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Short Communication

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

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Highlights

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

Abstract

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

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

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

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

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

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

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

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

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

TB positive cases (PC) were defined as animals with TBL and positive MTC isolation, while TB negative cases (NC) were defined as animals with no visible lesions and negative MTC isolation (Aurtenetxe et al., 2008). Sensitivity (Se), specificity (Sp) and 95% confidence intervals (CI_{95}) were assessed using the software WinEpi 2.0[1].

[1] See: http://www.winepi.net/ (accessed 17 July 2014).

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

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

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

Porcine tuberculosis is characterised by paucibacillary lesions, which result in 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 (κ = 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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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at 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

		Sensitivity		Specificity		Concordance	
Diagnostic tests	Positive/tested samples [a]	%	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.

Table 2

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

		Sensitivity		Specificity		Concordance	
Diagnostic tests	Positive/tested samples [a]	%	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

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

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

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

[d] qPCR, quantitative (real-time) PCR.

Appendix A

Supplementary figure legend

Supplementary Fig. 1. Submandibular lymph node. (A) Granulomatous inflammation of the submandibular lymph node of an affected pig. Bar = 1.3 cm. (B) Submandibular lymph node. Typical tuberculous granuloma made up of numerous epithelioid cells, multinucleated giant cells (*), interspersed lymphocytes and a thin, poorly defined, connective tissue capsule. Haematoxylin and eosin staining. Bar = 30 μm. (C) Submandibular lymph node. Extensive area of necrosis with multifocal areas of mineralisation. Haematoxylin and eosin staining. Bar = 300 μm. Inset: Acid-fast bacilli (arrow) identified within a focus of necrosis. Ziehl-Neelsen staining. Bar = 10 μm.

Appendix A

Supplementary Table 1

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

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

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

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

[c] qPCR, quantitative (real-time) PCR.

242