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Validity of the Enzyme-linked Immunoelectrotransfer Blot (EITB) for naturally acquired porcine cysticercosis

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<PrePrint>Title: Validity of the Enzyme-linked Immunoelectrotransfer Blot (EITB) for naturally acquired porcine cysticercosis

Short title: EITB for naturally acquired porcine cysticercosis

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Abstract

The Enzyme-linked Immunoelectrotransfer Blot (EITB) has been used widely as a screening test for *Taenia solium* cysticercosis in swine. However, the relation between seropositivity and infection in pig populations from endemic areas has not been well defined. The aim of this study is to relate EITB seropositivity with infection and infection burden, analyse the trade-off between sensitivity and specificity with various cut-off points for the EITB assay, and finally describe the serology changes in a cohort of rural pigs raised under natural conditions. A group of 107 pigs that were used as controls during a vaccination field trial in Peru was our study population. The prevalence of porcine cysticercosis determined by necropsy examination was 16.82% (18/107) in these animals. Using EITB reactivity to ≥ 1 band as a cut-off point for the assay, the sensitivity was 88.89% (65.29-98.62, 95% CI) and the specificity was 48.31% (37.59-59.16, 95% CI). Comparing other cut-off points, involving up to as many as 7 reactive bands, a reactivity of ≥ 3 bands provided the best trade-offs in sensitivity and specificity. Using this cut-off point for the assay, the sensitivity was 77.77% (52.36 - 93.59, 95% CI) and the specificity was 76.40% (66.22 - 84.76, 95% CI). A significant association was found between cyst counts over 100 cysts and reactivity to ≥ 3 bands in the EITB assay (Fisher's exact test, $p<0.05$). The results of this study suggest that the use of the EITB assay to study porcine cysticercosis may require setting different cut-offs under field and experimental conditions, and depending upon the objective of the screening process.

Keywords: porcine cysticercosis; Enzyme-linked Immunoelectrotransfer Blot (EITB) assay; Receiver operating characteristic (ROC) curve; sensitivity; specificity

1. Introduction

The original publication describing the Enzyme-linked Immunoelectrotransfer Blot (EITB) for serological diagnosis of porcine cysticercosis found that any positive number of bands of reactivity (i.e. one or more bands), between a pig serum sample and glycoprotein antigens used in the assay, was highly sensitive (100%) and specific (100%) for *Taenia solium* infection (Gonzalez et al., 1990). EITB assay has been used for epidemiological surveys as one of the main techniques to quantify infection in porcine rural populations in Peru as well as elsewhere in the world (Diaz et al., 1992; Garcia et al., 1999; Garcia et al., 2003a; Gonzalez et al., 1990; Krecek et al., 2008; Lescano et al., 2007; Rodriguez-Hidalgo et al., 2006; Sakai et al., 1998). Studies on the epidemiology of porcine cysticercosis worldwide have also used other diagnostic and screening techniques, such as tongue inspection (Phiri et al., 2002; Pouedet et al., 2002; Praet et al., 2010; Secka et al., 2010; Sikasunge et al., 2008); Enzyme-linked Immunosorbent assay for antibody detection (Phiri et al., 2002; Praet et al., 2010; Sikasunge et al., 2007; Sikasunge et al., 2008) and antigen detection (Dorny et al., 2004; Nguekam et al., 2003). Necropsy examination has been used by several authors as a method to determine and quantify infection intensity in experimentally *T. solium*-infected pigs (Flisser et al., 2004; Gonzalez et al., 2005; Huerta et al., 2001; Plancarte et al., 1999). In naturally infected populations necropsy data is limited if we compare it to the data generated by serological studies. Some of the studies reporting infection rates assessed by necropsy include comparisons between necropsy and tongue and meat inspection techniques to detect porcine cysticercosis infection in naturally infected pigs (Phiri et al., 2006); studies to determine the age at which pigs get infected (de Aluja et al., 1998) and the distribution of cysts in the pig carcass (Boa et al., 2002) and studies that

71 estimated the sensitivity and specificity of EITB for porcine cysticercosis (Sciutto et al.,
72 1998; Taico et al., 2003).

73 Since the original description of the EITB technique for diagnosis of porcine
74 cysticercosis by Gonzalez *et al.* (1990), a number of researchers have endeavoured to
75 verify a correlation between EITB results and necropsy in naturally infected pigs
76 (Devleesschauwer et al., 2013; Gavidia et al., 2013; Sciutto et al., 1998; Taico et al., 2003).
77 Sciutto *et al.* (1998) found a high proportion of pigs to be EITB positive and yet had no
78 cysticerci detected when they were subjected to 'complete necropsy'. Taico *et al.* (2003)
79 developed a simulation to establish the number of positive bands to EITB to which pigs
80 were positive to estimate the real prevalence of infection. The latter was reflected in
81 subsequent studies that attempted to adjust EITB results by number of bands and by
82 binomial simulations (Aybar, 2002; Turin et al., 2005); or by using the Rogan and Gladen
83 (1978) formula (Garcia et al., 2003b). A seroprevalence study undertaken using EITB as
84 the screening test did not use any correction or adjustment for the results of EITB (Lescano
85 et al., 2007). Interestingly, in this study 50% (73/146) of EITB positive animals were found
86 to revert to EITB negative when assessed four months after initially tested. While this
87 included animals that were more than 4 months of age at the time of initial testing, the
88 change in EITB status could have been the result of and hence the positive EITB results of
89 maternally derived antibodies (Gonzalez et al., 1999), rather than representing variability in
90 EITB status of the individual animals *per se*.. Lescano et al. (2007) referred to these pigs as
91 serorevertors, while humans showing similar serological results have been identified as
92 transient positives (Garcia et al., 2001). In our study, adopting the same terminology used
93 in humans, we investigated the occurrence of transient positives in the sampled population.

Initial investigations identified a rural area in Peru where porcine cysticercosis was endemic (Morropon, seroprevalence 45.6%) using EITB as the screening test (Jayashi et al., 2012a). Following this study, a cohort of pigs, that did not receive any anthelmintic treatment to affect the natural development of infection, was used to compare the necropsy results versus EITB assay results. The animals were raised under common rearing practices from the endemic area (free-roaming and food scavenging) and were exposed to conditions in which porcine cysticercosis naturally occurs. This study compares the results of the EITB assay undertaken on sequential bleeds taken from each animal during the study and necropsy examination, and aims to provide an insight into use and interpretation of EITB results in animals raised naturally. Our study aims to improve the epidemiological interpretation and inferences made based on the EITB assay results in field populations. With this study, we aim to increase the validity of EITB results when quantifying porcine cysticercosis infection under rural/field conditions.

2. Materials and methods

2.1. Ethics statement

The study complied with the "National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (7th edition, 2004) ethics standard. The study protocol was approved by the scientific boards at the Veterinary Faculty of the University of Melbourne, Australia and at the Veterinary Faculty, San Marcos National University, Peru. Study permissions were obtained from the Municipality of Morropon, from village leaders and from the pig owners.

2.2. **Animals**

The animals were part of a vaccination field trial against porcine cysticercosis (Jayashi et al., 2012b). This trial included 274 mixed-breed (criollo) piglets, ranging from 8 to 16 weeks of age that were sourced from farms within the department of Piura, Peru. Animals were randomly assigned to the treatment (vaccinated) and control groups and distributed to households in pairs of one vaccinated and one control animal (137 pairs in total). Only the control animals were considered while analysing the EITB assay versus necropsy examination. The original cohort included 137 control pigs. However, 30 control animals were lost before the end of the study (eaten, stolen, or disappeared) and were not available for necropsy examination. The remaining 107 control pigs were used for the analysis of EITB and necropsy results. Pigs were distributed to households in Morropon, Piura, an area that was determined to be endemic (Jayashi et al., 2012a). These households were selected in a first instance, if they had animals reacting to ≥ 4 bands or were within 50 metres of houses with 4-band reactor pigs determined previously by a serological survey (Jayashi et al., 2012a). The animals were kept and raised for 4 months in rural households under common rearing practices. At the end of this period, the animals were gathered and transported to a secure compound in Tumbes, Peru, managed by the CWGP in Peru and that was considered to have no contamination with *T. solium* eggs. The pigs were maintained there for 9-12 weeks, by which time any *T. solium* infection that may have been acquired in the village in the period shortly before the animals were moved to Tumbes would have had the opportunity to grow into cysticerci that would be identifiable at post-mortem examination.

