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Evaluation of ELISA coupled with Western blot as a surveillance tool for *Trichinella* infection in wild boar (*Sus scrofa*)

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Abstract

*Trichinella* surveillance in wildlife relies on muscle digestion of large samples which are logistically difficult to store and transport in remote and tropical regions as well as labour-intensive to process. Serological methods such as enzyme-linked immunosorbent assays (ELISAs) offer rapid, cost-effective alternatives for surveillance but should be paired with additional tests because of the high false-positive rates encountered in wildlife. We investigated the utility of ELISAs coupled with Western blot (WB) in providing evidence of *Trichinella* exposure or infection in wild boar. Serum samples were collected from 673 wild boar from a high- and low-risk region for *Trichinella* introduction within mainland Australia, which is considered *Trichinella*-free. Sera were examined using both an ‘in-house’ and a commercially available indirect-ELISA that used excretory-secretory (E/S) antigens. Cut-off values for positive results were determined using sera from the low-risk population. All wild boar from the high-risk region (352) and 139/321 (43.3%) of the wild boar from the low-risk region were tested by artificial digestion. Testing by Western blot using E/S antigens, and a *Trichinella*-specific real-time PCR was also carried out on all ELISA-positive samples. The two ELISAs correctly classified all positive controls as well as one naturally-infected wild boar from Gabba Island in the Torres Strait. In both the high- and low-risk populations, the ELISA results showed substantial agreement ($\kappa$-value= 0.66) that increased to very good ($\kappa$-value= 0.82) when WB-positive only samples were compared. The results of testing sera collected from the Australian mainland showed the *Trichinella* seroprevalence was 3.5% (95% C.I. 0.0-8.0) and 2.3% (95% C.I. 0.0-5.6) using the in-house and commercial ELISA coupled with WB respectively. These estimates were significantly higher (P < 0.05) than the artificial digestion estimate of 0.0% (95% C.I. 0.0-1.1). Real-time PCR testing of muscle from seropositive animals did not detect *Trichinella* DNA in any mainland animals, but did reveal the presence of a second larvae-positive wild boar on Gabba Island, supporting its utility as an alternative, highly sensitive method in muscle examination. The serology results suggest Australian wildlife may have been exposed to *Trichinella* parasites. However, because of the possibility of non-specific reactions with
other parasitic infections, more work using well-defined cohorts of positive and negative samples is
required. Even if the specificity of the ELISAs is proven to be low, their ability to correctly classify
the small number of true positive sera in this study indicates utility in screening wild boar
populations for reactive sera which can be followed up with additional testing.

Keywords: Trichinella, ELISA, Western Blot, serosurveillance, wildlife, real-time PCR, zoonosis
1. Introduction

Parasitic nematodes of the genus *Trichinella* are the causative agents of trichinellosis – a global, food-borne zoonosis that is also a recognised public health problem in South-East Asia (SE Asia) and some Indo-Pacific regions (Conlan et al., 2011; Khumjui et al., 2008; Kusolsuk et al., 2010; Owen et al., 2005). *Trichinella* parasites have never been reported on mainland Australia although there are nearby foci of *Trichinella papuae* in Papua New Guinea (PNG) and an uninhabited island in the Torres Strait, as well as the Australian genotype of *Trichinella pseudospiralis* in the south-eastern Australian island of Tasmania (Cuttell et al., 2012a; Obendorf et al., 1990; Pozio et al., 1999). The close geographical proximity of these reservoirs to mainland Australia suggests there is potential for introduction via natural or illegal movements of infected animals or meat products. To date, surveillance data for the presence of *Trichinella* infection in Australian wildlife has relied on extensive testing of muscle samples by artificial digestion (AD) from game animals for export and to a lesser extent from samples collected during limited surveys in wildlife (AHA, 2009; Bearup, 1949; DAFF, 2007; Oakwood and Spratt, 2000; Waddell, 1969). Surveillance in Australia could be enhanced through improved diagnostic capacity for *Trichinella* infections in animals as well as targeting surveillance to areas at high risk of introduction.

Testing for *Trichinella* infection in animals in Australia is currently performed by muscle examination by AD of large (minimum of 10 g but greater is recommended) samples to ensure adequate sensitivity. However, the process is labour-intensive and it can be logistically difficult to transport and store muscle samples, particularly in remote, tropical areas. Serological methods such as enzyme-linked immunosorbent assays (ELISAs) may offer a rapid, cost-effective alternative and are the most commonly used method in serodiagnosis of *Trichinella* infection in animals (Gamble et al., 2004). In domestic pigs, ELISAs provide acceptable levels of sensitivity and specificity as well as cost-efficiency, however they should be applied with caution in wildlife because of...
insufficient information regarding their diagnostic performance (Gamble et al., 2004; OIE, 2012).

The high levels of sensitivity attained with ELISAs (pigs with worm burdens as low as 0.01
larvae/g are detectable) (Gamble et al., 1983) indicates their usefulness in detecting low-level
infections such as those encountered in wildlife. Test specificity may be reduced however because
of the possibility of parasitic, fungal, bacterial and viral infections in wildlife that increase the risk
of non-specific reactions (Gamble et al., 2004).

