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

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1 **Evaluation of ELISA coupled with Western blot as a surveillance tool**
2 **for *Trichinella* infection in wild boar (*Sus scrofa*)**
3

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27 **Abstract**

28 *Trichinella* surveillance in wildlife relies on muscle digestion of large samples which are
29 logistically difficult to store and transport in remote and tropical regions as well as labour-intensive
30 to process. Serological methods such as enzyme-linked immunosorbent assays (ELISAs) offer
31 rapid, cost-effective alternatives for surveillance but should be paired with additional tests because
32 of the high false-positive rates encountered in wildlife. We investigated the utility of ELISAs
33 coupled with Western blot (WB) in providing evidence of *Trichinella* exposure or infection in wild
34 boar. Serum samples were collected from 673 wild boar from a high- and low-risk region for
35 *Trichinella* introduction within mainland Australia, which is considered *Trichinella*-free. Sera were
36 examined using both an 'in-house' and a commercially available indirect-ELISA that used
37 excretory-secretory (E/S) antigens. Cut-off values for positive results were determined using sera
38 from the low-risk population. All wild boar from the high-risk region (352) and 139/321 (43.3%) of
39 the wild boar from the low-risk region were tested by artificial digestion. Testing by Western blot
40 using E/S antigens, and a *Trichinella*-specific real-time PCR was also carried out on all ELISA-
41 positive samples. The two ELISAs correctly classified all positive controls as well as one naturally-
42 infected wild boar from Gabba Island in the Torres Strait. In both the high- and low-risk
43 populations, the ELISA results showed substantial agreement (κ-value= 0.66) that increased to very
44 good (κ-value= 0.82) when WB-positive only samples were compared. The results of testing sera
45 collected from the Australian mainland showed the *Trichinella* seroprevalence was 3.5% (95% C.I.
46 0.0-8.0) and 2.3% (95% C.I. 0.0-5.6) using the in-house and commercial ELISA coupled with WB
47 respectively. These estimates were significantly higher ($P < 0.05$) than the artificial digestion
48 estimate of 0.0% (95% C.I. 0.0-1.1). Real-time PCR testing of muscle from seropositive animals
49 did not detect *Trichinella* DNA in any mainland animals, but did reveal the presence of a second
50 larvae-positive wild boar on Gabba Island, supporting its utility as an alternative, highly sensitive
51 method in muscle examination. The serology results suggest Australian wildlife may have been
52 exposed to *Trichinella* parasites. However, because of the possibility of non-specific reactions with

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53 other parasitic infections, more work using well-defined cohorts of positive and negative samples is
54 required. Even if the specificity of the ELISAs is proven to be low, their ability to correctly classify
55 the small number of true positive sera in this study indicates utility in screening wild boar
56 populations for reactive sera which can be followed up with additional testing.

57

58 *Keywords:* Trichinella*, ELISA, Western Blot, serosurveillance, wildlife, real-time PCR, zoonosis*

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59
60 **1. Introduction**

61

62 Parasitic nematodes of the genus *Trichinella* are the causative agents of trichinellosis – a global,
63 food-borne zoonosis that is also a recognised public health problem in South-East Asia (SE Asia)
64 and some Indo-Pacific regions (Conlan et al., 2011; Khumjui et al., 2008; Kusolsuk et al., 2010;
65 Owen et al., 2005). *Trichinella* parasites have never been reported on mainland Australia although
66 there are nearby foci of *Trichinella papuae* in Papua New Guinea (PNG) and an uninhabited island
67 in the Torres Strait, as well as the Australian genotype of *Trichinella pseudospiralis* in the south-
68 eastern Australian island of Tasmania (Cuttell et al., 2012a; Obendorf et al., 1990; Pozio et al.,
69 1999). The close geographical proximity of these reservoirs to mainland Australia suggests there is
70 potential for introduction via natural or illegal movements of infected animals or meat products. To
71 date, surveillance data for the presence of *Trichinella* infection in Australian wildlife has relied on
72 extensive testing of muscle samples by artificial digestion (AD) from game animals for export and
73 to a lesser extent from samples collected during limited surveys in wildlife (AHA, 2009; Bearup,
74 1949; DAFF, 2007; Oakwood and Spratt, 2000; Waddell, 1969). Surveillance in Australia could be
75 enhanced through improved diagnostic capacity for *Trichinella* infections in animals as well as
76 targeting surveillance to areas at high risk of introduction.