2.3. **EITB serology**

Blood samples (6-8 ml) were taken from the cranial vena cava. Samples were taken at days 0 (2 - 4 months old), 28, 45, 90, 120, and 210 (9 - 11 months old). Serum samples were obtained by centrifugation at 1000g for 5 minutes. Serum was frozen and stored until tested. All serum samples were assessed with the EITB assay to detect antibodies against *T. solium* cysticercosis infection. The results of the serologic test and the necropsy results were compared and analysed to determine specificity and sensitivity of the EITB assay. The EITB results of the study animals were analysed to determine the changes of EITB reactivity through time.

2.4. **Enzyme linked Immunoelectrotransferblot (EITB)**

The original interpretation the EITB assay for diagnosis human cysticercosis identifies as being positive any sample having reactivity with any one of seven lentil-lectin, affinity-purified *T. solium* metacestode glycoprotein antigens: GP50, GP42-39, GP24, GP21, GP18, GP14 and GP13 (Tsang et al., 1989) and for porcine cysticercosis (Gonzalez et al., 1990). In our study we defined the cut-off points, determined by number of reactive bands and described the trade-offs in sensitivity and specificity of the assay. Serum samples were processed at the diagnostic laboratory of the Unidad de Investigacion en Enfermedades Parasitarias del Sistema Nervioso de la Universidad Cayetano Heredia (Lima, Peru) to perform the EITB assay, following the methodology precisely as described by Tsang et al. (1989) and Gonzalez et al. (1990).

2.5. **Transient positive reactivity**

In this study, transient positive reactivity was defined as serology in an individual pig that changed from negative to positive and back to negative in an animal that had no parasites detected at the necropsy examination. Some animals were positive at the initial

bleed, possibly because of the presence of maternal antibodies (Gonzalez et al., 1999). An animal was considered serologically positive due to the presence of maternal antibodies where the serology results were positive at the initial bleed with subsequent bleeds showing reduced reactivity leading to negative serology resulting in an animal having no cysts at the necropsy examination.

2.6. **Necropsy examination**

Necropsy examination was used as a gold standard to determine porcine cysticercosis infections in the study animals. The whole carcass of the animal was examined to determine the presence/absence of cysts. Muscles were sliced with sagittal cuts, endeavouring not to exceed 3 mm between cuts. Cysts were classified into viable and non-viable cysts. Viable cysts were translucent vesicles filled with transparent fluid. These cysts had sizes that varied from 0.5 to 1.5 cm in diameter and were oval or round shaped. A visible white scolex could be found in the viable cysts. Non-viable cysts were small vesicles that varied from whitish to yellowish colour and had a dense fluid. Non-viable cysts were smaller than viable cysts and had the appearance similar to that of a grain of rice.

2.7. **Data analysis**

Data was entered on Microsoft Office Excel 2010 datasheets. Statistical calculations of prevalence and proportions were performed using the software STATA 10.0 (StataCorp LP, USA). EITB results where plotted in a ROC curve to compare them to the gold-standard technique, necropsy. The ROC curve was calculated and plotted using SigmaPlot 12.0 (Systat, USA). The ROC curve then was used to determine the optimal cut-off for the test. We used two methods to determine the optimal cut-off point for the test. The first

method was selecting the point that after balancing the sensitivity and specificity of the test was closest (*C*) to the (0,1) point of ROC curve. In this method the optimal diagnostic sensitivity and specificity are defined as the one who yielded the minimal value for (1 - sensitivity)^2 + (1 - specificity)^2 (Akobeng, 2007). As a second method we used the Youden index (*J*). Following this measure, the cut-off point at which J = (sensitivity + specificity - 1) is maximised is taken as the optimal cut-off point (Akobeng, 2007).

3. Results

3.1. Prevalence and seroprevalence

The total number of infected pigs (as determined by necropsy) was 18, giving a prevalence of 16.8% (18/107, 9.61 – 24.03, 95% CI). Following the current interpretation of the EITB test, where an animal is considered positive if has ≥ 1 band reactivity (Gonzalez et al., 1990), the seroprevalence was 57.9% (62/107, 48.43 – 67.45, 95% CI). Comparing the EITB to the gold standard technique (necropsy), the assay achieved a sensitivity of 88.9% (16/18) and a specificity of 48.3% (43/89). The positive and negative predictive values were 25.4% (16/62) and 97.7% (43/45), respectively.

3.2. ROC curve and optimal cut-off point

Table 1 shows a comparison of the sensitivity and specificity of the assay using various cut-offs (numbers of reactive bands) to determine seropositivity. The sensitivity ranged from 88.9% (≥ 1 band) to 38.9% (= 7 bands), and specificity ranged from 48.3% (≥ 1 band) to 98.9% (= 7 bands) when using the highest and lowest cut-off points of the EITB results.

The area under the ROC curve was 0.84 (0.72 - 0.95, 9% CI). Based on closest point to (0,1) and the Youden indexes, together with the results described in Table 1 and Figure 1

we choose the reactivity to ≥ 3 bands cut-off point because it had the best trade off in sensitivity and specificity amongst all the cut-off points (different number of reactivity bands). The cut-off for the reactivity to 3 bands yielded the closest point (C = 0.1050) to (0,1) in the ROC curve as well as the maximum J = 0.5418. The cut-off for the reactivity to 4 bands had the second best trade-off after the 3 bands with (C = 0.1574; J = 0.5324). Figure 1 shows the trade-off between sensitivity and specificity with various cut-off points for the EITB assay. Based on these results we decided to analyse the ≥ 3 bands results and compared it to the current cut-off point, reactivity to ≥ 1 bands.

Considering the reactivity to ≥ 3 as a cut-off point for the assay, the sensitivity decreased from 88.9% to 77.8%, while the specificity increased from 48.3% to 76.4%. When using the ≥ 3 bands cut-off the seroprevalence of porcine cysticercosis at day 210 (date when necropsies were performed) was 32.7% (35/107). As an anecdotic coincidence, when using the ≥ 4 bands cut-off, the seroprevalence was 16.8%, which was identical to the prevalence of infection found by the necropsy, but results did not match exactly on an individual animal basis.

3.3. Intensity of infection and EITB results

Among the 18 pigs detected as being infected at necropsy, 14 (77.8%) were positive to ≥ 3 bands in the EITB assay. Three of these animals had non-viable cysts only. Seven of the 8 animals (87.8%) that had more than 100 cysts were positive to 7 bands in the EITB assay. Six animals with less than 100 cysts reacted to 3 - 6 bands; 2 reacted to 1 - 2 bands, and 2 did not react (0 bands). In the non-infected pigs, 23.6% (21/89) were positive to ≥ 3 bands, 28.1% (25/89) were positive to 1 - 2 bands and 48.3% (43/89) were negative (0 bands).

Considering the animals that were found to have viable cysts, 80.0% (12/15) reacted to ≥ 3 bands; and 13.3% (2/15) were negative (0 bands), both animals had 1 viable cyst each. There was a significant association between cyst counts over 100 cysts and reactivity to ≥ 3 in the EITB assay (Fisher's exact test, $p<0.05$).

3.4. EITB serology in infected animals and intensity of infection through time

Two (11.1%) of the 18 positive animals in the control group were positive to 7 bands at the beginning of the study and remained positive to 7 bands until the end of the trial. This may indicate that the animals were infected before the start of the trial. Six of these animals that started with 0 bands, become positive to ≥ 3 bands during the trial and kept or increased their reactivity until the necropsy examination. These animals had between 1 - 3474 cysts. From all the infected animals 94.4% (17/18) were positive to ≥ 3 band at some point during the trial.

3.5. EITB serology reactivity through time

EITB reactivity over time for the cohort of animals in the study is shown in Figure 2. Among all the animals of the study, 27.1% (29/107) were initially determined to be EITB positive to ≥ 3 bands. The highest number of EITB negative animals (67) was found at day 28. From day 28 onwards there was sustained increase in the number of seropositive animals considering either reactivity to ≥ 1 band or ≥ 3 bands. From day 28 to day 210, the increase in animals reacting to ≥ 3 bands was 20.7% (35/29) and it was more dramatic in the animals reacting to ≥ 4 bands, 157.1% (18/7).

