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Title: Evaluation of rapid methods for diagnosis of tuberculosis in slaughtered free-range pigs

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1 **Short Communication**

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3 **Evaluation of rapid methods for diagnosis of tuberculosis in slaughtered free-**
4 **range pigs**

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25 **Highlights**

- 26 • Rapid and accurate diagnostic tests are important for control of tuberculosis.
- 27 • This study evaluated methods for diagnosis of tuberculosis in domestic free-
28 range pigs.
- 29 • Quantitative real-time PCR (qPCR) on tissues is an accurate method for
30 diagnosis of tuberculosis in pigs.
- 31 • A combination of qPCR and histopathology provides optimal diagnostic value.
- 32 • Diagnosis of tuberculosis by ELISA in domestic free-range pigs was also
33 evaluated.

34 **Abstract**

35 Free-range pigs can be infected by *Mycobacterium tuberculosis* complex (MTC)
36 and may contribute to the spread of bovine tuberculosis (bTB). In the present study, the
37 diagnostic values of bacteriological culture, a duplex real-time quantitative PCR and an
38 antibody ELISA were evaluated in an abattoir study of submandibular lymph nodes and
39 serum samples from 73 pigs with and without lesions consistent with bTB. The duplex
40 qPCR was an accurate method for diagnosis of TB in pigs (specificity 100%; sensitivity
41 80%). Combining qPCR with histopathology improved sensitivity and had very good
42 concordance ($\kappa = 0.94$) with the reference method. Serological results suggest that the
43 antibody ELISA can be used for monitoring herds but not individuals.

44

45 **Keywords:** Tuberculosis; Pigs; Diagnosis; Real-time quantitative PCR; ELISA

46 In the Iberian Peninsula, wild boar (*Sus scrofa*) are a reservoir for
47 *Mycobacterium bovis*, the cause of bovine tuberculosis (bTB), along with other
48 members of the *Mycobacterium tuberculosis* complex (MTC) (Parra et al., 2003; Santos
49 et al., 2010). Post-mortem diagnostic tests for TB in livestock include gross pathology,
50 histopathology for detection of tuberculosis-like lesions (TBL) or acid-fast bacilli
51 (AFB) by Ziehl-Neelsen (ZN) staining, bacteriology and PCR (Santos et al., 2010).

52

53 Although bacteriology is considered to be the gold standard for TB
54 confirmation, this technique is time consuming and may produce false negative results
55 (Boadella et al., 2011; Corner et al., 2012). Antibody ELISAs have been used to
56 complement the diagnosis of TB in wild boar (Aurtenetxe et al., 2008; Richomme et al.,
57 2013). The aim of the present study was to evaluate quantitative real-time PCR (qPCR)
58 and an antibody ELISA as diagnostic tools for TB in slaughtered free-range pigs in
59 relation to histopathology and culture.

60

61 Submandibular lymph nodes, the most frequently affected site in cases of *M.*
62 *bovis* infection in pigs (Di Marco et al., 2012), were collected at an abattoir from 100
63 free-range pigs > 14 months of age without clinical signs raised on Southern Spanish
64 farms with a history of condemnation due to TBL. Pigs were divided into animals with
65 TBL and animals with no visible lesions on gross examination (Di Marco et al., 2012).

66

67 Blood samples were collected into plain tubes, allowed to clot and the serum
68 was harvested and stored at -70 °C until testing. Serum samples were tested by means of
69 an indirect ELISA to detect specific antibodies against bovine tuberculin purified
70 protein derivative (bPPD) of *M. bovis* (TB ELISA-VK; Vacunek S.L.)

71

72 The presence of epithelioid cells and multinucleated giant (MNG) cells, in the
73 absence of foreign bodies or fungal structures, was considered to be indicative of TB in
74 routine histological sections stained with haematoxylin and eosin (histopathology I) and
75 the presence of AFB was recorded (histopathology II) (see Appendix: Supplementary
76 Fig. 1).

77

78 Samples were decontaminated with 0.75% hexa-decyl-pyridinium chloride
79 (Sigma Aldrich) and inoculated in Lowenstein-Jensen medium with pyruvate (Oxoid)
80 (Corner et al., 2012). Colonies consistent with MTC were identified by a multiplex PCR
81 assay based on a MTC-specific 23S ribosomal DNA fragment, *gyrB* DNA sequence
82 polymorphisms and the RD1 deletion of *M. bovis* BCG (GenoType MTBC, Hain
83 Lifescience) (Richter et al., 2004).

84

85 DNA was extracted from 25 mg homogenised tissue from each sample
86 (NucleoSpin Tissue, Macherey-Nagel). A duplex qPCR for MTC and *Mycobacterium*
87 *avium* complex (MAC) was performed as described by Gómez-Laguna et al. (2010),
88 except that the DNA template was diluted 1:10 in nuclease free-water. All reactions
89 were run in duplicate.