77

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

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86 insufficient information regarding their diagnostic performance (Gamble et al., 2004; OIE, 2012).
87 The high levels of sensitivity attained with ELISAs (pigs with worm burdens as low as 0.01
88 larvae/g are detectable) (Gamble et al., 1983) indicates their usefulness in detecting low-level
89 infections such as those encountered in wildlife. Test specificity may be reduced however because
90 of the possibility of parasitic, fungal, bacterial and viral infections in wildlife that increase the risk
91 of non-specific reactions (Gamble et al., 2004).
92
93 When ELISAs have been used to screen wild animals, seroprevalence is often reported at higher
94 levels than estimates obtained by AD, which is attributed to either the increased sensitivity or
95 reduced specificity of the serological method (Chavez-Larrea et al., 2005; Davidson et al., 2009;
96 Richomme et al., 2010; Wacker et al., 1999). The pairing of ELISAs with a test such as Western
97 blot (WB) to improve *Trichinella* diagnosis has been proposed and applied in humans (Gomez-
98 Morales et al., 2008) as well as domestic and wild animals (Davidson et al., 2009; Frey et al., 2009;
99 Noeckler et al., 2009; Pozio et al., 2002). Western blot offers higher specificity by allowing
100 visualisation of specific *Trichinella* proteins reacting with host antibodies, but is too costly to
101 perform with large sample sizes or for routine screening. WB using E/S antigens offers greater
102 specificity and ease of interpretation compared to blots using crude worm extracts. Distinctive
103 immunogenic profiles for *Trichinella* infection in pig sera have described a three-band pattern
104 between 40-53 kDa (Wee et al., 2001) or 48-72 kDa (Gómez-Morales et al., 2012).
105
106 As indirect methods, serological tests cannot be used to gauge actual zoonotic risk but have been
107 proposed as suitable for epidemiological studies when the objective is to estimate the level of
108 exposure to *Trichinella* parasites (Noeckler and Kapel, 2007). Therefore, the aim of this study was
109 to establish the value of serological methods in *Trichinella* surveillance of wild boar (*Sus scrofa*) in
110 Australia. This was achieved by comparing the performances of a commercial and in-house E/S
111 antigen indirect-ELISA and further investigation of ELISA-positive sera by WB. Sera samples were

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112 paired with muscle and also tested by AD. An alternative direct method of detection using
113 *Trichinella*-specific real-time PCR for amplification of DNA extracted from 10 g of muscle was
114 also applied in ELISA-positive sera.
115
116 **2 Materials and methods**
117
118 2.1 Sera and muscle sample collection
119 Details of the sera used in the study are provided in Table 1. Sera from 321 wild boar from south-
120 western Western Australia (WA) were classified as 'low-risk' for *Trichinella* infection because it is
121 an isolated region with limited opportunity for the introduction of *Trichinella* and no history of the
122 existence of pigs prior to European colonisation in the 1800's (Figure 1). One hundred and thirty
123 nine (43.3%) sera had matching samples of diaphragm tissue (5-10 g) which were tested using the
124 pooled magnetic stirrer AD method with a sieve modification described previously (Zimmer et al.,
125 2008). There was insufficient material to test the remaining 182 samples. *Metastrongylus* sp.
126 (lungworm) infections were detected in the lungs of 103/321 (32.0%) of the pigs from WA. Other
127 parasites that were detected in the WA population, but for which data are not available for
128 individuals, included *Ascaris* sp. (prevalence ~20%) and protozoans including *Cryptosporidium* sp.,
129 *Balantidium* sp., *Blastocystis* sp. and *Entamoeba* sp. (prevalence ~5-15%) (P. Adams, pers. comm.).
130 Of 311/321 (96.8%) wild boar for which age was estimated, 55.6% (173/311) were adults and
131 44.3% (138/311) were juveniles.
132
133 Sera were collected from 352 wild boars from a region of northern Australia that is assumed to be at
134 high-risk for the introduction of *Trichinella*. Sampling sites included 7 locations within the Cape
135 York Peninsula (CYP) of north-eastern Australia and 2 islands in the Torres Strait (Figure 1). A
136 sample of approximately 50 g of diaphragm or tongue muscle was collected from each animal and
137 stored for 1-3 years at -20 ^{o}C before testing. For each animal, 20 g of muscle was then tested for the