3.6. The phenomenon of transient positive pigs

Among the animals that were found not to be infected with *T. solium* at necropsy, 29.0% (9/31) of those that started with reactivity to ≥ 1 band became and remained seronegative during the trial. Twenty (64.5%, 20/31) of these animals subsequently re-developed EITB positive status and hence were categorised as transient positives. Among the non-infected animals that were initially seronegative, 25.9% (15/58) remained seronegative throughout all the bleeds, while the remaining 74.1% (43/58) were seropositive at one or more time points; and hence were also categorised as transient positives. In total, the proportion of transient positives was 56.6% (64/113), with 63 non-infected animals and 1 infected animal that fitted into this classification. The infected animal was categorised as being transient positive because over the duration of assessment its status changed from seronegative to positive and back again to negative. Figure 3 illustrates EITB results through time for selected animals representing various patterns of reactivity including negative throughout, positive due to maternally derived antibody, transient positive, and positive, infected animals.

4. Discussion

Applying an interpretation of the EITB assay wherein the test is 100% specific and 100% sensitive, the area under the ROC curve should equal 1. The area under the ROC curve found in this study, 0.84, indicates that the EITB test has a moderate accuracy (Fischer et al., 2003). In the investigations described here the levels of sensitivity and specificity of the EITB test for porcine cysticercosis, that were reported by Gonzalez et al (1990), could not be reproduced using any of the cut-off points that were analysed. Animals in our study were transported to a controlled environment and remained there for an

273 average of three months (enough time for cyst development); this weakens the argument
274 proposed previously, that immature or hidden cysts were present in pigs stimulating a
275 humoral response (Gonzalez et al., 1990). While the EITB for human diagnosis has been
276 assessed extensively for the potential influence of parasitic diseases other than *T. solium,*
277 and been found to be highly specific (Tsang et al. 1989), potential for non-specific
278 reactivity in the assay has been assessed for relatively few porcine parasitic infections. A
279 number of cestode infections of pigs, or exposure to cestode parasites, have yet to be
280 assessed for their effects on the EITB. For these reasons, EITB positive/necropsy negative
281 animals identified in the studies described here are considered to be false positives. Further
282 investigations are required on the effect, if any, of parasitic infections other than *T. solium*
283 before a clear interpretation can be made about the specificity of the test for *T. solium*
284 infection or *T. solium* exposure.

285 During the period that the animals were housed at the CWGP campus they were
286 held in circumstances where there was minimal risk that they may have been exposed to *T.*
287 *solium* eggs. Nevertheless, this risk was not eliminated entirely. Gonzalez (personal
288 communication) has evidence that direct ingestion of eggs carried by flies or dung beetles
289 could potentially lead to contamination of food and/or infection in animals. These potential
290 sources of extraneous infection are considered to have posed a minimal risk, unlikely to
291 have contributed to the serological results that were recorded. The CWGP campus is
292 enclosed, has no access to any source of human waste, and staff working with the animals
293 was validated as not having taeniasis.

294 While describing the variation of reactivity and its relation with intensity of
295 infection we found that the EITB reactivity was inconsistent in animals with a relatively

296 low-cyst burden (≤ 75 cysts); even comparing animals with only viable cysts versus
297 animals with only degenerated cysts. On the other hand, EITB results were more consistent
298 in animals with a relative high burden (168 - 18598 cysts). As an example, one pig had 200
299 degenerated cysts only and still reacted to 7 bands consistently through time. It appears that
300 even in the absence of viable cysts the reactivity of sera in EITB may remain strong if a
301 relatively large number of non-viable cysts are present in the animal. The evidence found in
302 our study suggests that the more cysts in an animal the more consistent the EITB results
303 will be in subsequent bleeds. It has been reported elsewhere that the probability of pigs
304 having cysts increased with the number of EITB bands (Gonzalez et al., 2006), which
305 coincides with the results presented here. We agree with Gavidia et al. (2013) in that the
306 intensity of infection must be taken into consideration when looking at the EITB assay
307 outcome, since we found that 91.7% (11/12) of animals with a burden of ≥ 10 cysts were
308 found to react consistently to ≥ 3 bands. It is difficult to make an accurate interpretation of
309 the results from relatively high proportion (35.5% 38/107) of animals that did not have
310 cysts but reacted to ≥ 3 bands in the EITB assay at the necropsy time point. Arguably this
311 reactivity could have been triggered by exposure and an aborted infection while the animals
312 were in the field; however, it is difficult to determine accurately these events and non-
313 specific factors cannot be excluded.

314 In this study we determined an optimal cut-off point for the EITB to be used in
315 naturally reared pigs. From the different cut-offs (1 – 7 bands of reactivity), we found that
316 the optimal cut-off was the reactivity to ≥ 3 bands. However reactivity to ≥ 4 bands was
317 very close to the optimal value and provided the EITB test with high specificity (94.38%).
318 Increasing the assay cut-off point from ≥ 1 bands to ≥ 3 bands increased the assay's

validity, but a proportion of false positives and false negatives were still found. Despite this, we consider that using the ≥ 3 band cut-off point could provide researchers with better estimation of the porcine cysticercosis infection in field conditions. Other factors could be taken into account when selecting the optimal cut-off point for the EITB test. Seroprevalence and risk factor studies have determined that certain age groups in a pig population have lower/higher risk to be infected (Jayashi et al., 2012a; Pondja et al., 2010; Sarti et al., 1992). Using a ≥ 3 band cut-off in high risk groups (old animals) could increase the sensitivity of the assay and most likely detect the true positive animals. On the same scope, the ≥ 4 band cut-off could be used for low risk groups (young animals).

Epidemiological studies aiming to quantify transmission and estimate the level of exposure in pigs in an endemic area may use low cut-offs to capture any exposure, even if this exposure does not lead to active infection. On the other hand, public health studies which purpose is to analyse the risk for human infection based on the level of porcine cysticercosis may be better served by using higher cut-offs that will be more correlated with higher likelihoods of finding infectious forms of *T. solium* in pigs. Serologic tests such as EITB (using the current interpretation positivity = reactivity to ≥ 1 band) are useful in places like Morropon where the prevalence is high. Using the assay as screening test, sensitivity will be 89.9%, but almost 50% of the seropositive animals will be negative at necropsy in places with similar prevalence to that of the study setting. Following diagnostic test and disease concepts (Altman and Bland, 1994; Deeks and Altman, 2004), it is noticeable that negative likelihoods ratio found for EITB (≥ 1 band) suggest that the assay would be more useful for "ruling out" the disease, while the positive likelihood ratio found for the adjusted EITB (≥ 3 bands) assay would be more useful for "ruling in" the disease.

342 Relatively few cysticercotic pigs were identified in the study group, which limits the
343 confidence with which the most effective diagnostic cut-off points could be determined.
344 The major limitation on the number of animals enrolled in the study was determined by the
345 requirement to undertake full-body slicing of muscle and other tissues in order to
346 enumerate the cysticercus burden in individual animals. The region in which the study was
347 undertaken was chosen because it was known to be endemic for porcine cysticercosis and
348 have a high prevalence of EITB-positive pigs. Having completed the work, the actual
349 prevalence of cysticercotic pigs was less than may have been anticipated based upon the
350 serological evidence, resulting in there being fewer animals in the infected group than
351 would have been preferred for the purpose of estimating accurately the specificity and
352 sensitivity of the EITB as a serological test. Nevertheless, the studies incorporating full
353 necropsy evaluation of porcine cysticercosis are few and the present work provides
354 valuable additional data to assist with interpretation of EITB serological data for diagnosis
355 of porcine cysticercosis. Whether using an optimal cut-off for EITB assay is the best
356 approach to quantify the prevalence of porcine cysticercosis remains to be determined;
357 however, a clear finding is that the optimal cut-off point results showed a closer
358 concordance with the necropsy examinations. The necropsy results of our study are
359 significant because all animals were reared, exposed and infected under natural conditions.
360 These animals did not receive any antihelmintic treatment that could have affected the
361 outcome of the necropsy and the complete carcass was examined to determine infection in
362 comparison with other studies that only examined certain parts of the carcass (Assana et al.,
363 2010; Phiri et al., 2002; Sciutto et al., 1998; Sikasunge et al., 2008).