90

91 TB positive cases (PC) were defined as animals with TBL and positive MTC
92 isolation, while TB negative cases (NC) were defined as animals with no visible lesions
93 and negative MTC isolation (Aurtenetxe et al., 2008). Sensitivity (Se), specificity (Sp)
94 and 95% confidence intervals (CI₉₅) were assessed using the software WinEpi 2.0¹.

¹ See: <http://www.winepi.net/> (accessed 17 July 2014).

95 Inter-rate agreement between the different diagnostic methods was calculated by means
96 of Cohen's κ coefficient (GraphPad Software). A combination of tests was also
97 evaluated.

98

99 Seventy-three animals matched one of two case definitions and were included in
100 the study; 46/73 were classified as PC and 27/73 were classified as NC. Only *M. bovis*
101 was detected. MTC was detected by duplex qPCR in 40/73 cases; 10/46 samples from
102 PC animals were negative in the duplex qPCR. MTC DNA was amplified from 4/27 NC
103 pigs. AFB or consistent TBL were detected histologically in these samples (see
104 Appendix: Supplementary Table 1). These cases were considered to be false negative
105 bacteriology results but true positive TB cases by means of qPCR and histopathology.
106 All samples were negative for MAC by both culture and qPCR. In the ELISA, 34/46
107 (74%) PC and 7/27 (26%) NC had *M. bovis*-specific antibodies. Se and Sp, along with
108 95% confidence intervals (95% CI) and concordance values, are summarised in Table 1.

109

110 In the light of previous studies (Gómez-Laguna et al., 2010; Santos et al., 2010;
111 Corner et al., 2012) and because qPCR showed four false negative bacteriology results,
112 a new criterion was established to describe TB positive cases (Table 2). Use of the
113 duplex qPCR had 100% Sp, 80% Se (95% CI 69-91%) and a good concordance with the
114 case definitions ($\kappa = 0.72$). In comparison, PCR on tissue homogenates from wild boar
115 had a lower Se (67%, 95% CI 41-86%) and a similar Sp (100%, 95% CI 95-100%) to
116 the present study (Santos et al., 2010).

117

118 Porcine tuberculosis is characterised by paucibacillary lesions, which result in
119 low levels of extraction of mycobacterial DNA (Santos et al., 2010). This could explain

120 the lack of successful mycobacterial DNA amplification from 10/46 PC samples in the
121 present study. Combining qPCR and histopathology improved Se (98-100%), while
122 maintaining good Sp (96-91%) and concordance ($\kappa = 0.93-0.94$) with respect to the
123 second criterion established for a TB positive case (Table 2).

124

125 Serodiagnosis has been proposed for large scale and individual TB testing of
126 wild boar (Boadella et al., 2011; Richomme et al., 2013). In the present study, Se was
127 similar, but Sp was lower than reported by Aurtenetxe et al. (2008) (Table 2). Although
128 our results do not support the use of the antibody ELISA for diagnosis of Tb in
129 individual pigs, it could be a valuable tool for the monitoring the TB status of domestic
130 pigs at the herd level.

131

132 The results of this study suggest that the duplex qPCR is an accurate method for
133 diagnosis of TB in slaughtered free-range pigs when compared with bacteriology as the
134 reference method. Future efforts should focus on improving Se, while maintaining high
135 Sp. Combining qPCR with histopathology resulted in high diagnostic accuracy.

136

137 **Conflict of interest statement**

138 None of the authors has any financial or personal relationships that could
139 inappropriately influence or bias the content of the paper.

140

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148

149 **Appendix. Supplementary material**

150 Supplementary data associated with this article can be found, in the online version, at
151 doi: ...

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198 **Table 1**

199 Estimates of sensitivity and specificity with 95% confidence intervals (95% CI) and concordance
 200 values for each diagnostic test with criteria for positive cases based on gross lesions, bacterial culture
 201 and PCR.

202

| Diagnostic tests | Positive/tested samples ^a | Sensitivity | | Specificity | | Concordance | |
|--------------------------------|--------------------------------------|-------------|-----------|-------------|-----------|-------------|-----------|
| | | % | 95% CI | % | 95% CI | κ | Agreement |
| ELISA | 41/73 | 73.9 | 61.2-86.6 | 74.1 | 57.5-90.6 | 0.46 | Moderate |
| Histopathology I ^b | 38/73 | 73.9 | 61.2-86.6 | 85.2 | 71.8-98.6 | 0.56 | Moderate |
| Histopathology II ^c | 43/73 | 80.4 | 69-91.9 | 77.8 | 62.1-93.5 | 0.57 | Moderate |
| qPCR ^d | 40/73 | 77.3 | 66.3-90.2 | 85.2 | 71.8-98.6 | 0.67 | Good |
| qPCR + Histopathology I | 49/73 | 97.8 | 93.6-100 | 85.2 | 71.8-98.6 | 0.87 | Very good |
| qPCR + Histopathology II | 51/73 | 97.9 | 93.7-100 | 77.8 | 62.1-93.5 | 0.78 | Good |

203

204 ^a Tuberculosis positive cases, pigs with compatible gross lesions at post-mortem inspection and *Mycobacterium tuberculosis*
 205 complex (MTC) identification by bacterial culture and PCR confirmation.