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138 presence of *Trichinella* as described previously (Cuttell et al., 2012a). Data including ~age, sex and
139 body condition were recorded for all animals. Data of infection with other nematodes was available
140 for approximately 70% of the wild boar. A total of 43.0% were infected with *Stephanurus dentatus*
141 (kidney worm), 14.5% with *Macracanthorhynchus hirudinaceus* (Thorny-headed worm) and 32.0%
142 with *Spirometra* sp. (sparganosis) (Table 1). Of 344/350 (98%) wild boar for which age was
143 estimated, 89.5% (308/344) were adults and 10% (36/344) were juveniles.
144
145 Positive and negative reference sera used in both the ELISA and WB were obtained from the
146 European Union Reference Laboratory for Parasites (EURLP), Rome, Italy. Positive sera were
147 collected from 4 specific pathogen free (spf) pigs experimentally infected with 20,000-30,000 *T.*
148 *spiralis* muscle larvae. Negative sera were collected from 3 non-infected spf pigs. Serum from a
149 wild boar from the low-risk population and confirmed as larvae-negative by AD of 10 g of
150 diaphragm was also used as a negative control in the WB.
151
152 2.2 Western blot
153 Purified E/S antigen was obtained from *T. spiralis* muscle larvae prepared at the EURLP according
154 to an established protocol (Gamble et al., 1988). E/S antigens produced at the EURLP have
155 previously been evaluated for cross-reactivity with *Trichuris suis*, *Oesophagostomum* sp.,
156 *Metastrongylus* sp., *Ascaris suum* and the protozoa *Eimeria* with negative results (Gomez-Morales
157 et al., 2009). E/S proteins were separated on pre-cast 10% Tris-HCl Ready-Gels (Bio-rad Pty Ltd,
158 Hercules, CA) under reducing conditions before transfer to nitrocellulose (pore size 0.45 µm, Bio-
159 Rad). A pre-stained protein standard (Precision Plus Protein WesternC Standard, Bio-Rad Pty Ltd)
160 evaluated the quality of the transfer and protein size. The membrane was blocked with 5% skim
161 milk in PBS with 1% Tween-20 (PBS-T), cut into strips and then each strip incubated with
162 individual sera at a 1:50 dilution in blocking solution for 1 hour at RT. After washing 3 times with
163 PBS-T for 15 mins, strips were incubated for 1 hour with a 1:5000 dilution of goat anti-swine IgG-