When ELISAs have been used to screen wild animals, seroprevalence is often reported at higher
levels than estimates obtained by AD, which is attributed to either the increased sensitivity or
reduced specificity of the serological method (Chavez-Larrea et al., 2005; Davidson et al., 2009;
Richomme et al., 2010; Wacker et al., 1999). The pairing of ELISAs with a test such as Western
blot (WB) to improve Trichinella diagnosis has been proposed and applied in humans (Gomez-
Morales et al., 2008) as well as domestic and wild animals (Davidson et al., 2009; Frey et al., 2009;
Noeckler et al., 2009; Pozio et al., 2002). Western blot offers higher specificity by allowing
visualisation of specific Trichinella proteins reacting with host antibodies, but is too costly to
perform with large sample sizes or for routine screening. WB using E/S antigens offers greater
specificity and ease of interpretation compared to blots using crude worm extracts. Distinctive
immunogenic profiles for Trichinella infection in pig sera have described a three-band pattern
between 40-53 kDa (Wee et al., 2001) or 48-72 kDa (Gómez-Morales et al., 2012).

As indirect methods, serological tests cannot be used to gauge actual zoonotic risk but have been
proposed as suitable for epidemiological studies when the objective is to estimate the level of
exposure to Trichinella parasites (Noeckler and Kapel, 2007). Therefore, the aim of this study was
to establish the value of serological methods in Trichinella surveillance of wild boar (Sus scrofa) in
Australia. This was achieved by comparing the performances of a commercial and in-house E/S
antigen indirect-ELISA and further investigation of ELISA-positive sera by WB. Sera samples were
paired with muscle and also tested by AD. An alternative direct method of detection using
Trichinella-specific real-time PCR for amplification of DNA extracted from 10 g of muscle was
also applied in ELISA-positive sera.

2 Materials and methods

2.1 Sera and muscle sample collection
Details of the sera used in the study are provided in Table 1. Sera from 321 wild boar from south-western Western Australia (WA) were classified as ‘low-risk’ for Trichinella infection because it is
an isolated region with limited opportunity for the introduction of Trichinella and no history of the
existence of pigs prior to European colonisation in the 1800’s (Figure 1). One hundred and thirty
nine (43.3%) sera had matching samples of diaphragm tissue (5-10 g) which were tested using the
pooled magnetic stirrer AD method with a sieve modification described previously (Zimmer et al.,
2008). There was insufficient material to test the remaining 182 samples. Metastrongylus sp.
(lungworm) infections were detected in the lungs of 103/321 (32.0%) of the pigs from WA. Other
parasites that were detected in the WA population, but for which data are not available for
individuals, included Ascaris sp. (prevalence ~20%) and protozoans including Cryptosporidium sp.,
Balantidium sp., Blastocystis sp. and Entamoeba sp. (prevalence ~5-15%) (P. Adams, pers. comm.).
Of 311/321 (96.8%) wild boar for which age was estimated, 55.6% (173/311) were adults and
44.3% (138/311) were juveniles.

Sera were collected from 352 wild boars from a region of northern Australia that is assumed to be at
high-risk for the introduction of Trichinella. Sampling sites included 7 locations within the Cape
York Peninsula (CYP) of north-eastern Australia and 2 islands in the Torres Strait (Figure 1). A
sample of approximately 50 g of diaphragm or tongue muscle was collected from each animal and
stored for 1-3 years at -20 °C before testing. For each animal, 20 g of muscle was then tested for the
presence of *Trichinella* as described previously (Cuttell et al., 2012a). Data including age, sex and body condition were recorded for all animals. Data of infection with other nematodes was available for approximately 70% of the wild boar. A total of 43.0% were infected with *Stephanurus dentatus* (kidney worm), 14.5% with *Macracanthorhynchus hirudinaceus* (Thorny-headed worm) and 32.0% with *Spirometra* sp. (sparganosis) (Table 1). Of 344/350 (98%) wild boar for which age was estimated, 89.5% (308/344) were adults and 10% (36/344) were juveniles.

Positive and negative reference sera used in both the ELISA and WB were obtained from the European Union Reference Laboratory for Parasites (EURLP), Rome, Italy. Positive sera were collected from 4 specific pathogen free (spf) pigs experimentally infected with 20,000-30,000 *T. spiralis* muscle larvae. Negative sera were collected from 3 non-infected spf pigs. Serum from a wild boar from the low-risk population and confirmed as larvae-negative by AD of 10 g of diaphragm was also used as a negative control in the WB.