206 ^b Histopathology I, animals with granulomas, with presence of epithelioid cells or multinucleated giant (MNG) cells, in the
 207 absence of foreign bodies or fungal structures.

208 ^c Histopathology II, animals with acid-fast bacilli (AFB) detected by Ziehl-Neelsen (ZN) staining.

209 ^d qPCR, quantitative (real-time) PCR.

210 **Table 2**

211 Estimates of sensitivity and specificity with 95% confidence intervals (95% CI) and concordance
 212 values for each diagnostic test with criteria for positive cases based on gross lesions, bacterial culture
 213 and PCR or qPCR.

214

| Diagnostic tests | Positive/tested samples ^a | Sensitivity | | Specificity | | Concordance | |
|--------------------------------|--------------------------------------|-------------|-----------|-------------|-----------|-------------|-----------|
| | | % | 95% CI | % | 95% CI | κ | Agreement |
| ELISA | 41/73 | 72 | 59.6-84.4 | 78.3 | 61.4-95.1 | 0.46 | Moderate |
| Histopathology I ^b | 38/73 | 74 | 61.8-86.2 | 95.7 | 87.3-100 | 0.61 | Good |
| Histopathology II ^c | 43/73 | 82 | 71.4-92.6 | 91.3 | 79.8-100 | 0.68 | Good |
| qPCR ^d | 40/73 | 80 | 68.9-91.1 | 100 | - | 0.72 | Good |
| qPCR + Histopathology I | 50/73 | 98 | 94.1-100 | 95.7 | 87.3-100 | 0.94 | Very good |
| qPCR + Histopathology II | 52/73 | 100 | - | 91.3 | 79.8-100 | 0.93 | Very good |

215

216 ^a Tuberculosis positive cases, pigs with compatible gross lesions at post-mortem inspection and *Mycobacterium tuberculosis*
 217 complex (MTC) identification by bacterial culture and PCR confirmation or qPCR genome amplification from tissue.

218 ^b Histopathology I, animals with granulomas, with presence of epithelioid cells or multinucleated giant (MNG) cells, in the
 219 absence of foreign bodies or fungal structures.

220 ^c Histopathology II, animals with acid-fast bacilli (AFB) detected by Ziehl-Neelsen (ZN) staining.

221 ^d qPCR, quantitative (real-time) PCR.

222 **Appendix A**223 **Supplementary figure legend**

224

225 Supplementary Fig. 1. Submandibular lymph node. (A) Granulomatous inflammation of the
 226 submandibular lymph node of an affected pig. Bar = 1.3 cm. (B) Submandibular lymph node.

227 Typical tuberculous granuloma made up of numerous epithelioid cells, multinucleated giant
 228 cells (*), interspersed lymphocytes and a thin, poorly defined, connective tissue capsule.

229 Haematoxylin and eosin staining. Bar = 30 μm . (C) Submandibular lymph node. Extensive

230 area of necrosis with multifocal areas of mineralisation. Haematoxylin and eosin staining. Bar

231 = 300 μm . Inset: Acid-fast bacilli (arrow) identified within a focus of necrosis. Ziehl-Neelsen

232 staining. Bar = 10 μm .

233 **Appendix A**234 **Supplementary Table 1**

235 Diagnostic tests profiles for animals with uncertain results in some of the diagnostic tests.

236

| Sample identification (ID) | Gross lesions + Bacteriology | Histopathology I ^a | Histopathology II ^b | qPCR ^c | ELISA |
|----------------------------|------------------------------|-------------------------------|--------------------------------|-------------------|-------|
| ID-87 | - | + | + | + | + |
| ID-89 | - | - | + | + | - |
| ID-94 | - | + | + | + | + |
| ID-103 | - | + | + | + | - |

237

238 ^a Histopathology I, animals with granulomas, with presence of epithelioid cells or multinucleated giant (MNG) cells, in the
 239 absence of foreign bodies or fungal structures.

240 ^b Histopathology II, animals with acid-fast bacilli (AFB) detected by Ziehl-Neelsen (ZN) staining.

241 ^c qPCR, quantitative (real-time) PCR.

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