5. Conclusions

Despite being a useful and relatively fast serology assay, EITB showed moderate sensitivity and specificity under field conditions, and caution should be made when making inferences from the assay's results. The use of EITB in combination with other tests could be evaluated to find ways to improve overall validity. We found that different cut offs may be useful to improve the sensitivity and specificity of the assay. The latter may increase the accuracy of results and provide with a better estimation of porcine cysticercosis infection in rural populations or under field conditions.

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7. Cited references

Akobeng, A.K., 2007. Understanding diagnostic tests 3: Receiver operating characteristic curves. Acta paediatr. 96, 644-647.

Altman, D.G., Bland, J.M., 1994. Diagnostic tests 2: Predictive values. BMJ. 309, 102.

Assana, E., Kyngdon, C.T., Gauci, C.G., Geerts, S., Dorny, P., De Deken, R., Anderson, G.A., Zoli, A.P., Lightowlers, M.W., 2010. Elimination of *Taenia solium* transmission to pigs in a field trial of the TSOL18 vaccine in Cameroon. Int J Parasitol 40. 515-519.

Aybar, V., 2002. [Seroprevalence of porcine cysticercosis in the villages of Nueva Esperanza, Matapuquio y Turpo province of Andahuaylas departament of Apurimac]. San Marcos National University, Lima, Peru.

Boa, M.E., Kassuku, A.A., Willingham, A.L., 3rd, Keyyu, J.D., Phiri, I.K., Nansen, P., 2002. Distribution and density of cysticerci of *Taenia solium* by muscle groups and organs in naturally infected local finished pigs in Tanzania. Vet Parasitol. 106, 155-164.

de Aluja, A.S., Martinez, M.J., Villalobos, A.N., 1998. *Taenia solium* cysticercosis in young pigs: age at first infection and histological characteristics. Vet Parasitol. 76, 71-79.

Deeks, J.J., Altman, D.G., 2004. Diagnostic tests 4: likelihood ratios. BMJ. 329, 168-169.

Devleesschauwer, B., Aryal, A., Tharmalingam, J., Joshi, D.D., Rijal, S., Speybroeck, N., Gabriel, S., Victor, B., Dorny, P., 2013. Complexities in using sentinel pigs to study *Taenia solium* transmission dynamics under field conditions. Vet Parasitol. 193, 172-178.

Diaz, F., Garcia, H.H., Gilman, R.H., Gonzales, A.E., Castro, M., Tsang, V.C., Pilcher, J.B., Vasquez, L.E., Lescano, M., Carcamo, C., et al., 1992. Epidemiology of taeniasis and cysticercosis in a Peruvian village. The Cysticercosis Working Group in Peru. Am J Epidemiol. 135, 875-882.

Dorny, P., Phiri, I.K., Vercruysse, J., Gabriel, S., Willingham, A.L., 3rd, Brandt, J., Victor, B., Speybroeck, N., Berkvens, D., 2004. A Bayesian approach for estimating values for

409 prevalence and diagnostic test characteristics of porcine cysticercosis. Int J Parasitol. 34,
410 569-576.
411 Fischer, J.E., Bachmann, L.M., Jaeschke, R., 2003. A readers' guide to the interpretation of
412 diagnostic test properties: clinical example of sepsis. Intens Care Med. 29, 1043-1051.
413 Flisser, A., Gauci, C.G., Zoli, A., Martinez-Ocana, J., Garza-Rodriguez, A., Dominguez-Alpizar,
414 J.L., Maravilla, P., Rodriguez-Canul, R., Avila, G., Aguilar-Vega, L., Kyngdon, C., Geerts,
415 S., Lightowlers, M.W., 2004. Induction of protection against porcine cysticercosis by
416 vaccination with recombinant oncosphere antigens. Infect Immun. 72, 5292-5297.
417 Garcia, H.H., Gilman, R.H., Gonzalez, A.E., Pacheco, R., Verastegui, M., Tsang, V.C., 1999.
418 Human and porcine *Taenia solium* infection in a village in the highlands of Cusco, Peru.
419 The Cysticercosis Working Group in Peru. Acta Trop. 73, 31-36.
420 Garcia, H.H., Gilman, R.H., Gonzalez, A.E., Verastegui, M., Rodriguez, S., Gavidia, C., Tsang,
421 V.C., Falcon, N., Lescano, A.G., Moulton, L.H., Bernal, T., Tovar, M., Cysticercosis
422 Working Group in, P., 2003a. Hyperendemic human and porcine *Taenia solium* infection in
423 Peru. The Am J Trop Med Hyg. 68, 268-275.
424 Garcia, H.H., Gonzalez, A.E., Gavidia, C., Falcon, N., Bernal, T., Verastegui, M., Rodriguez, S.,
425 Tsang, V.C., Gilman, R.H., Cysticercosis Working Group in, P., 2003b. Seroincidence of
426 porcine *T. solium* infection in the Peruvian highlands. Prev Vet Med. 57, 227-236.
427 Garcia, H.H., Gonzalez, A.E., Gilman, R.H., Palacios, L.G., Jimenez, I., Rodriguez, S., Verastegui,
428 M., Wilkins, P., Tsang, V.C., 2001. Short report: transient antibody response in *Taenia*
429 *solium* infection in field conditions-a major contributor to high seroprevalence. Am J Trop
430 Med Hyg. 65, 31-32.
431 Gavidia, C.M., Verastegui, M.R., Garcia, H.H., Lopez-Urbina, T., Tsang, V.C., Pan, W., Gilman,
432 R.H., Gonzalez, A.E., Cysticercosis Working Group in, P., 2013. Relationship between
433 Serum Antibodies and *Taenia solium* Larvae Burden in Pigs Raised in Field Conditions.
434 PLoS Neg Trop Dis. 7, e2192.

435 Gonzalez, A.E., Cama, V., Gilman, R.H., Tsang, V.C., Pilcher, J.B., Chavera, A., Castro, M.,
436 Montenegro, T., Verastegui, M., Miranda, E., et al., 1990. Prevalence and comparison of
437 serologic assays, necropsy, and tongue examination for the diagnosis of porcine
438 cysticercosis in Peru. Am J Trop Med Hyg. 43, 194-199.
439 Gonzalez, A.E., Gauci, C.G., Barber, D., Gilman, R.H., Tsang, V.C., Garcia, H.H., Verastegui, M.,
440 Lightowlers, M.W., 2005. Vaccination of pigs to control human neurocysticercosis. Am J
441 Trop Med Hyg. 72, 837-839.
442 Gonzalez, A.E., Lopez-Urbina, T., Tsang, B., Gavidia, C., Garcia, H.H., Silva, M.E., Ramos, D.D.,
443 Manzanedo, R., Sanchez-Hidalgo, L., Gilman, R.H., Tsang, V.C., cysticercosis working
444 group in, P., 2006. Transmission dynamics of *Taenia solium* and potential for pig-to-pig
445 transmission. Parasit Int. 55 Suppl, S131-135.
446 Gonzalez, A.E., Verastegui, M., Noh, J.C., Gavidia, C., Falcon, N., Bernal, T., Garcia, H.H., Tsang,
447 V.C., Gilman, R.H., Wilkins, P.P., 1999. Persistence of passively transferred antibodies in
448 porcine *Taenia solium* cysticercosis. Cysticercosis Working Group in Peru. Vet Parasitol.
449 86, 113-118.
450 Huerta, M., de Aluja, A.S., Fragoso, G., Toledo, A., Villalobos, N., Hernandez, M., Gevorkian, G.,
451 Acero, G., Diaz, A., Alvarez, I., Avila, R., Beltran, C., Garcia, G., Martinez, J.J., Larralde,
452 C., Sciutto, E., 2001. Synthetic peptide vaccine against *Taenia solium* pig cysticercosis:
453 successful vaccination in a controlled field trial in rural Mexico. Vaccine 20, 262-266.
454 Jayashi, C.M., Arroyo, G., Lightowlers, M.W., Garcia, H.H., Rodriguez, S., Gonzalez, A.E., 2012a.
455 Seroprevalence and risk factors for *Taenia solium* cysticercosis in rural pigs of northern
456 Peru. PLoS Neg Trop Dis. 6, e1733.
457 Jayashi, C.M., Kyngdon, C.T., Gauci, C.G., Gonzalez, A.E., Lightowlers, M.W., 2012b. Successful
458 immunization of naturally reared pigs against porcine cysticercosis with a recombinant
459 oncosphere antigen vaccine. Vet Parasitol. 188, 261-267.

