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Diagnostic Accuracy of Two DNA-based Molecular Assays for Detection of Porcine Circovirus 3 in Swine Population using Bayesian Latent Class Analysis

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Running headline:

Evaluation of PCV-3 diagnostic assays

28 **Significance and Impact of Study**

29 The continuous discovery of new pathogens poses a challenge in the development and evaluation of
30 adequate diagnostic tools. In fact, since molecular-based tools sometimes are the only available
31 laboratory techniques, it is typically difficult to evaluate their diagnostic performances in absence of a
32 gold standard. The present study assess this issue, demonstrating the excellent performances of two
33 PCR-based assays for PCV-3 detection using a Bayesian latent class analysis approach.
34 Therefore, the molecular tests evaluated under this study constitute reliable tools for the routine
35 diagnosis and surveillance programs of PCV-3 circulating in swine populations.

36

37 **Abstract**

38 **Aims:** Molecular-based tools sometimes are the only laboratory techniques available to detect a
39 recently discovered agent and their validation without the existence of previously described "gold
40 standard" methods poses a challenge for the diagnosticians. A good example within this scenario is the
41 recently described *Porcine circovirus 3* (PCV-3) in the swine population worldwide, from which only
42 few PCR methods have been described. Therefore, the primary objective of this study was to estimate
43 the diagnostic accuracy of a direct-PCR (dPCR) and a real-time qPCR (qPCR) for detection of PCV-3
44 in Italian swine population. **Methods and Results:** Bayesian latent class analysis approach was used to
45 rigorously assess their features and applicability in routine diagnostic activity. Data on dPCR and
46 qPCR were available from 116 domestic pigs, which were randomly selected from 55 farms located at
47 different regions in northern Italy. The sensitivity (Se) estimates of dPCR (94%; posterior credibility
48 interval [PCI%] 84-100) and qPCR (96%; PCI% 90-100) were high and similar. The estimated
49 specificity (Sp) of both dPCR and qPCR assays was around 97%. **Conclusions:** dPCR and qPCR
50 assays showed a high and comparable sensitivity and specificity estimates for the detection of PCV-3
51 in Italian swine population.

52 **Keywords:** porcine circovirus 3; direct-PCR; quantitative PCR; diagnostic test evaluation; sensitivity
53 and specificity**;** latent class modelling

54

55 ## Introduction

56 The genus Circovirus rose to veterinary medicine attention by the 90s', when viruses responsible for
57 different avian diseases were discovered and classified into this group (Todd, 2004). Since then, many
58 new circovirus species have been identified in different hosts, being in most of the cases responsible for
59 infections of negligible clinical and economic relevance (Delwart and Li, 2012; Rosario et al., 2017).
60 *Porcine circovirus 2* (PCV-2) represents a very relevant exception due to its ability to cause one of the
61 most damaging and widespread swine infections (Allan et al., 2012), responsible for several clinical
62 syndromes collectively named as porcine circovirus diseases (PCVDs) (Segalés et al., 2013). More
63 recently, a new swine circovirus species, named *Porcine circovirus 3* (PCV-3) has been (Palinski et al.,
64 2017). Its genome includes two major open reading frames (ORFs), ORF1 and ORF2, located in
65 different strands of the viral replicative form, oriented in opposite direction and encoding the replicase
66 (Rep) and capsid (Cap) proteins, respectively (Palinski et al., 2017). Although the genomic data
67 availability is still limited, the Rep gene appears more conserved than the Cap one, likely due to the
68 different action of functional constraints and effect of immune response, as it has been proposed for
69 PCV-2 (Franzo et al., 2016). Up to now, no definitive evidence of PCV-3 ability to cause disease in
70 pigs is currently available (Franzo et al., 2018c; Klaumann et al., 2018a). However, PCV-3 detection in
71 presence of different disease conditions such as porcine dermatitis and nephropathy syndrome (PDNS),
72 reproductive and respiratory disorders (Ku et al., 2017; Palinski et al., 2017; Shen et al., 2018) and
73 myocarditis (Phan et al., 2016) has elicited a remarkable interest in the scientific community.