### 2.2 Western blot

Purified E/S antigen was obtained from *T. spiralis* muscle larvae prepared at the EURLP according to an established protocol (Gamble et al., 1988). E/S antigens produced at the EURLP have previously been evaluated for cross-reactivity with *Trichuris suis*, *Oesophagostomum* sp., *Metastrongylus* sp., *Ascaris suum* and the protozoa *Eimeria* with negative results (Gomez-Morales et al., 2009). E/S proteins were separated on pre-cast 10% Tris-HCl Ready-Gels (Bio-rad Pty Ltd, Hercules, CA) under reducing conditions before transfer to nitrocellulose (pore size 0.45 µm, Bio-Rad). A pre-stained protein standard (Precision Plus Protein WesternC Standard, Bio-Rad Pty Ltd) evaluated the quality of the transfer and protein size. The membrane was blocked with 5% skim milk in PBS with 1% Tween-20 (PBS-T), cut into strips and then each strip incubated with individual sera at a 1:50 dilution in blocking solution for 1 hour at RT. After washing 3 times with PBS-T for 15 mins, strips were incubated for 1 hour with a 1:5000 dilution of goat anti-swine IgG-
horseradish peroxidase conjugate (Bio-rad Pty Ltd) in PBS-T. Reactive protein bands were revealed
by Immun-Star HRP chemiluminescent substrate (Bio-rad Pty Ltd). The banding pattern of a
*Trichinella*-positive serum by WB was established using sera from 4 *T. spiralis* experimentally-
infected pigs and the naturally-infected wild boar from the Torres Strait. Profiles of the signal
intensities and relative migration values of seroreacting bands were compared with a positive and
negative control included on the same blot. A subset of 7 ELISA- and WB-positive samples from
the high-risk population, including 6 sera from the CYP and the serum from the larvae-positive
animal from Gabba Island were also forwarded to the EURLP for testing by ELISA and WB under
validated conditions.

2.3 In-house ELISA

The in-house ELISA used a standard methodology (Gomez-Morales et al., 2009) with some
modifications and E/S antigens were the same as used in WB. The protocol included coating plates
(96-well micro-titre, Greiner Bio One Pty Ltd, Frickenhausen, Germany) for 1 hour with 100
µl/well of E/S antigen (5 µg/mL) in carbonate buffered saline (pH 9.6). Plates were blocked (2%
BSA, 0.05% Tween 20) at 37 °C for 1 hour before 100 µl/well of an optimal serum dilution of 1:200
was added in duplicate and the plates incubated at 37°C for 30 mins. After manually washing plates
3 times in PBS containing 0.05% Tween 20 (PBS-T), 100 µl/well of anti-swine IgG horseradish
peroxidase labelled antibody diluted 1:5000 (Kirkegaard & Perry Laboratories, Gaithersburg, MD)
was added and the plates further incubated at 37°C for 1 hr. Plates were washed a final time before
100 µl/well of 1x tetramethylbenzidine (TMB) Ready-Set-Go substrate (eBioscience, San Diego
CA) was added and plates incubated at room temperature for 10 mins. 50 µl/well of 5N H₂SO₄ stop
solution was added to wells and the adsorbance read at a wavelength of 450 nm. Two positive and
two negative duplicated reference sera were included on each plate, as well as duplicated blank
wells with no serum added. OD values were normalised between plates by expressing the results as
a function of the reactivity of the positive control serum with the highest value of the two controls.
included in each run of the assay. The mean OD values of the control sera, as well as the mean OD values of the duplicated test sera were then calculated, and for each serum, an ELISA sample/positive ratio (S/P) expressed as a percentage of positivity was calculated according to the following formula:

\[
S/P = \frac{\text{OD mean duplicate sample} - \text{OD mean duplicate blanks}}{\text{OD mean duplicate highest positive control} - \text{OD mean duplicate blanks}} \times 100\% 
\]

If the coefficient of variation (CV) of a duplicated OD mean for any sample exceeded 10%, it was repeated.

2.4 Commercial ELISA

Sera were tested using the commercially produced PrioCHECK Trichinella-antibody indirect-ELISA kit (Prionics Pty Ltd, Zurich, Switzerland) approximately one year after samples were tested with the in-house ELISA. The kit was used according to the manufacturer’s instructions except the S/P cut-off was determined using methodology below. Briefly, sera were diluted to 1/50 and incubated on E/S antigen pre-coated plates for 30 mins. A peroxidase-labelled anti-swine secondary antibody was added for 30 mins before TMB substrate added to visualise the reaction. The absorbance of each duplicated sample was read at 450 nm.

2.5 Molecular methods

Diaphragm muscle was prepared and tested by the real-time PCR method described in Cuttell et al. (2012b). This test generically targets the small subunit of the ribosomal RNA (SSU-rRNA) gene of Trichinella parasites and the analytical sensitivity is 0.1 larvae/g when 10 g of muscle is tested. Trichinella species identification of real-time PCR positive samples was performed using the same DNA preparation (a co-extraction of genomic parasite and host DNA) and a PCR assay that targets the expansion segment five (ES5) region of the large subunit ribosomal DNA (LSU rRNA)
(Zarlenga et al., 1999; Zarlenga and Dame, 1992). Amplicons were sequenced and aligned with ES5 sequences of reference strains of *T. papuae* originating from the Bula Plain (Bensbach River region) (GenBank accession no. FJ493493) and Kikori region (GenBank accession no. FJ493494) of PNG.

