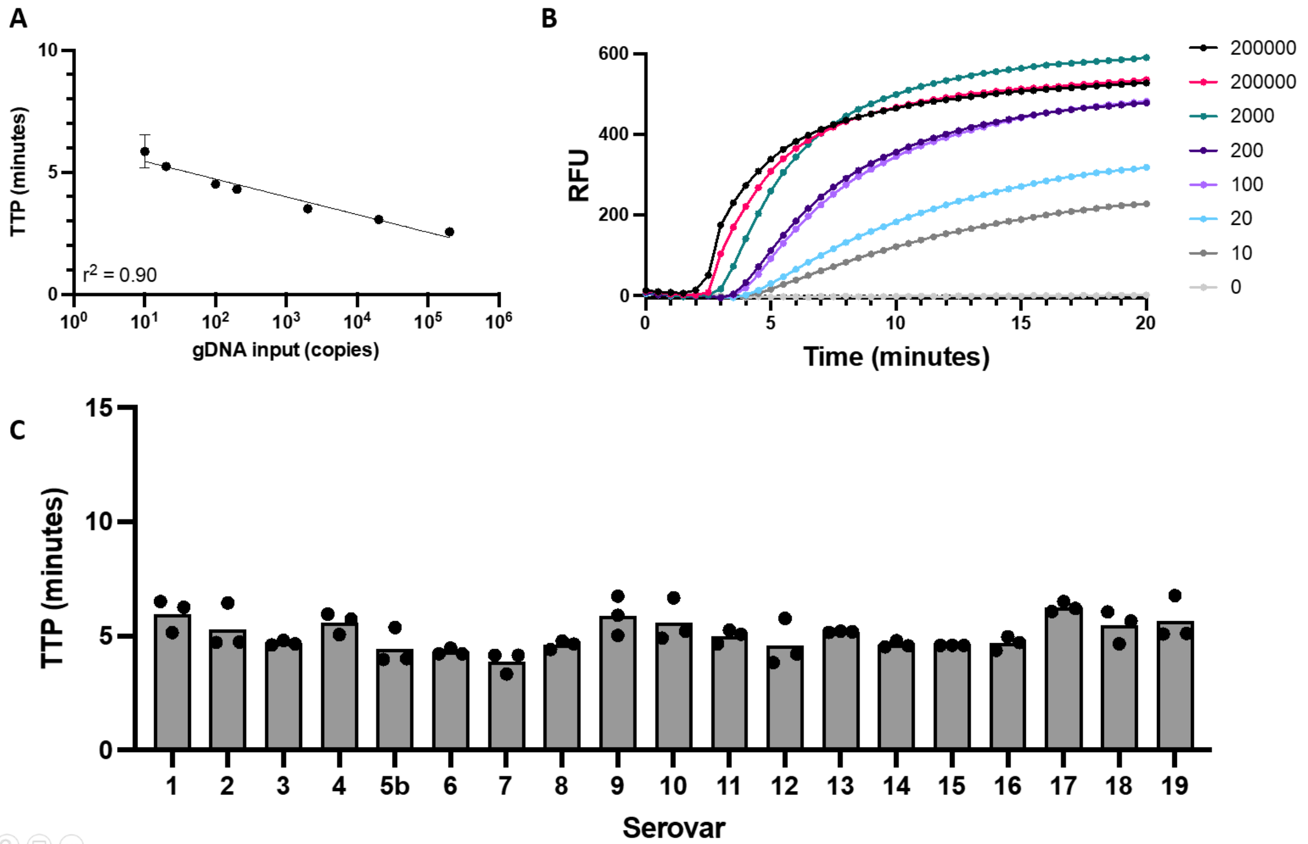
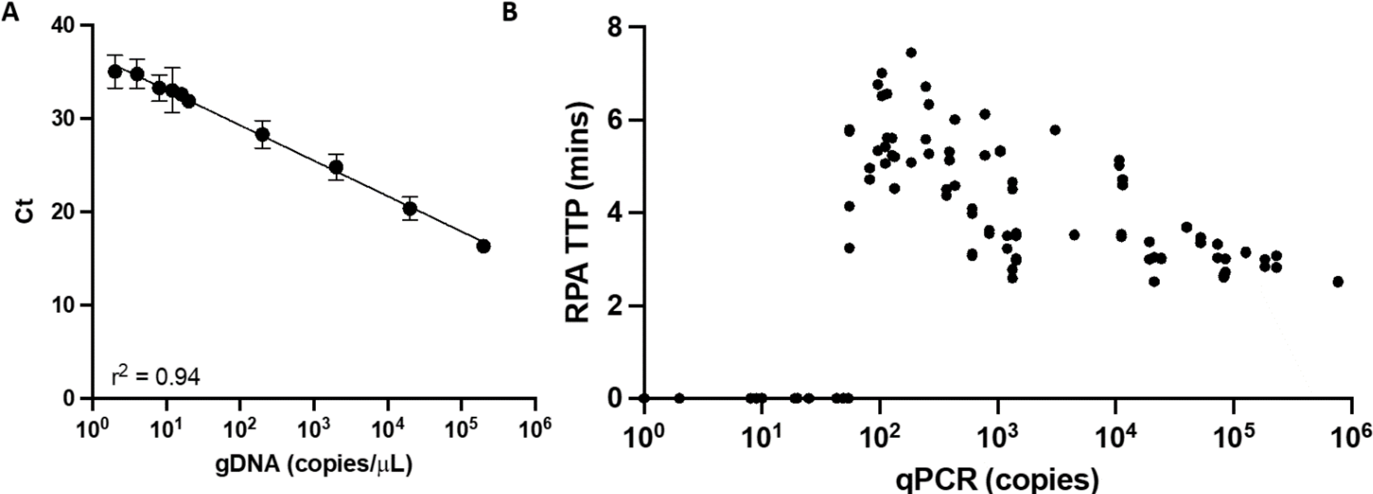


**Figure 2**



**Supplementary Table 1.** Species tested against APP-RPA in specificity experiments.

| Species | Isolates (n) |
|---|---|
| *Actinobacillus equuli* | 4 |
| *“Actinobacillus porcitonsilarium”* | 4 |
| *Actinobacillus suis* | 3 |
| *Glaesserlla parasuis* | 3 |
| *Mycoplasma hyopneumoniae* | 3 |
| *Pasteurella multocida* | 4 |
| *Pasteurella pneumotropica* | 2 |
| *Streptococcus suis* | 4 |

**Supplementary Figure 1.** Graphical depiction of *apxIVA* locus of isolates with untypical *apxIVA* gene arrangement.

MIDG2311 contains two hypothetical proteins in opposite directions dispersing a partial duplication of *apxIVA*, denoted as *apxIVSS*. MIDG3396 contains a transposable element, WP_005599960, flanked by a partial duplication of *apxIVA*, denoted *apxIVS'* and a fragmented *apxIVA* gene containing three open reading frames. MIDG2206 contains a hypothetical protein, WP-017357847, between *lacZ* and *apxIVA*. Red arrows depict APP-RPA primer binding sites, alternative binding sites may contain single-nucleotide polymorphisms (not shown).