74

75 PCV-3 has been reported in North and South America (Palinski et al., 2017; Tochetto et al., 2018),
76 Asia (Kwon et al., 2017; Shen et al., 2018) and Europe (Stadejek et al., 2017; Franzo et al., 2018b),
77 suggesting its ubiquity and prolonged circulation over time (Klaumann et al., 2018b). Moreover, its
78 high frequency of detection in wild boar populations has been recently reported (Franzo et al., 2018c;
79 Franzo et al., 2019; Klaumann et al., 2019). Based on these premises, the pivotal role of effective and
80 accurate diagnostic tools, finding application for both research and diagnostic purposes, appears of
81 interest. So far, the only techniques developed to detect PCV-3 are molecular methods such as direct-
82 PCR (dPCR), real-time quantitative PCR (qPCR), and in situ hybridization (Chen et al., 2018; Franzo
83 et al., 2018a; Li et al., 2018). The latest one, however, is available just in a few laboratories worldwide.
84

85 Molecular based tools have become of great importance as diagnostic assays in veterinary virology
86 because of their high sensitivity, specificity and rapidity (Belák, 2007; Hoffmann et al., 2009; Pestana
87 et al., 2010). Test validation studies assuming perfect reference tests are common, but with a potential
88 to introduce bias in estimation of index test(s) performances (Lijmer et al., 1999). Paradoxically, the
89 high sensitivity of PCR methods compared to other assays makes almost impossible to define a proper
90 "gold standard" for diagnostic performance evaluation. In fact, their actual higher sensitivity could be
91 misclassified as an inadequate specificity (Drigo et al., 2014a). Latent class analysis (LCA) allows for
92 the simultaneous estimation of test parameters in populations where the underlying true infection status
93 is unknown (Hui and Walter, 1980). The true infection status in LCA is regarded as an existing, but
94 unknown (latent) variable, and test accuracy and prevalence are subsequently parametrized according
95 to this latent variable. Therefore, the application of an appropriate statistical framework allowing the
96 estimation of the diagnostic performances in a "gold-standard independent fashion" is essential.
97 The primary objective of this study was to estimate the diagnostic accuracy of recently developed
98 molecular-based PCR assays including dPCR and qPCR (Franzo et al., 2018a) for detection of PCV-3

99 in domestic pig populations within a Bayesian framework. The findings of this study will serve to
100 illustrate the tests' potential for routine field use in PCV-3 screening.
101
102
103 **Results and Discussion**
104 This is the first study estimating the diagnostic Se and Sp estimates of dPCR and qPCR for detection of
105 PCV-3 in domestic pig populations sampled under natural field conditions without the assumption of a
106 gold standard. Results of detection of PCV-3 DNA using dPCR and qPCR were available for 116
107 porcine samples from different pig populations in northern Italy representing different sample type
108 including 39 lungs, 33 sera, 32 organ pools, 8 oral fluids, 4 nasal swabs samples. The data of both
109 dPCR and qPCR results were subjected for the LCA analysis. Descriptive statistics showed that 33.6%
110 (n=39) samples were positive for PCV-3 DNA by dPCR, whereas 34.5% (n=40) samples were positive
111 by qPCR. Results of cross-tabulated (contingency) of the dichotomous outcome of dPCR and qPCR for
112 detection of PCV-3 DNA are shown in Table 1. The estimates of posterior median and 95% PCI of true
113 prevalence and Se and Sp of dPCR and qPCR are shown in Table 2.
114 The Se estimates of both dPCR and qPCR was high and similar at 95% PCI. Se of dPCR was 94%
115 (95% PCI: 84-100), whereas Se of qPCR 96% (95% PCI: 90-100). The estimated Sp of both molecular
116 assays was high and comparable at 97%. The estimated true prevalence of PCV-3 was varied among
117 the tested porcine subpopulations, ranging from 16% (95% PCI: 0.007-0.62) in Piemonte to 38% (95%
118 PCI: 0.27-0.50) in Lombardia (Table 2). The covariance parameters ($\sigma_{se}$and σ_{sp}) differed significantly
119 from zero "i.e. the 95% PCI did not cover 0" suggesting conditional dependence between the tests.
120 That was further confirmed based on comparison between the DIC values. For these reasons, the
121 model assuming conditional covariance (COC) between dPCR and qPCR was preferred over other CID