### 2.6 Cut-off values

The initial cut-off values for the in-house and commercial ELISA were estimated by averaging the S/P ratios of the low-risk population and adding three standard deviations plus 10% for additional robustness. In the in-house ELISA, 316 sera were used in the analysis and 289 sera used in the commercial ELISA due to insufficient materials. The low-risk sera were screened with the results that samples with S/P ratios below the cut-off were considered negative, and values above the cut-off considered positive. Seropositive samples, as well as borderline samples (within 1% of S/P cut-off), were tested by WB to confirm the presence of *Trichinella*-specific proteins and the optimum cut-off values selected from these results.

### 2.7 Data analysis

Two tailed *t*-tests, correlation analysis and Chi-squared tests were performed in GraphPad Prism (Version 5, GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)) with a significance level of 5% (*P* value = 0.05) for all tests. Seroprevalence estimates when random sampling was assumed were calculated using EpiTools and the ‘estimated true prevalence and predictive values from survey testing’ function with a selected test sensitivity and specificity of 0.9 and Wilson’s 95% confidence interval (Sergeant, 2009). The selected test sensitivity and specificity was a conservative estimate based on the International Commission for Trichinellosis’ guidelines on the use of serological tests for *Trichinella* infections (Gamble et al., 2004). The WinPepi program (Abramson, 2011) and the ‘estimate prevalence using cluster, stratified or pooled samples’ function and Cochran’s method to estimate 95% confidence intervals were used for clustered sampling. The
Kappa statistic (κ-value) to measure test agreement was calculated in GraphPad QuickCalcs (http://www.graphpad.com/quickcalcs/) and the interpretation by Landis and Koch (1977) followed.

3 Results

3.1 Artificial digestion results
None of the samples of muscle from south-western WA wild boar were found to contain *Trichinella* larvae. The results of the AD testing of wild boar from the high-risk region were previously published by Cuttell et al. (2012a). All wild boar were larvae-negative except for an animal collected from Gabba Island in the Torres Strait which had a larval burden of ~14 larvae/g in diaphragm. The isolate was characterised by molecular methods as *T. papuae*.

3.2 Western blot
The common profile for *Trichinella* infection by WB was derived from 4 domestic pigs experimentally infected with *T. spiralis* and the wild boar naturally infected with *T. papuae* identified by the AD testing in which there was a clear consensus with a ‘triplicate’ group of bands localizing at around 40, 45 and 49 kDa. One or both of a further two bands at 60 and 65 kDa were also present in a proportion of the sera. The combined results of WB-positive sera showed 100% recognized all three bands between 40-49 kDa, 75% recognised the 60kDa band and 81% recognised the 65kDa band (Table 2).

3.3 ELISA performance and cut-off values
The cut-off value for positive sera was established using sera collected from the low-risk population. The initial cut-off for the in-house ELISA was determined to be 18.4% of which 2 (0.6%) out of 316 sera were reactive and yielded a 40-49 kDa banding pattern by WB that was consistent with the *Trichinella*-positive controls (Figure 2). No muscle tissue was available for
these two samples and therefore the presence of *Trichinella* larvae could not be confirmed. In the commercial ELISA, an initial cut-off of 11.4% was selected. The two sera from the low-risk population that tested positive with the in-house ELISA were also positive in the commercial test although one of these sera was borderline (S/P ratio 11.1%). The cut-off values were re-calculated following WB analysis and set as 25% for the in-house ELISA and 11% for the commercial assay. Graphical display of the distribution of the investigated sera confirmed the validity of the cut-offs (Figure 3). There were no significant correlations between ELISA positivity and the presence of either *S. dentatus*, *M. hirudinaceus* or *Spirometra* sp. in the high-risk population and *Metastrongylus* sp. in the low-risk population ($R^2$ range: -0.036-0.048, $P >0.05$).

The mean OD and S/P ratios of the in-house ELISA were significantly higher than the commercial ELISA values in both the low- and high-risk populations (unpaired t-test, $P< 0.05$). A higher proportion of samples from the high-risk population were positive (including borderline samples) when tested with the in-house ELISA (25/352, 7.1%) compared to the commercial test (14/321, 4.3%). There was substantial agreement between the results of the two ELISAs when used to test sera from both the high- and low-risk populations ($\kappa$-value= 0.66). The level of agreement between the tests increased when only ELISA- and WB-positive samples were included in the analysis ($\kappa$-value= 0.82).

### 3.4 Investigation of serum from wild boar in a high-risk region

The distribution of ELISA S/P values in wild boar sera from the CYP and Torres Strait are shown in Figure 4. The results of each diagnostic method and estimates of prevalence are provided in Table 3. In the small number of samples collected from the Torres Strait, 2/12 (16.6%) sera from wild boar of Gabba Island tested positive in both the in-house and commercial ELISA and WB. One of these reactive sera was the larvae-positive animal from Gabba Island identified in Cuttell et al.
(2012a) (Figure 2) and which also tested positive by ELISA and WB at the EURLP. There were no positive sera from Prince of Wales Island which was the only other island tested in the Torres Strait.