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164 horseradish peroxidase conjugate (Bio-rad Pty Ltd) in PBS-T. Reactive protein bands were revealed
165 by Immun-Star HRP chemiluminescent substrate (Bio-rad Pty Ltd). The banding pattern of a
166 *Trichinella*-positive serum by WB was established using sera from 4 *T. spiralis* experimentally-
167 infected pigs and the naturally-infected wild boar from the Torres Strait. Profiles of the signal
168 intensities and relative migration values of seroreacting bands were compared with a positive and
169 negative control included on the same blot. A subset of 7 ELISA- and WB-positive samples from
170 the high-risk population, including 6 sera from the CYP and the serum from the larvae-positive
171 animal from Gabba Island were also forwarded to the EURLP for testing by ELISA and WB under
172 validated conditions.
173
174 2.3 In-house ELISA
175 The in-house ELISA used a standard methodology (Gomez-Morales et al., 2009) with some
176 modifications and E/S antigens were the same as used in WB. The protocol included coating plates
177 (96-well micro-titre, Greiner Bio One Pty Ltd, Frickenhausen, Germany) for 1 hour with 100
178 µl/well of E/S antigen (5 µg/mL) in carbonate buffered saline (pH 9.6). Plates were blocked (2%
179 BSA, 0.05% Tween 20) at 37 ^{o}C for 1 hour before 100 µl/well of an optimal serum dilution of 1:200
180 was added in duplicate and the plates incubated at 37°C for 30 mins. After manually washing plates
181 3 times in PBS containing 0.05% Tween 20 (PBS-T), 100 µl/well of anti-swine IgG horseradish
182 peroxidase labelled antibody diluted 1:5000 (Kirkegaard & Perry Laboratories, Gaithersburg, MD)
183 was added and the plates further incubated at 37°C for 1 hr. Plates were washed a final time before
184 100 µl/well of 1x tetramethylbenzidine (TMB) Ready-Set-Go substrate (eBioscience, San Diego
185 CA) was added and plates incubated at room temperature for 10 mins. 50 µl/well of 5N H_2SO_4 stop
186 solution was added to wells and the adsorbance read at a wavelength of 450 nm. Two positive and
187 two negative duplicated reference sera were included on each plate, as well as duplicated blank
188 wells with no serum added. OD values were normalised between plates by expressing the results as
189 a function of the reactivity of the positive control serum with the highest value of the two controls

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190 included in each run of the assay. The mean OD values of the control sera, as well as the mean OD
191 values of the duplicated test sera were then calculated, and for each serum, an ELISA
192 sample/positive ratio (S/P) expressed as a percentage of positivity was calculated according to the
193 following formula:
194

195–196

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

197

198 If the coefficient of variation (CV) of a duplicated OD mean for any sample exceeded 10%, it was
199 repeated.
200

201 2.4 Commercial ELISA

202 Sera were tested using the commercially produced PrioCHECK *Trichinella*-antibody indirect-
203 ELISA kit (Prionics Pty Ltd, Zurich, Switzerland) approximately one year after samples were tested
204 with the in-house ELISA. The kit was used according to the manufacturer's instructions except the
205 S/P cut-off was determined using methodology below. Briefly, sera were diluted to 1/50 and
206 incubated on E/S antigen pre-coated plates for 30 mins. A peroxidase-labelled anti-swine secondary
207 antibody was added for 30 mins before TMB substrate added to visualise the reaction. The
208 absorbance of each duplicated sample was read at 450 nm.
209

210 2.5 Molecular methods

211 Diaphragm muscle was prepared and tested by the real-time PCR method described in Cuttell et al.
212 (2012b). This test generically targets the small subunit of the ribosomal RNA (SSU-rRNA) gene of
213 *Trichinella* parasites and the analytical sensitivity is 0.1 larvae/g when 10 g of muscle is tested.
214 *Trichinella* species identification of real-time PCR positive samples was performed using the same
215 DNA preparation (a co-extraction of genomic parasite and host DNA) and a PCR assay that targets
216 the expansion segment five (ES5) region of the large subunit ribosomal DNA (LSU rRNA)

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217 (Zarlenga et al., 1999; Zarlenga and Dame, 1992). Amplicons were sequenced and aligned with ES5
218 sequences of reference strains of *T. papuae* originating from the Bula Plain (Bensbach River region)
219 (GenBank accession no. FJ493493) and Kikori region (GenBank accession no. FJ493494) of PNG.
220

221 2.6 Cut-off values

222 The initial cut-off values for the in-house and commercial ELISA were estimated by averaging the
223 S/P ratios of the low-risk population and adding three standard deviations plus 10% for additional
224 robustness. In the in-house ELISA, 316 sera were used in the analysis and 289 sera used in the
225 commercial ELISA due to insufficient materials. The low-risk sera were screened with the results
226 that samples with S/P ratios below the cut-off were considered negative, and values above the cut-
227 off considered positive. Seropositive samples, as well as borderline samples (within 1% of S/P cut-
228 off), were tested by WB to confirm the presence of *Trichinella*-specific proteins and the optimum
229 cut-off values selected from these results.
230