122 model scenario (DIC = 26.1 for a model assuming COC, and DIC = 27.6 for a model assuming CID
123 between the two tests).
124 These findings showed that both dPCR and qPCR assays are highly sensitive and specific for detection
125 of PCV-3 DNA from different sample types of domestic pigs. These results are in agreement with
126 previous studies that consistently reported an extremely high Se of PCR-based methods, being able to
127 detect up to 1 genome copy/reaction when properly designed and optimized (Hoffmann et al., 2009;
128 Mijatovic-Rustempasic et al., 2013; Parker et al., 2015; Kralik and Ricchi, 2017). Additionally, since
129 successful target amplification and detection rely on multiple specific interactions between assays
130 oligonucleotides and target genome, a high-test specificity is typically expected, especially for qPCR.
131 Nevertheless, this virtue comes at the cost of a potential susceptibility to mismatches among designed
132 oligonucleotides and target genome, which can significantly affect the assays diagnostic sensitivity
133 (Drigo et al., 2014b). However, the remarkable performances herein proven for both assays exclude
134 this limitation. The quite modest PCV-3 genetic variability at present (maximum genetic distance of
135 3.4% in the Rep gene, based on available sequences) (Klaumann et al., 2018a) and the selection of the
136 more conserved genomic region for primer/probe design can probably justify the observed results.
137 Although only Italian samples were screened, the *in silico* evaluation of published sequences obtained
138 from virus sequences collected in several countries revealed the presence of few mismatches compared
139 to the designed oligonucleotides, suggesting that these results could be confidently extended to the
140 worldwide scenario (Franzo et al., 2018a).

141 In the previous study where the evaluated PCR techniques were described (Franzo et al., 2018a), it was
142 shown that the analytic Se and Sp of both dPCR and qPCR methods were high, with a Se of 10 viral
143 genome copies/μL. Current findings support the substantially perfect concordance between the two
144 assays and confirm the accuracy of dPCR assay. Consequently, both assays can find a practical
145 application for reliable PCV-3 DNA detection and their use as laboratory tools to monitor the infection,

146 even though with different advantages. The dPCR can provide a new, automatable and cheap tool for
147 massive PCV-3 screening, while the more "traditional" qPCR, although slightly more expensive and
148 laborious, could find application when viral quantification is of interest.

149 When applying LCA to estimate test performance of diagnostic tests, there are model assumptions and
150 conditions to consider. The first assumption of the LCA model is that the two tests are conditionally
151 independent given positivity status. In our study, dPCR and qPCR were considered as conditionally
152 dependent because they are measuring the same target "DNA of PCV3". This assumption was further
153 confirmed based on the DIC value for the different models (COC & CID), where the model accounting
154 for covariance "COC" between dPCR and qPCR was preferred (DIC = 26.1). The second assumption
155 when using LCA is that Se and Sp of each test are constant throughout the tested populations. To
156 justify that assumption, we repeated the BLCM analysis with exclusion of each of the 5 regions based-
157 populations, one at a time (Mahmmod et al., 2013). The obtained test estimates (Se, Sp) for both PCR
158 tests showed no substantial changes, which supports that the assumption was not violated. The final
159 assumption is that prevalence of infection/disease status should differ between populations. It was
160 assumed *a priori* that the apparent prevalence of PCV-3 differed among the study populations because
161 of different features of regional farming in Italy and efforts devoted to infectious diseases spreading
162 control. Additionally, fluctuations in PCV-3 detection over time has already been reported (Klaumann
163 et al., 2018b). That assumption was also confirmed in the present study, as posterior estimates of
164 prevalence median were different among regions (Table 2). This evidence is of particular interest since
165 Toft et al. (2005) reported that the larger the difference between disease prevalence in the populations,
166 the higher the precision in the estimates (Toft et al., 2005).