In the Cape York Peninsula of the Australian mainland, the apparent Trichinella seroprevalence was higher using the in-house ELISA (6.8%) than the commercial ELISA (3.9%). Western blot eliminated 47.8% and 41.6% of ELISA-positive sera in the in-house and commercial test respectively. The seroprevalence estimates using either ELISA coupled with WB (2.3% – 3.5%) were significantly higher than the AD estimate of 0.0 % (95% C.I 0.0-1.1) ($P$= < 0.05). Three samples that were in-house ELISA-/WB-positive were negative when tested by the commercial ELISA. There was insufficient material to test 2 other sera using the commercial ELISA that were WB-positive in the in-house test. Of the subset of ELISA- and WB-positive sera sent for analysis at the EURLP, 5/7 animals tested positive by both ELISA and WB.

The estimated seroprevalence (using either ELISA) was significantly higher in the north-eastern regions (Princess Charlotte Bay, Silver Plains and Lockhart River) of the CYP compared to other regions (unpaired $t$-test: in-house ELISA $P$=0.006; Commercial $P$=0.02) (Figure 5). Only in Aurukun was the seroprevalence estimate using the in-house ELISA (13%, 95% C.I. 4.5-32.1) significantly higher than the estimate from the commercial ELISA (0%, 95% C.I. 0.0-18.4) ($P$< 0.05). All wild boar positive by WB, irrespective of ELISA result, were adults and there was no significant difference in prevalence between males (56.2%) and females (43.7%) (unpaired $t$-test, $P$=> 0.05).

Real-time PCR was performed on muscle samples from all ELISA-positive and borderline samples except for 2 of 4 borderline high-risk region samples identified by the in-house ELISA (but which were WB negative). Only 2 (7.7%) out of 26 samples produced positive signals by real-time PCR, including the larvae-positive wild boar from Gabba Is. and a second, newly identified animal from
Gabba Is. that was weakly positive (Ct 30.52 ±0.1). A single melt curve peak in this sample was matched to a *Trichinella* positive control, and ES5 PCR and sequence analysis identified the species as *T. papuae*, Kikori strain. This animal had been sampled in 2008 and 20 g of diaphragm muscle tested by AD in 2010 did not detect the presence of *Trichinella* muscle larvae. A further 10 g of tissue from the animal’s tongue was re-tested by AD following the real-time PCR result but this also failed to detect intact larvae. No seropositive wild boar from the Australian mainland were positive using the real-time PCR method.

4 Discussion

In mainland Australia there is a possibility that *Trichinella* parasites will enter based on the presence of nearby non-encapsulated *Trichinella* reservoirs in south-western PNG and the southern Australian island state of Tasmania, coupled with the potential movements of infected wildlife or human-mediated exchange of infected animals or meat products. Targeted surveillance in regions at high risk of introduction and the application of ELISAs could improve Australia’s ability to provide more rapid, cost-effective and sensitive analyses for monitoring possible *Trichinella* incursions. A serosurvey for *Trichinella* infection in wild boar in north-eastern Australia was conducted in order to assess the diagnostic value of serological methods for use in surveillance. This study also represents the first large-scale serological investigation for *Trichinella* infection in mainland Australia and some islands of the Torres Strait.

The results from this study provided serological evidence of exposure to *Trichinella* parasites in wild boar of the Australian mainland although there was a lack of evidence of infection using AD or real-time PCR. The discrepancy between serology and AD has been noted in previous studies including in red foxes (Wacker et al., 1999) and pigs in Ecuador (Chavez-Larrea et al., 2005). Overestimation of the true prevalence by serological methods is sometimes attributed to differences between the persistence of muscle larvae and the antibody response. Persistent immune responses
have been described in experimental infections of the non-encapsulated *T. pseudospiralis* in domestic and wild pigs where antibodies were still detectable at 40 weeks post infection (p.i.) even though larval persistence had decreased to 0 or very low (< 0.003 larvae/g) (Kapel, 2000, 2001). Failure of larvae to establish in muscle may also occur when sylvatic *Trichinella* species are poorly adapted to swine yet anti-*Trichinella* antibody titres may be detectable (Kapel et al., 1998). Of the two most likely *Trichinella* species to be introduced to Australia, *T. papuae* appears sufficiently well-adapted to swine hosts based on its common isolation from wild pigs and role in several trichinellosis outbreaks in SE Asia (Khumjui et al., 2008; Kusolsuk et al., 2010). In contrast, an experimental infection study has suggested the Australian genotype of *T. pseudospiralis* is poorly infective to swine hosts (Kapel, 2001) which is supported by the absence of this parasite in a domestic cycle in Tasmania, despite its high prevalence (30%) in sylvatic carnivorous marsupials (Jackson, 1996; Obendorf et al., 1990).