231 2.7 Data analysis

232 Two tailed *t*-tests, correlation analysis and Chi-squared tests were performed in GraphPad Prism
233 (Version 5, GraphPad Software, San Diego California USA, www.graphpad.com) with a
234 significance level of 5% (*P* value=0.05) for all tests. Seroprevalence estimates when random
235 sampling was assumed were calculated using EpiTools and the 'estimated true prevalence and
236 predictive values from survey testing' function with a selected test sensitivity and specificity of 0.9
237 and Wilson's 95% confidence interval (Sergeant, 2009). The selected test sensitivity and specificity
238 was a conservative estimate based on the International Commission for Trichinellosis'guidelines on
239 the use of serological tests for *Trichinella* infections (Gamble et al., 2004). The WinPepi program
240 (Abramson, 2011) and the 'estimate prevalence using cluster, stratified or pooled samples' function
241 and Cochran's method to estimate 95% confidence intervals were used for clustered sampling. The

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242 Kappa statistic (κ-value) to measure test agreement was calculated in GraphPad QuickCalcs
243 (http://www.graphpad.com/quickcalcs/) and the interpretation by Landis and Koch (1977) followed.
244
245 **3 Results**
246
247 3.1 Artificial digestion results
248 None of the samples of muscle from south-western WA wild boar were found to contain *Trichinella*
249 larvae. The results of the AD testing of wild boar from the high-risk region were previously
250 published by Cuttell et al. (2012a). All wild boar were larvae-negative except for an animal
251 collected from Gabba Island in the Torres Strait which had a larval burden of ~14 larvae/g in
252 diaphragm. The isolate was characterised by molecular methods as *T. papuae*.
253
254 3.2 Western blot
255 The common profile for *Trichinella* infection by WB was derived from 4 domestic pigs
256 experimentally infected with *T. spiralis* and the wild boar naturally infected with *T. papuae*
257 identified by the AD testing in which there was a clear consensus with a 'triplicate' group of bands
258 localizing at around 40, 45 and 49 kDa. One or both of a further two bands at 60 and 65 kDa were
259 also present in a proportion of the sera. The combined results of WB-positive sera showed 100%
260 recognized all three bands between 40-49 kDa, 75% recognised the 60kDa band and 81%
261 recognised the 65kDa band (Table 2).
262
263 3.3 ELISA performance and cut-off values
264 The cut-off value for positive sera was established using sera collected from the low-risk
265 population. The initial cut-off for the in-house ELISA was determined to be 18.4% of which 2
266 (0.6%) out of 316 sera were reactive and yielded a 40-49 kDa banding pattern by WB that was
267 consistent with the *Trichinella*-positive controls (Figure 2). No muscle tissue was available for

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268 these two samples and therefore the presence of *Trichinella* larvae could not be confirmed. In the
269 commercial ELISA, an initial cut-off of 11.4% was selected. The two sera from the low-risk
270 population that tested positive with the in-house ELISA were also positive in the commercial test
271 although one of these sera was borderline (S/P ratio 11.1%). The cut-off values were re-calculated
272 following WB analysis and set as 25% for the in-house ELISA and 11% for the commercial assay.
273 Graphical display of the distribution of the investigated sera confirmed the validity of the cut-offs
274 (Figure 3). There were no significant correlations between ELISA positivity and the presence of
275 either *S. dentatus*, *M. hirudinaceus* or *Spirometra* sp. in the high-risk population and
276 *Metastrongylus* sp. in the low-risk population (R^2 range: -0.036-0.048, P >0.05).
277

278 The mean OD and S/P ratios of the in-house ELISA were significantly higher than the commercial
279 ELISA values in both the low- and high-risk populations (unpaired t-test, $P< 0.05$). A higher
280 proportion of samples from the high-risk population were positive (including borderline samples)
281 when tested with the in-house ELISA (25/352, 7.1%) compared to the commercial test (14/321,
282 4.3%). There was substantial agreement between the results of the two ELISAs when used to test
283 sera from both the high- and low-risk populations (κ-value= 0.66). The level of agreement between
284 the tests increased when only ELISA- and WB-positive samples were included in the analysis (κ-
285 value= 0.82).
286

287 3.4 Investigation of serum from wild boar in a high-risk region

288 The distribution of ELISA S/P values in wild boar sera from the CYP and Torres Strait are shown
289 in Figure 4. The results of each diagnostic method and estimates of prevalence are provided in
290 Table 3. In the small number of samples collected from the Torres Strait, 2/12 (16.6%) sera from
291 wild boar of Gabba Island tested positive in both the in-house and commercial ELISA and WB. One
292 of these reactive sera was the larvae-positive animal from Gabba Island identified in Cuttell et al.