460 Krecek, R.C., Michael, L.M., Schantz, P.M., Ntanjana, L., Smith, M.F., Dorny, P., Harrison, L.J.,
461 Grimm, F., Praet, N., Willingham, A.L., 3rd, 2008. Prevalence of *Taenia solium*
462 cysticercosis in swine from a community-based study in 21 villages of the Eastern Cape
463 Province, South Africa. Vet Parasitol. 154, 38-47.
464 Lescano, A.G., Garcia, H.H., Gilman, R.H., Guezala, M.C., Tsang, V.C., Gavidia, C.M., Rodriguez,
465 S., Moulton, L.H., Green, J.A., Gonzalez, A.E., Cysticercosis Working Group in, P., 2007.
466 Swine cysticercosis hotspots surrounding *Taenia solium* tapeworm carriers. Am J Trop Med
467 Hyg. 76, 376-383.
468 Nguekam, A., Zoli, A.P., Vondou, L., Pouedet, S.M., Assana, E., Dorny, P., Brandt, J., Losson, B.,
469 Geerts, S., 2003. Kinetics of circulating antigens in pigs experimentally infected with
470 *Taenia solium* eggs. Vet Parasitol. 111, 323-332.
471 Phiri, I.K., Dorny, P., Gabriel, S., Willingham, A.L., 3rd, Sikasunge, C., Siziya, S., Vercruysse, J.,
472 2006. Assessment of routine inspection methods for porcine cysticercosis in Zambian
473 village pigs. J Helminthol. 80, 69-72.
474 Phiri, I.K., Dorny, P., Gabriel, S., Willingham, A.L., 3rd, Speybroeck, N., Vercruysse, J., 2002. The
475 prevalence of porcine cysticercosis in Eastern and Southern provinces of Zambia. Vet
476 Parasitol. 108, 31-39.
477 Plancarte, A., Flisser, A., Gauci, C.G., Lightowlers, M.W., 1999. Vaccination against *Taenia solium*
478 cysticercosis in pigs using native and recombinant oncosphere antigens. Int J Parasitol. 29,
479 643-647.
480 Pondja, A., Neves, L., Mlangwa, J., Afonso, S., Fafetine, J., Willingham, A.L., 3rd, Thamsborg,
481 S.M., Johansen, M.V., 2010. Prevalence and risk factors of porcine cysticercosis in
482 Angonia District, Mozambique. PLoS Neg Trop Dis. 4, e594.
483 Pouedet, M.S., Zoli, A.P., Nguekam, Vondou, L., Assana, E., Speybroeck, N., Berkvens, D., Dorny,
484 P., Brandt, J., Geerts, S., 2002. Epidemiological survey of swine cysticercosis in two rural
485 communities of West-Cameroon. Vet. Parasitology. 106, 45-54.

Praet, N., Kanobana, K., Kabwe, C., Maketa, V., Lukanu, P., Lutumba, P., Polman, K., Matondo, P., Speybroeck, N., Dorny, P., Sumbu, J., 2010. *Taenia solium* cysticercosis in the Democratic Republic of Congo: how does pork trade affect the transmission of the parasite? PLoS Neg Trop Dis. 4.

Rodriguez-Hidalgo, R., Benitez-Ortiz, W., Praet, N., Saa, L.R., Vercruysse, J., Brandt, J., Dorny, P., 2006. Taeniasis-cysticercosis in Southern Ecuador: assessment of infection status using multiple laboratory diagnostic tools. Memorias do Instituto Oswaldo Cruz. 101, 779-782.

Rogan, W.J., Gladen, B., 1978. Estimating prevalence from the results of a screening test. Am J Epidemiol. 107, 71-76.

Sakai, H., Sone, M., Castro, D.M., Nonaka, N., Quan, D., Canales, M., Ljungstrom, I., Sanchez, A.L., 1998. Seroprevalence of *Taenia solium* cysticercosis in pigs in a rural community of Honduras. Vet. Parasitol. 78, 233-238.

Sarti, E., Schantz, P.M., Plancarte, A., Wilson, M., Gutierrez, I.O., Lopez, A.S., Roberts, J., Flisser, A., 1992. Prevalence and risk factors for *Taenia solium* taeniasis and cysticercosis in humans and pigs in a village in Morelos, Mexico. Am J Trop Med Hyg. 46, 677-685.

Sciutto, E., Martinez, J.J., Villalobos, N.M., Hernandez, M., Jose, M.V., Beltran, C., Rodarte, F., Flores, I., Bobadilla, J.R., Fragoso, G., Parkhouse, M.E., Harrison, L.J., de Aluja, A.S., 1998. Limitations of current diagnostic procedures for the diagnosis of *Taenia solium* cysticercosis in rural pigs. Vet Parasitol. 79, 299-313.

Secka, A., Marcotty, T., De Deken, R., Van Marck, E., Geerts, S., 2010. Porcine cysticercosis and risk factors in the gambia and senegal. J Parasitol Res. 2010, 823892.

Sikasunge, C.S., Phiri, I.K., Phiri, A.M., Dorny, P., Siziya, S., Willingham, A.L., 3rd, 2007. Risk factors associated with porcine cysticercosis in selected districts of Eastern and Southern provinces of Zambia. Vet Parasitol. 143, 59-66.

510 Sikasunge, C.S., Phiri, I.K., Phiri, A.M., Siziya, S., Dorny, P., Willingham, A.L., 3rd, 2008.
511 Prevalence of *Taenia solium* porcine cysticercosis in the Eastern, Southern and Western
512 provinces of Zambia. Vet J. 176, 240-244.
513 Taico, F., López, T., González, A., García, H., Gilman, R., 2003. [Epidemiology of porcine
514 cysticercosis in three villages of Zarumilla province – Tumbes]. Rev Invest Vet Per. 14,
515 166-173.
516 Tsang, V.C., Brand, J.A., Boyer, A.E., 1989. An enzyme-linked immunoelectrotransfer blot assay
517 and glycoprotein antigens for diagnosing human cysticercosis (*Taenia solium*). J Infect Dis.
518 159, 50-59.
519 Turin, R., López, T., González, A., 2005. [Prevalence of porcine cysticercosis in the porcine park of
520 of Ventanilla "Pampa de los Perros", Callao]. Rev Invest Vet Per. 16, 82-89.

521

Table 1. Performance measures of the EITB test considering the number of reactive bands to the EITB test at the necropsy time point (Morropon, Peru).

Test measures	EITB reactive bands						
	≥ 1B	≥ 2B	≥ 3B	≥ 4B	≥ 5B	≥ 6B	≥ 7B
Sensitivity (95% CI)	88.89 (65.29-98.62)	83.33 (58.58-96.42)	77.77 (52.36-93.59)	61.11 (35.75-82.70)	50.00 (26.02-73.98)	44.44 (21.53-69.24)	38.89 (17.30-64.25)
Specificity (95% CI)	48.31 (37.59-59.16)	66.29 (55.49-75.97)	76.40 (66.22-84.76)	92.13 (84.46-96.78)	94.38 (87.37-98.15)	97.75 (92.12-99.73)	98.88 (93.90-99.97)
PPV	25.40	33.33	40.00	61.11	64.29	80.00	87.50
NPV	97.73	95.16	94.44	92.13	90.32	89.69	88.89
FPR	11.11	16.67	22.22	38.90	50.00	55.55	61.1
FNR	51.69	33.71	23.59	7.80	5.61	2.24	1.12
LRP	1.72	2.47	3.30	7.76	8.90	19.75	34.72
LRN	0.23	0.25	0.29	0.42	0.53	0.57	0.62

PPV: positive predictive value. NPV: negative predictive value. FPR: false positive rate. FNR: false negative rate. LRP: Likelihood ratio positive. LRN: likelihood ratio negative.

527

528 **Figure 1.** Receiver Operating Characteristic curve (ROC) of the EITB assay for naturally
529 infected pigs.