In this study, the failure to recover muscle larvae by AD in seroreactors could also be related to lower test sensitivity or a loss in quality of the muscle samples due to the extended freezing before testing. Long term storage of muscle by freezing is thought to affect larval integrity and may have been a factor in the inability to recover intact larvae (International Commission for Trichinellosis, 2012). This possibility is supported by the fact that no muscle larvae were recovered from one Gabba Island wild pig identified as positive by serology and real-time PCR despite digestion of up to 40 g of tissue. The use of a more sensitive direct method of detection such as real-time PCR, which is also able to detect *Trichinella* DNA in samples that are frozen for long periods of time or are degraded, gives added confidence of the absence of *Trichinella* muscle larvae in the majority of the wild pigs sampled in this study.

Cross-reactivity to E/S antigens by other infections in Australian wild pigs is a plausible explanation for the high seroprevalence estimates, particularly by ELISA. The use of ELISAs for surveillance of *Trichinella* in wildlife has not been recommended previously because of a poor
understanding of test performance and often unacceptably high rates of false positives due to non-specific cross-reactions (Gamble et al., 2004). Although infections of wild boar with parasites such as *Metastrongylus* sp., *M. hirudinaceus* and *Spirometra* sp. were investigated for serological cross-reactivity, future work could also investigate the possibility of cross-reaction with sera infected with a closely-related parasite such as *Trichurus* sp. Non-specific cross-reactions could also occur when there is a reduction in serum sample quality through haemolysis or bacterial or fungal contamination, although in this study most sera tested were of high quality. In future work, in order to employ tests such as ELISA with accuracy, well-defined cohorts of positive and negative reference samples are needed to establish the test parameters before assumptions regarding infection status and/or prevalence can be made. In this study, a lack of well-defined reference samples hampered the ability to validate the ELISAs, rather, WB was used to further determine the infection status of animals that tested positive with ELISA.

WB has been recommended for confirmation of *Trichinella* infection in ELISA-positive sera as a common profile for trichinellosis distinct from other infections have been established when panels of *Trichinella*-positive sera are compared (Frey et al., 2009; Gómez-Morales et al., 2012; Robert et al., 1996). In this study, only a small panel of experimentally-infected (*n*=4) and naturally-infected (*n*=1) sera could be used to establish the *Trichinella* profile. The E/S antigens used were prepared under a quality assurance system at the EURLP and which were the same used recently to validate a WB that defined a triplicate banding pattern of 48-72kDa with 100% specificity in infected pig sera (Gómez-Morales et al., 2012). In the study by Gómez-Morales et al. (2012), false positive wild boar sera could be differentiated from true positives as although cross-reaction with one or two bands of the triplet were observed, there was never an occurrence of cross-reaction with all three. Here, test sera were only regarded as WB-positive if they produced the established triplicate pattern between 40-50 kDa. The molecular weight variations of the diagnostic bands characterised in this study from those in the EURLP findings are most likely due to differences in protein standards and gel type,
although our results are almost identical to those described by others (Wee et al., 2001). The absence of a further two bands at 60 and 63 kDa in a small proportion of the sera could be related to a reduced antibody titre in these samples. Furthermore, the corroboration of results in 5/7 seropositive, larvae-negative sera from mainland wild boar that tested ELISA- and WB-positive at the EURLP adds further support to the accuracy of our method.

The specificities of the serological methods used in this study were further evaluated in sera from wild boar infected with *Metastrongylus* spp., *S. dentatus, M. hirudinaceus* and *Spirometra* sp.. The lack of observable correlation between ELISA seropositivity or specific band pattern by WB suggested that there was little or no cross-reactivity with these parasites. There was also poor to no correlation between the ELISA-positive animals from the low-risk population and evidence of infection with *Ascaris* (20%), *Blastocystis, Entamoeba, Balantidium* and *Cryptosporidium* (all 5-15%).

The detection of *T. papuae* in a second wild boar from Gabba Island is further evidence of the endemic nature of the parasite in the local population of this island. As no animals from a second Torres Strait island (Prince of Wales) were positive by either serological or parasitological methods, the distribution of *T. papuae* may not be widespread on islands of the strait. On the Australian mainland, three neighbouring, north-eastern sampling locations in the CYP including Princess Charlotte Bay, Silver Plains and Lockhart River were identified as regions with the highest *Trichinella* seroprevalence. These areas are now the focus of ongoing surveillance. All three locations are situated on intertidal and alluvial plain wetlands which are areas where fresh and saltwater ecosystems converge and have a greater diversity of animal and plant species. Marine plains are plausible areas for the maintenance of transmission cycles for *Trichinella* as they support some of the highest wild pig densities in this region as well as large populations of migratory
shorebirds and saltwater crocodiles that may be part of a non-encapsulated *Trichinella* cycle (CYWFAP, 2006).

There was generally good agreement between the two ELISAs used in the study. However, the in-house ELISA identified a number of seroreacting samples in the Aurukun region which were not corroborated by the commercial test. Aurukun is another large estuarine embayment comprising of tidal flats and swamps that support high densities of wild pigs. It is possible that the poor test agreement in these samples was due to the lower analytical sensitivity of the commercial ELISA compared to the in-house ELISA. Lower test sensitivity using an E/S antigen commercial assay compared to an in-house developed assay has also been reported previously (Akisu et al., 2006) and indeed, the results of this study showed that the commercial ELISA produced a significantly lower mean S/P ratio in all populations tested. In addition, it is also possible that there had been a reduction in antibody titre in sera from this area that were collected over a prolonged period of time and stored at -20 °C for several years.