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293 (2012a) (Figure 2) and which also tested positive by ELISA and WB at the EURLP. There were no
294 positive sera from Prince of Wales Island which was the only other island tested in the Torres Strait.
295
296 In the Cape York Peninsula of the Australian mainland, the apparent *Trichinella* seroprevalence was
297 higher using the in-house ELISA (6.8%) than the commercial ELISA (3.9%). Western blot
298 eliminated 47.8% and 41.6% of ELISA-positive sera in the in-house and commercial test
299 respectively. The seroprevalence estimates using either ELISA coupled with WB (2.3% – 3.5%)
300 were significantly higher than the AD estimate of 0.0 % (95% C.I 0.0-1.1) ($P= < 0.05$). Three
301 samples that were in-house ELISA-/WB-positive were negative when tested by the commercial
302 ELISA. There was insufficient material to test 2 other sera using the commercial ELISA that were
303 WB-positive in the in-house test. Of the subset of ELISA- and WB-positive sera sent for analysis at
304 the EURLP, 5/7 animals tested positive by both ELISA and WB.
305
306 The estimated seroprevalence (using either ELISA) was significantly higher in the north-eastern
307 regions (Princess Charlotte Bay, Silver Plains and Lockhart River) of the CYP compared to other
308 regions (unpaired *t*-test: in-house ELISA P=0.006; Commercial P=0.02) (Figure 5). Only in
309 Aurukun was the seroprevalence estimate using the in-house ELISA (13%, 95% C.I. 4.5-32.1)
310 significantly higher than the estimate from the commercial ELISA (0%, 95% C.I. 0.0-18.4) ($P< 0.05$).
311 All wild boar positive by WB, irrespective of ELISA result, were adults and there was no
312 significant difference in prevalence between males (56.2%) and females (43.7%) (unpaired *t*-test,
313 $P=> 0.05$).
314
315 Real-time PCR was performed on muscle samples from all ELISA-positive and borderline samples
316 except for 2 of 4 borderline high-risk region samples identified by the in-house ELISA (but which
317 were WB negative). Only 2 (7.7%) out of 26 samples produced positive signals by real-time PCR,
318 including the larvae-positive wild boar from Gabba Is. and a second, newly identified animal from

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319 Gabba Is. that was weakly positive (*Ct* 30.52 ±0.1). A single melt curve peak in this sample was
320 matched to a *Trichinella* positive control, and ES5 PCR and sequence analysis identified the species
321 as *T. papuae*, Kikori strain. This animal had been sampled in 2008 and 20 g of diaphragm muscle
322 tested by AD in 2010 did not detect the presence of *Trichinella* muscle larvae. A further 10 g of
323 tissue from the animal's tongue was re-tested by AD following the real-time PCR result but this
324 also failed to detect intact larvae. No seropositive wild boar from the Australian mainland were
325 positive using the real-time PCR method.

326

327 4 Discussion

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

338

339 The results from this study provided serological evidence of exposure to *Trichinella* parasites in
340 wild boar of the Australian mainland although there was a lack of evidence of infection using AD or
341 real-time PCR. The discrepancy between serology and AD has been noted in previous studies
342 including in red foxes (Wacker et al., 1999) and pigs in Ecuador (Chavez-Larrea et al., 2005).
343 Overestimation of the true prevalence by serological methods is sometimes attributed to differences
344 between the persistence of muscle larvae and the antibody response. Persistent immune responses