530

531

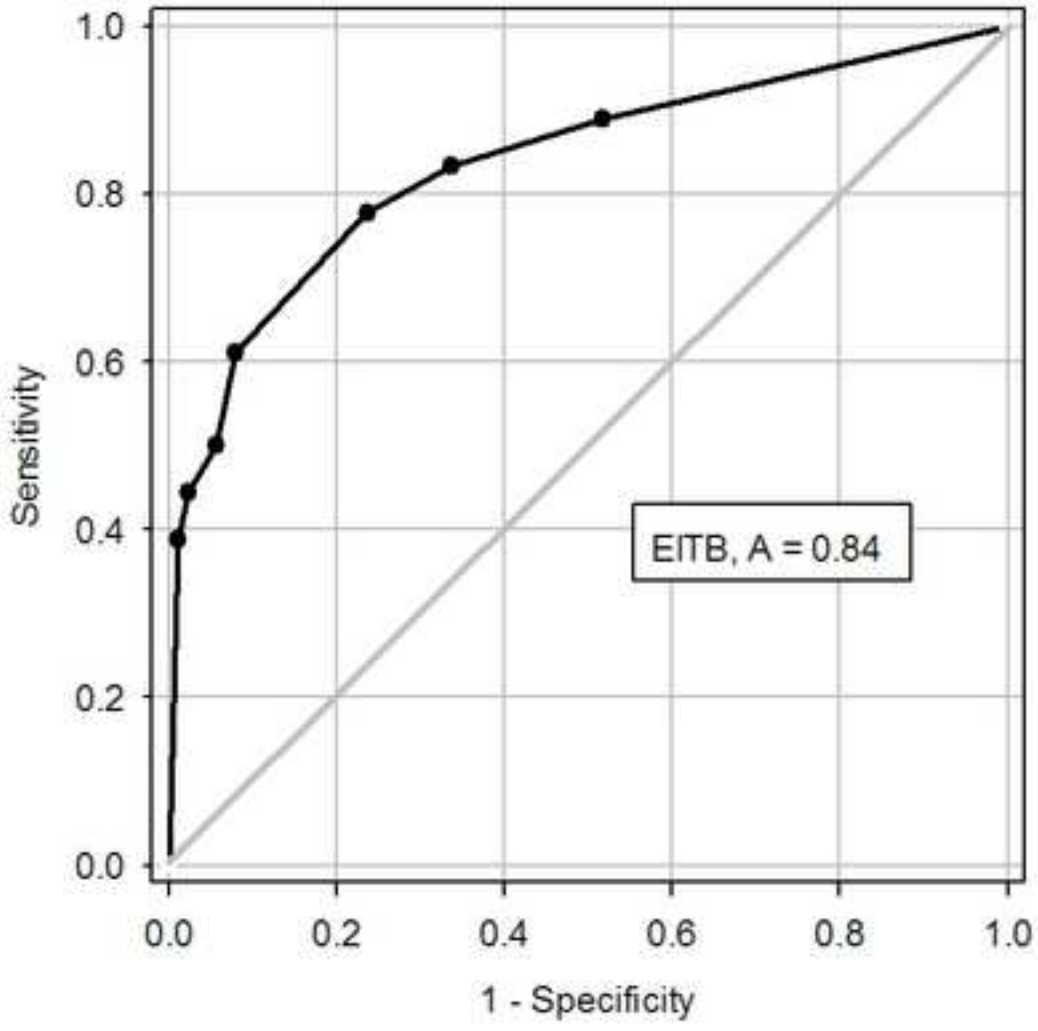
532 Each point on the ROC curve marks the sensitivity and (1-specificity) corresponding to
533 each number of EITB reactive bands (1 - 7) as cut-off points.

534

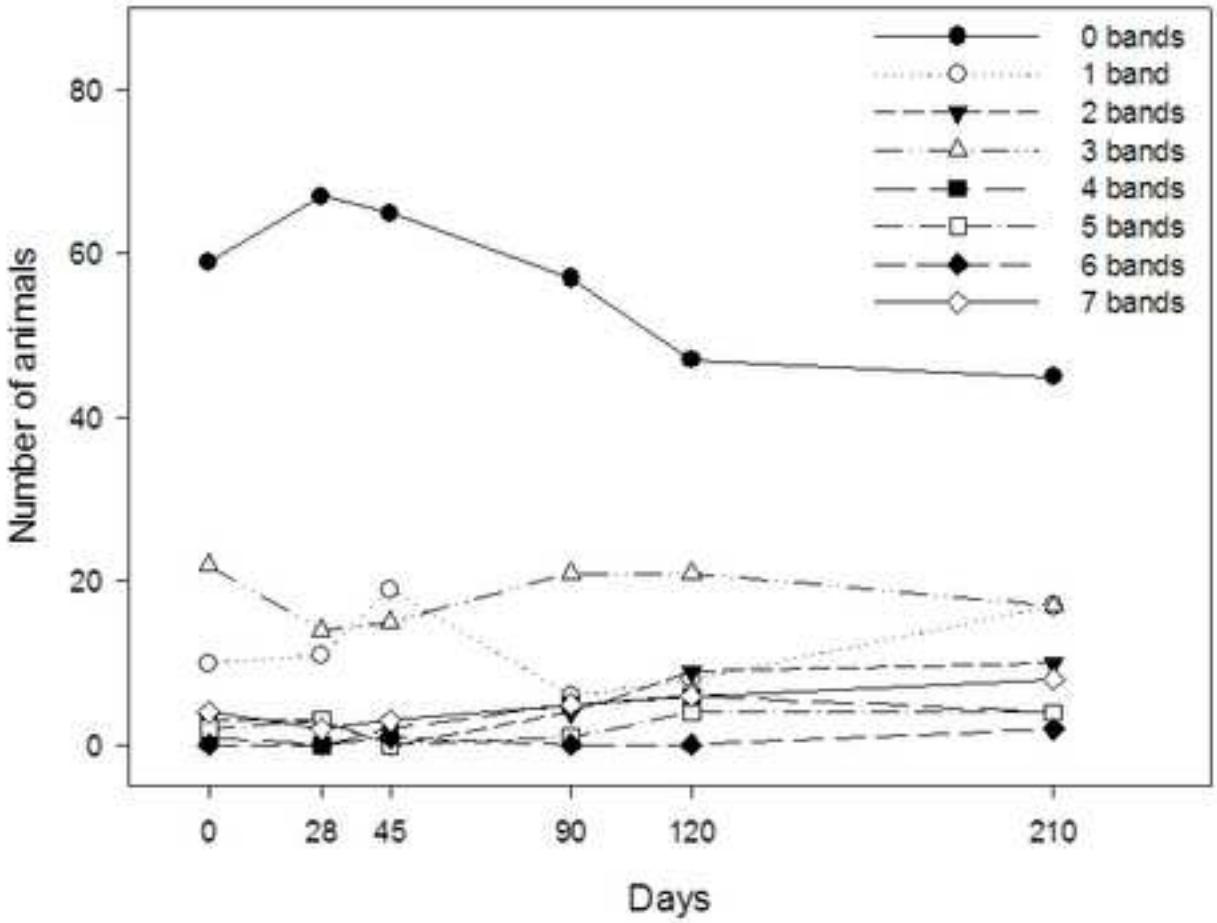
534
535 **Figure 2.** EITB reactivity of all pigs (n=107) through time, arranged by the number of
536 reacting bands (0-7) (Morropon, Peru)
537
538
539

Figure 3. Typical patterns of EITB reactivity in negative, maternal antibody reactivity, transient positive, and infected animals