Evidence of *Trichinella* exposure in wild pigs originating from the low-risk population in southwestern WA was a surprising result. This population was originally selected as a negative reference cohort based on the remoteness of the area in the Australian continent, but the presence of two seroreactors showed that however unlikely, *Trichinella* exposure in wildlife could not be ruled out. Pigs from this population were collected from Jarrah forest in an area incorporating numerous water catchments and land uses including forestry, bauxite mining and recreation. The region also supports an abundance of native fauna as well as introduced species such as feral cats, foxes and wild pigs (Williams and Mitchell, 2001). The relative proximity of this region to the metropolitan city of Perth gives rise to the possibility of *Trichinella* parasite introduction via illegal human-mediated movement of infected animals or meat products from other endemic regions (Spencer and Hampton, 2005). Historical records of national disease surveillance data have also reported an
isolated case of trichinosis in 1963-1964 from WA, although more information regarding the exact location, *Trichinella* species or host cannot be obtained (National Disease Surveillance Report 1917-1971, Commonwealth Yearbook, unpublished data). In addition, although the large biomass of *T. pseudospiralis* in the south-eastern island state of Tasmania is thought to be geographically restricted, transmission via migrating carnivorous birds infected with *T. pseudospiralis* from Tasmania could be a possibility (Obendorf and Clarke, 1992). In particular, *T. pseudospiralis* infections have been reported in Tasmanian swamp harriers (*Circus approximans*), which is a species that migrates annually from Tasmania to overwinter in a broad swath of eastern Australia as far north as the CYP (Baker-Gabb and Steele, 1996). The presence of a second, isolated breeding population of this species in south-western WA, that also undertakes annual northwards migrations, could give rise to the possibility of transmission to WA via returning harriers.

In conclusion, the diagnostic value of E/S antigen ELISAs in wildlife surveillance in Australia can only be properly assessed when adequate numbers of positive and negative control sera are available to establish estimates of test sensitivity and specificity. WB was a sensitive, specific method that eliminated false-positive sera to provide a better estimate of the seroprevalence. Confirmation regarding the presence of *Trichinella* parasites on the Australian mainland, and subsequently establishing actual zoonotic risk, must rely on muscle examination by direct methods. There was no evidence of current infection of wild boar on the Australian mainland detected through this survey, which is supported by the lack of confirmed human cases of trichinellosis in Australia despite widespread pig hunting and consumption practises in some areas of northern Australia. For future surveillance, real-time PCR may be a suitable option to test seroreactors for current *Trichinella* infections as it offers greater sensitivity and requires a smaller sample size than AD.

Acknowledgements
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Legends to Figure

Figure 1. Study areas from which 673 Australian wild boar sera examined by enzyme-linked immunosorbent assay and Western blot for *Trichinella* infection were collected. Australian continent inset shows the location of collection of 321 sera from wild boar in an assumed ‘low risk’ region in south-western Australia (1) and collection of 352 sera from wild boar in an assumed ‘high-risk’ region in the Cape York Peninsula and Torres Strait (2). The origin and year of collection of test sera in each collection area of the high-risk region are shown.

Figure 2 Signal intensities and relative migration values of cross-reacting proteins in wild boar sera to *Trichinella* excretory-secretory antigens by Western blot. Panels ‘A’ are samples from a single blot and are therefore comparable. Samples include enzyme-linked immunosorbent assay (ELISA)- and Western blot-positive sera (PA3240 and PA3310) from the assumed low-risk population. Panels B are comparable samples from a single blot and include test sera from the assumed high-risk population.

Figure 3. Scatterplot of signal to positive (S/P) ratio values from the in-house (A) and commercial (B) enzyme-linked immunosorbent assays in wild boar sera from the low-risk population. Lines indicate the cut-off (c) and borderline cut-off (b). Seroreacting samples also subsequently positive by Western blot are labelled.

Figure 4. Scatterplot of enzyme-linked immunosorbent assay (ELISA) signal to positive (S/P) ratio values for wild boar sera arranged by geographic location in the assumed high-risk regions of the Cape York Peninsula and Torres Strait for the in-house (A) and commercial (B) ELISA. Lines indicate the cut-off (c) and borderline cut-off (b). Two samples identified as *Trichinella* larvae-positive by direct methods of either artificial digestion or real-time PCR are labelled.
Figure 5. Seroprevalence estimates for *Trichinella* infection by location in wild boar sera with an enzyme-linked immunosorbent assay- (ELISA) and Western blot-positive result from the high-risk region of mainland Australia and the Torres Strait. Red shaded boxes indicate regions with a seroprevalence greater than 0% in either ELISA.
Table 1. Description of the wild boar and control samples examined in the study