167 Using the LCA approach, Se and Sp of both dPCR and qPCR assays for detection of PCV-3 DNA were
168 estimated in different tissues of pig populations in Northern Italy. Both dPCR and qPCR assays were

169 highly sensitive and specific methods for detection of PCV-3; hence, they elucidate reliable tools for
170 the routine diagnostic and surveillance programs against PCV-3.
171

172 Materials and methods

173 Study population and sampling procedures

174 Data on test results of two PCR-based assays were obtained from a previous study (Franzo et al.,
175 2018a), which developed and validated the analytic performance of dPCR and qPCR assays for
176 detection of PCV-3 in pig populations. Porcine serum and tissue samples were collected from domestic
177 pigs originating from 55 farms located in Northern Italy. The samples were delivered to the Veterinary
178 Infectious Disease laboratory (Dept. Animal Medicine, Production and Health, Padua University, Italy)
179 for diagnostic purposes between 2014 and 2017. Samples were collected from 5 Northern Italy regions,
180 including Emilia-Romagna, Friuli Venezia Giulia, Lombardia, Piemonte and Veneto (Table 1). The
181 investigated populations can be considered representative of the Italian swine production industry since
182 (with negligible variation over time) 7.5 out of about 9 million Italian pigs are raised in Northern Italy:
183 18% in Emilia-Romagna, 3% in Friuli Venezia Giulia, 56% in Lombardia, 13% in Piemonte and 10%
184 in Veneto (ISTAT). The sample size was initially selected in order to estimate the infection prevalence
185 with at least a 10% precision and 95% confidence interval, assuming an infinite population size and
186 50% prevalence.
187 The samples were processed as previously described in Franzo et al. (2018a). Briefly, the tissues were
188 mechanically homogenized in phosphate buffer saline (10 mL of PBS/g of tissue) before further
189 processing. Similarly, swabs and sponges were diluted in 500 μL of PBS and vortexed. DNA was
190 extracted from 200 μL of liquid matrices (ExtractSpin TS kit, BIOLAB, Gorizia, Italy), setting the final
191 elution volume to 100 μL. All samples were subjected for testing using the optimized dPCR and qPCR

192 protocols. To ensure blindness, the samples were split in two aliquots and a randomized ID was
193 assigned. The link between different tests was disclosed at the end of the experiment.
194
195 **Diagnostic assays**
196 **Direct PCR assay**
197 The dPCR was developed and optimized as previously described (Franzo et al., 2018a). Briefly, several
198 primer pairs were designed to cover a region of ~500 bp located in the PCV-3 *rep* region. PCR was
199 performed using Thermo Scientific™ Phire™ Animal Tissue Direct PCR kit (Thermo Fisher Scientific,
200 Waltham, MA). To evaluate assay performance, various thermal protocols and reagent concentrations
201 were attempted and compared by testing a 10-fold plasmid dilution (10^8–1 copy/μL) performed in
202 different tissue matrices. The assay was developed using the selected matrices directly as templates
203 because the DNA extraction step was not required. Reactions were performed on a 2720 Thermal
204 Cycler (Applied Biosystems™, Foster City, CA), and amplification and specificity of the bands were
205 visualized after electrophoresis on 2% agarose gel and staining.
206 The assay limit of detection (LOD, defined as the lowest viral amount that can be detected in at least
207 50% of replicates) and the absence of nonspecific amplification products were selected as criteria to
208 evaluate and compare different assay settings.
209
210 **Real-time quantitative qPCR assay**
211 Similarly, the qPCR assay was developed and performed as previously described (Franzo et al., 2018a).
212 Briefly, primers and probes were designed based on the *rep* gene. Additionally, a commercially
213 available exogenous internal control was also implemented in the qPCR validation. To minimize the
214 interference between the IC and viral target amplification, different IC plasmid and primer–probe
215 combinations were evaluated to maximize PCV3 detection sensitivity while consistently detecting IC,