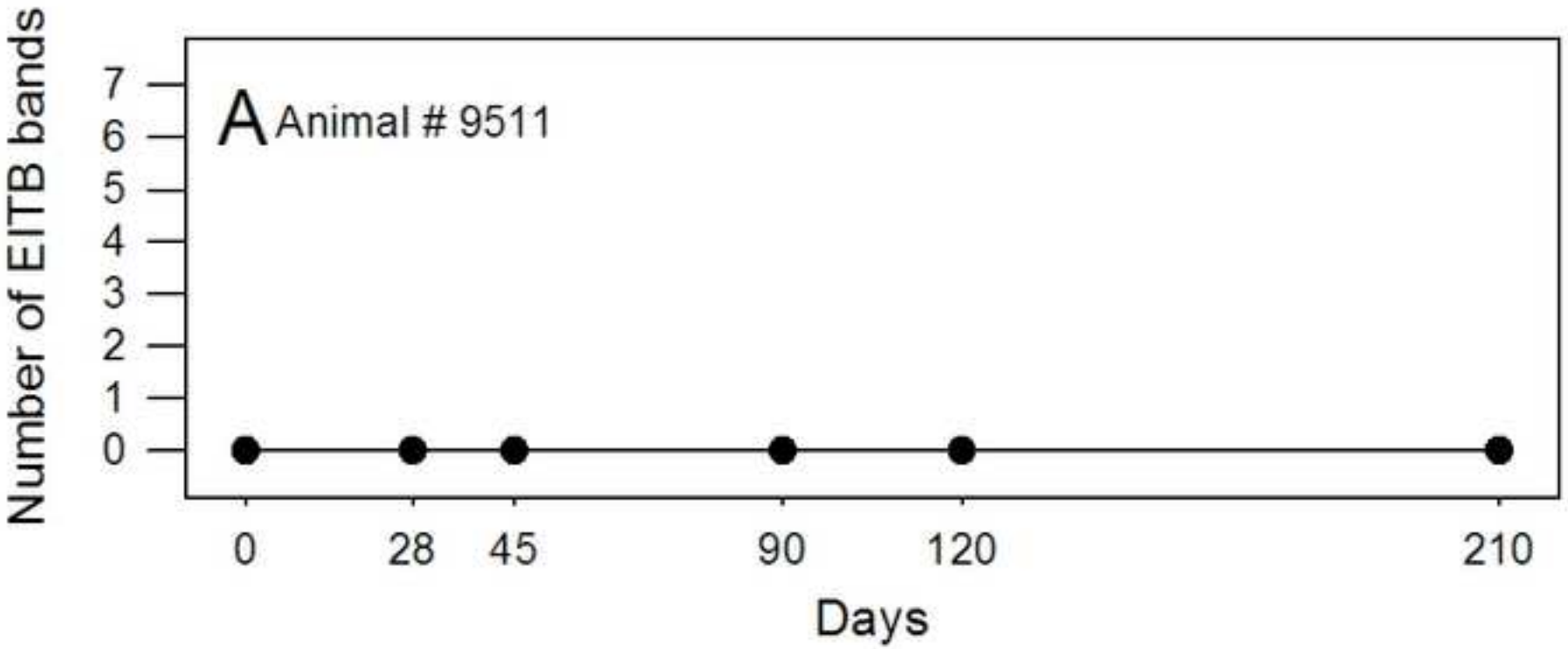
A, Negative throughout animal, no cysts at necropsy. B, Maternally derived antibodies, no cysts at necropsy. C, Transient positive, no cysts at necropsy. D, Transient positive, no cysts at necropsy. E, Positive, infected animal, 168 cysts at necropsy. Line plots show the EITB results of an individual pig through time.



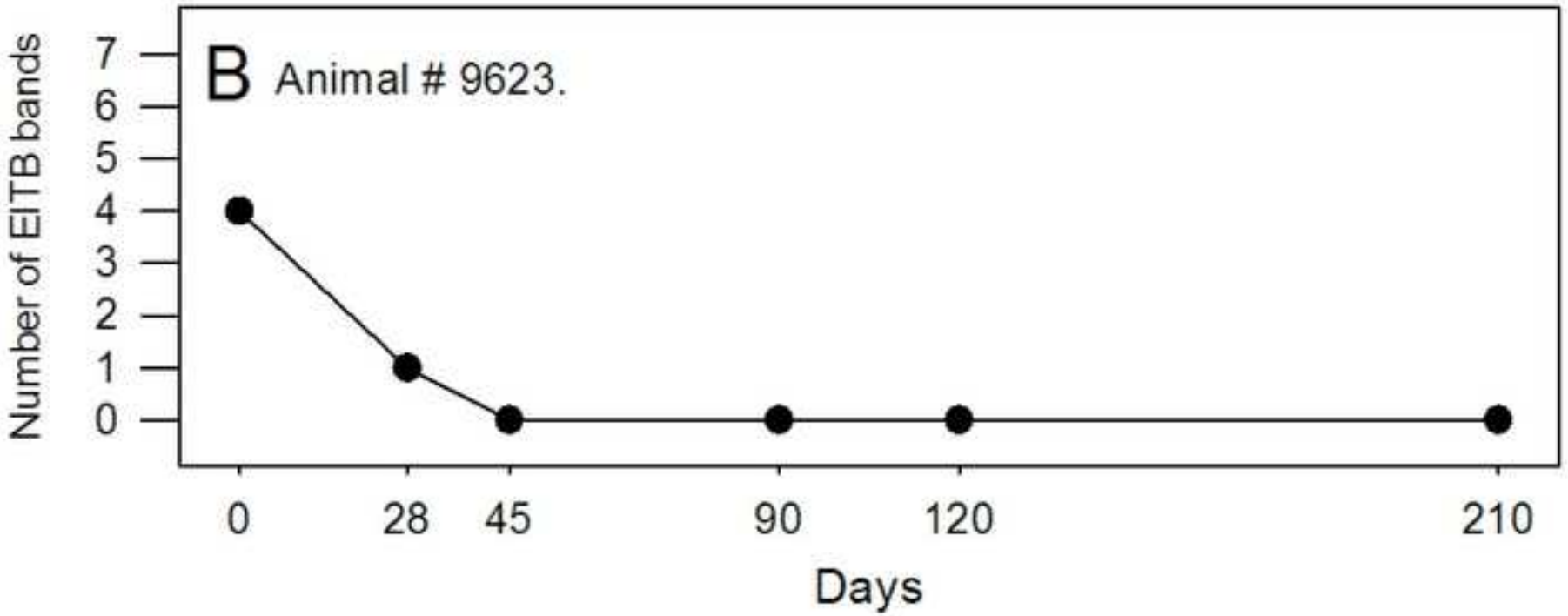
Figure


0 bands
1 band
2 bands
3 bands
4 bands
5 bands
6 bands
7 bands
Number of animals
80
60
40
20
0
0
28
45
90
120
210
Days