<table>
<thead>
<tr>
<th>Category</th>
<th>Origin of sample and other parasitic infections</th>
<th>Samples collected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive</strong> (spf pig)</td>
<td>European Union Reference Laboratory for Parasites</td>
<td>Sera</td>
</tr>
<tr>
<td><strong>Negative</strong> (spf pig)</td>
<td>European Union Reference Laboratory for Parasites</td>
<td>3</td>
</tr>
<tr>
<td><strong>Low-risk</strong> (Wild boar)</td>
<td>South-western Western Australia</td>
<td>321</td>
</tr>
<tr>
<td><strong>High-risk</strong> (Wild boar)</td>
<td>Torres Strait</td>
<td>340</td>
</tr>
<tr>
<td></td>
<td>Stephanurus dentatus</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Macracanthorhynchus hirudinaceus</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Spirometra sp.</td>
<td>119</td>
</tr>
</tbody>
</table>

*Specific pathogen-free (spf) pigs experimentally infected with 20,000-30,000 T. spiralis larvae*
Table 2 Frequency of band pattern for the five *Trichinella*-specific proteins detected in Western-blot positive test sera

<table>
<thead>
<tr>
<th>Protein size (kDa)</th>
<th>40</th>
<th>43</th>
<th>50</th>
<th>60</th>
<th>63</th>
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<tbody>
<tr>
<td>Low-risk</td>
<td>2 (100%)</td>
<td>2 (100%)</td>
<td>2 (100%)</td>
<td>2 (100%)</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>High-risk</td>
<td>14 (100%)</td>
<td>14 (100%)</td>
<td>14 (100%)</td>
<td>10 (71%)</td>
<td>11 (78%)</td>
</tr>
<tr>
<td>Total</td>
<td>16 (100%)</td>
<td>16 (100%)</td>
<td>16 (100%)</td>
<td>12 (75%)</td>
<td>13 (81%)</td>
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</table>
Table 3 Results and prevalence estimates of *Trichinella* infection in wild pigs from a high-risk region of Australia using direct and indirect methods.

<table>
<thead>
<tr>
<th>Study area</th>
<th>Method</th>
<th>n</th>
<th>Result</th>
<th>Apparent Prevalence % (95 C.I.)a</th>
<th>Paired method</th>
<th>Result</th>
<th>Adjusted Prevalence % (95 C.I.)a</th>
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</thead>
<tbody>
<tr>
<td>Cape York Peninsula</td>
<td>ADb</td>
<td>340</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0 (0.0-1.1)</td>
</tr>
<tr>
<td></td>
<td>In-house ELISA</td>
<td>340</td>
<td>19</td>
<td>5.6 (3.6-8.6)</td>
<td>Western blot</td>
<td>12*</td>
<td>3.5 (2.0-6.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Real-time PCRc</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>Commercial ELISA</td>
<td>323</td>
<td>11</td>
<td>3.4 (1.9-6.0)</td>
<td>Western blot</td>
<td>7</td>
<td>2.2 (1.1-4.4)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Real-time PCR</td>
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<td>0</td>
</tr>
<tr>
<td>Torres Strait</td>
<td>ADb</td>
<td>12</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.3 (1.5-35.4)</td>
</tr>
<tr>
<td></td>
<td>In-house ELISA</td>
<td>12</td>
<td>2</td>
<td>16.7 (4.7-44.8)</td>
<td>Western blot</td>
<td>2</td>
<td>16.7 (4.7-44.8)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Real-time PCR</td>
<td>2</td>
<td>16.7 (4.7-44.8)</td>
</tr>
<tr>
<td></td>
<td>Commercial ELISA</td>
<td>12</td>
<td>2</td>
<td>16.7 (4.7-44.8)</td>
<td>Western blot</td>
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<td>16.7 (4.7-44.8)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Real-time PCR</td>
<td>2</td>
<td>16.7 (4.7-44.8)</td>
</tr>
</tbody>
</table>

*includes one borderline sample

*a* Apparent and adjusted prevalence and confidence intervals (C.I.) calculated for serological tests with sensitivity and specificity approximate to 90%. For artificial digestion, sensitivity at 90% and specificity at 99% were used

*b* Artificial digestion of 20 g

*c* testing of 10 g
Figure
Figure

(A) Positive

Intensity (INT)

0.041 0.143 0.281 0.441 0.468 0.529 0.567 0.633

(B) Positive

Intensity (INT)

0.020 0.307 0.520 0.550 0.618 0.860

(A) Negative

Intensity (INT)

0.089

(B) Negative

Intensity (INT)

0.028

(A) PA3240

Intensity (INT)

0.016 0.059 0.236 0.476 0.628 0.652

(B) T. papuae larvae-positive serum

Intensity (INT)

0.000 0.026 0.296 0.404 0.572 0.607

(A) PA3310

Intensity (INT)

0.049 0.463 0.496 0.551 0.597 0.718 0.792

(B) CYP ELISA- and WB-positive serum

Intensity (INT)

0.070 0.196 0.550 0.558 0.625 0.862

R

0.00 0.25 0.50 0.75 1.00