particularly at low PCV3 titers. Because qPCR requires purified DNA as template, the plasmid dilution curves were extracted (ExtractSpin TS kit, BIOLAB, Gorizia, Italy) before further processing. qPCR was performed using DyNamo™ColorFlash Probe qPCR kit (Thermo Fisher Scientific, Waltham, MA) on a LightCycler® Nano Instrument (Roche Life Science, Indianapolis, IN), and the analysis was done using LightCycler nano software v.1.1, (Roche). The assay analytic performances were evaluated using the same approach described for dPCR.

Target condition

The latent infection status (viraemia) targeted for detection by the PCR assays reflects a blood sample containing either the live PCV-3 virus or its DNA fragments at any concentration level, whether the tests detected it or not. More specifically, the infectious status was assessed by the successful amplification of a specific region of the viral genome. The positive result (i.e. infection presence) was identified by the fluorescence increase (qPCR assay) or detection of a specific band after gel electrophoresis run (dPCR assay).

Population stratification

The Northern Italian regions: Emilia-Romagna, Friuli Venezia Giulia, Lombardia, Piemonte and Veneto stratified the Italian swine population into the respective five subpopulations – similarly perceived to have different true prevalences of PCV-3.

Statistical analyses

A Bayesian latent class model (BLCM) fitted in OpenBUGS v3.2.2 (Lunn et al., 2009) but called from R software via the 'BRugs' package (Thomas et al., 2006) was used to derive the sensitivity (Se) and specificity (Sp) of the tests as well as the PCV-3 prevalences specific to the aforementioned swine

240 subpopulations. The analysis was closely guided by the standards for reporting diagnostic accuracy
241 studies that use BLCMs (STARD-BLCM) (Kostoulas et al., 2017), Table S1.
242
243 Fitting BLCMs calls for three assumptions: (1) the target population ought to constitute two or more
244 subpopulations with different prevalences, (2) the Se and Sp of the index tests should be constant
245 across the subpopulations and (3) the tests should be conditionally independent given the disease status
246 (Hui and Walter, 1980). However, considering that the tests employ similar detection mechanisms for
247 the virus, i.e. both tests target the virus DNA, the assumption on conditional independence was relaxed
248 to allow for correlation between the two tests by fixing two conditional covariance parameters, σ_{se} and
249 σ_{sp} between pairs of Se and Sp of the tests respectively as specified by Gardner et al. (2000). To verify
250 the importance of the assumption of conditional covariance, the model was compared to a model
251 assuming conditional independence (CID model) between the two tests. The relative fit between the
252 two models was compared using the Deviance Information Criteria (DIC) (Spiegelhalter et al., 2002).
253
254 Counts (O_p) of the different test combinations (e.g. +,+) were presumed to be multinomially distributed:
255
256 $$O_p \vee Se_i Sp_i P_p \; multinomial(prob_p, n_p)$$
257
258 Where Se_i and Sp_irepresent the respective test characteristics for test i (i = 1,2) and P_p is the specific
259 prevalence for the pth ($p = 1{:}5$) subpopulation. $Prob_p$ is a vector of probabilities of observing the
260 different combinations of test results whereas n_p reflects the total number of pigs tested for the pth
261 subpopulation. For instance, in the 1st subpopulation for an animal testing positive to each of the two
262 tests, $prob_1$ is given by:
263

$$prob_1 = (Pr(T_1^+T_2^+|D^+) + \ Pr(T_1^+T_2^+|D^-))$$

$$= (Se_1Se_2 + \sigma_{se})P_1 + \ \big([1 \quad Sp_1][1 \quad Sp_2] + \sigma_{sp}\big)[1 \quad P_1]$$