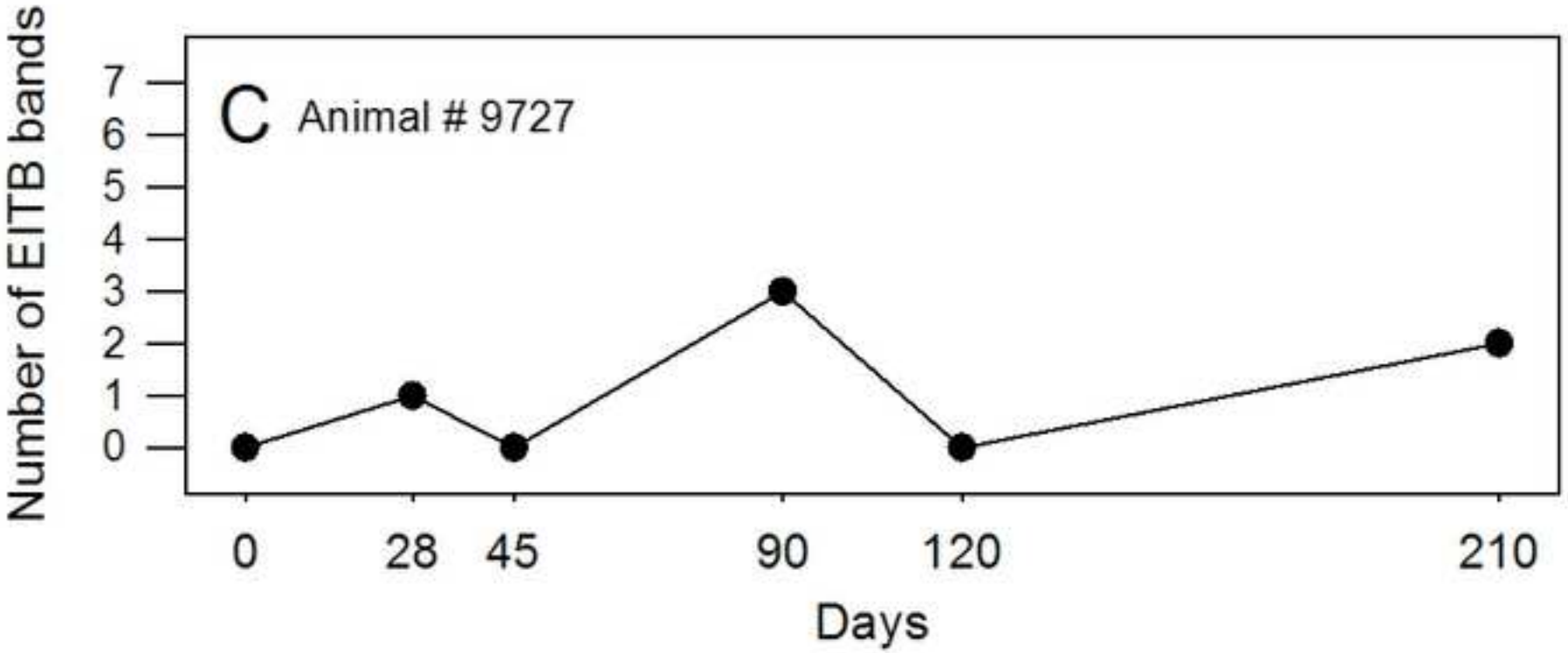
Figure


A Animal # 9511
Number of EITB bands
0
1
2
3
4
5
6
7
0
28
45
90
120
210
Days

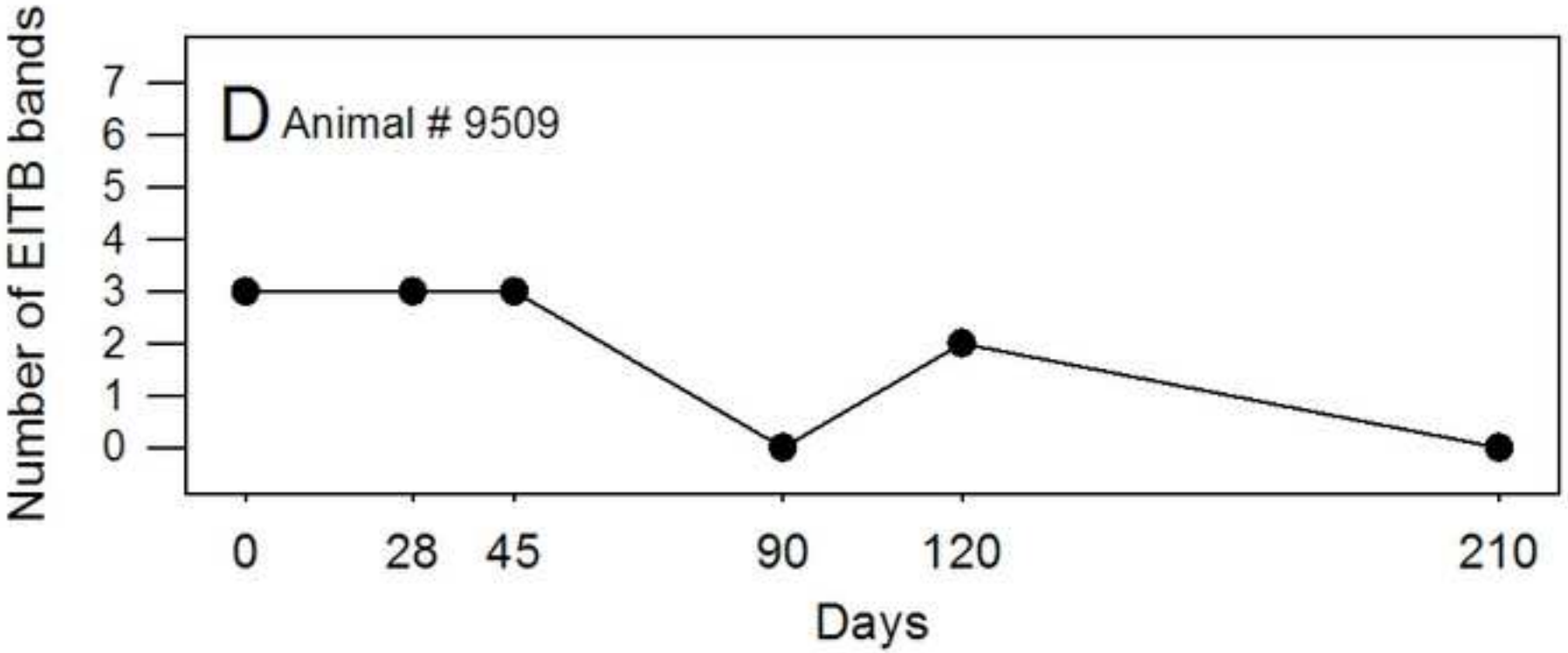
Figure


B Animal # 9623.
Number of EITB bands
0
1
2
3
4
5
6
7
0
28
45
90
120
210
Days

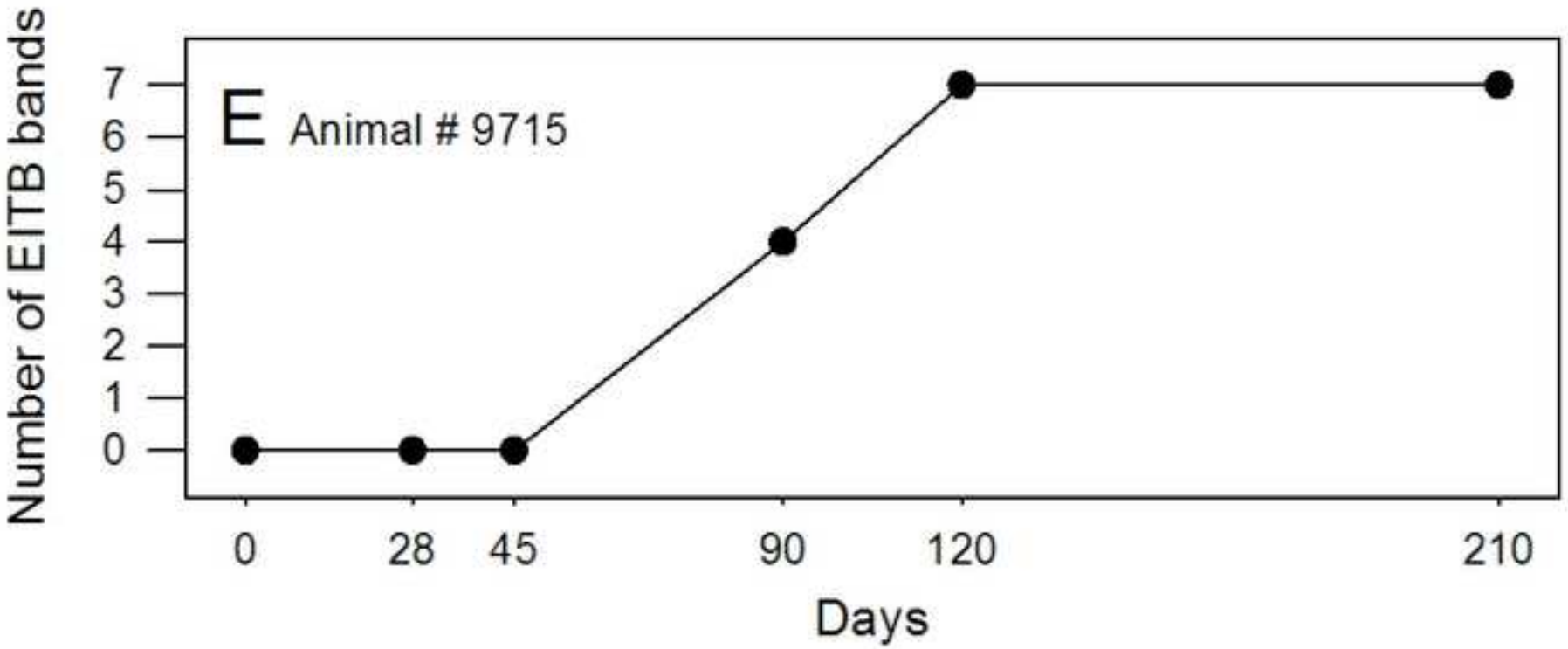
Figure


C
Animal # 9727
Number of EITB bands
0
1
2
3
4
5
6
7
0
28
45
90
120
210
Days

Figure


D Animal # 9509
Number of EITB bands
0
1
2
3
4
5
6
7
0
28
45
90
120
210
Days

Figure


E Animal # 9715
Number of EITB bands
0
1
2
3
4
5
6
7
0
28
45
90
120
210
Days