The five subpopulations yielded 15 degrees of freedom (df) sufficient to estimate 11 parameters (Se and Sp of the two tests, five subpopulation prevalences and two conditional covariances). To the best of authors' knowledge, there is no available literature/information on the diagnostic Se and Sp estimates for PCV-3 diagnostic tests. Thus, a vague uniform prior information was used for the Se and Sp of the quantitative PCR based on expert opinion [Sp and Se estimates in the range of 90-100%] on a TaqMan quantitative PCR assay. Such information was necessary to ensure identifiability of the model (Jones et al., 2010; Statisticat, 2015). The hypothesis: σ_{se}, σ_{sp} = 0, was evaluated using a Bayesian P value. The goodness-of-fit of the Bayesian model was evaluated using the posterior predictive P value.

The model was initialised with two Markov Chain Monte Carlo chains with different values. Each chain comprised 200,000 samples, with the first 100,000 being discarded as the burn-in. Convergence of the chains was evaluated by visual appraisal of the time series plots of selected variables and the Gelman-Rubin diagnostic plots. The posterior distribution of the subpopulation prevalences and the Se and Sp of the two tests were reported as the median and the corresponding 95% posterior credible intervals (PCI) (Table S2).

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Conflict of Interests

288 The authors declare no conflict of interest. No competing financial interests exist.

289

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291

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409

410 **Table 1.** Cross-tabulated results for combinations of real time PCR and direct PCR assays used for

411 identification of PCV3 in 116 samples collected from swine populations in Italy and stratified based on

412 the region into five subpopulations.

Population	Test combinations (T1; real time PCR and T2; direct PCR)				Total
	Pos/Pos	Pos/Neg	Neg/ Pos	Neg/ Neg	
Pop 1 (Emilia Romagna)	1	0	0	5	6
Pop 2 (Friuli Venezia Giulia)	0	0	0	2	2
Pop 3 (Lombardia)	31	1	0	50	82
Pop 4 (Piemonte)	0	0	0	3	3
Pop 5 (Veneto)	7	0	0	16	23
Total	39	1	0	76	116

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414

415 **Table 2.** Posterior median and 95% posterior credibility interval (PCI) of test estimates and true
416 prevalence of PCV3 diagnosed by real time PCR and direct PCR assays in 116 samples obtained from
417 five populations representing different Italian regions.

Item	Median estimate	95% PCI
Se_{qPCR}	0.96	0.90 - 1.00
Se_{dPCR}	0.94	0.84 - 1.00
Sp_{qPCR}	0.97	0.91 - 1.00
Sp_{dPCR}	0.98	0.91 - 1.00
σ_{Se}	0.02	0.005 - 0.15
σ_{Sp}	0.01	0.007 - 0.072
P_1 (Emilia Romagna)	0.22	0.02 - 0.59
P_2 (Friuli Venezia Giulia)	0.21	0.01 - 0.73
P_3 (Lombardia)	0.38	0.27 - 0.50
P_4 (Piemonte)	0.16	0.007 - 0.62
P_5 (Veneto)	0.31	0.14 - 0.52

418

419 **Supporting Information Captions**

420 **Table S1.** STARD-BLCM checklist. Based on Kostoulas et al., 2017. STARD-BLCM: Standards for
421 the Reporting of Diagnostic accuracy studies that use Bayesian Latent Class Models. Prev Vet Med,
422 138(2017)37–47.

423 **Table S2.** R script used for the present study analyses.