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345 have been described in experimental infections of the non-encapsulated *T. pseudospiralis* in
346 domestic and wild pigs where antibodies were still detectable at 40 weeks post infection (p.i.) even
347 though larval persistence had decreased to 0 or very low (< 0.003 larvae/g) (Kapel, 2000, 2001).
348 Failure of larvae to establish in muscle may also occur when sylvatic *Trichinella* species are poorly
349 adapted to swine yet anti-*Trichinella* antibody titres may be detectable (Kapel et al., 1998). Of the
350 two most likely *Trichinella* species to be introduced to Australia, *T. papuae* appears sufficiently
351 well-adapted to swine hosts based on its common isolation from wild pigs and role in several
352 trichinellosis outbreaks in SE Asia (Khumjui et al., 2008; Kusolsuk et al., 2010). In contrast, an
353 experimental infection study has suggested the Australian genotype of *T. pseudospiralis* is poorly
354 infective to swine hosts (Kapel, 2001) which is supported by the absence of this parasite in a
355 domestic cycle in Tasmania, despite its high prevalence (30%) in sylvatic carnivorous marsupials
356 (Jackson, 1996; Obendorf et al., 1990).
357
358 In this study, the failure to recover muscle larvae by AD in seroreactors could also be related to
359 lower test sensitivity or a loss in quality of the muscle samples due to the extended freezing before
360 testing. Long term storage of muscle by freezing is thought to affect larval integrity and may have
361 been a factor in the inability to recover intact larvae (International Commission for Trichinellosis,
362 2012). This possibility is supported by the fact that no muscle larvae were recovered from one
363 Gabba Island wild pig identified as positive by serology and real-time PCR despite digestion of up
364 to 40 g of tissue. The use of a more sensitive direct method of detection such as real-time PCR,
365 which is also able to detect *Trichinella* DNA in samples that are frozen for long periods of time or
366 are degraded, gives added confidence of the absence of *Trichinella* muscle larvae in the majority of
367 the wild pigs sampled in this study.
368
369 Cross-reactivity to E/S antigens by other infections in Australian wild pigs is a plausible
370 explanation for the high seroprevalence estimates, particularly by ELISA. The use of ELISAs for
371 surveillance of *Trichinella* in wildlife has not been recommended previously because of a poor

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372 understanding of test performance and often unacceptably high rates of false positives due to non-
373 specific cross-reactions (Gamble et al., 2004). Although infections of wild boar with parasites such
374 as *Metastrongylus* sp., *M. hirudinaceus* and *Spirometra* sp. were investigated for serological cross-
375 reactivity, future work could also investigate the possibility of cross-reaction with sera infected with
376 a closely-related parasite such as *Trichuris* sp. Non-specific cross-reactions could also occur when
377 there is a reduction in serum sample quality through haemolysis or bacterial or fungal
378 contamination, although in this study most sera tested were of high quality. In future work, in order
379 to employ tests such as ELISA with accuracy, well-defined cohorts of positive and negative
380 reference samples are needed to establish the test parameters before assumptions regarding infection
381 status and/or prevalence can be made. In this study, a lack of well-defined reference samples
382 hampered the ability to validate the ELISAs, rather, WB was used to further determine the infection
383 status of animals that tested positive with ELISA.
384
385 WB has been recommended for confirmation of *Trichinella* infection in ELISA-positive sera as a
386 common profile for trichinellosis distinct from other infections have been established when panels
387 of *Trichinella*-positive sera are compared (Frey et al., 2009; Gómez-Morales et al., 2012; Robert et
388 al., 1996). In this study, only a small panel of experimentally-infected (n=4) and naturally-infected
389 (n=1) sera could be used to establish the *Trichinella* profile. The E/S antigens used were prepared
390 under a quality assurance system at the EURLP and which were the same used recently to validate a
391 WB that defined a triplicate banding pattern of 48-72kDa with 100% specificity in infected pig sera
392 (Gómez-Morales et al., 2012). In the study by Gómez-Morales et al. (2012), false positive wild boar
393 sera could be differentiated from true positives as although cross-reaction with one or two bands of
394 the triplet were observed, there was never an occurrence of cross-reaction with all three. Here, test
395 sera were only regarded as WB-positive if they produced the established triplicate pattern between
396 40-50 kDa. The molecular weight variations of the diagnostic bands characterised in this study from
397 those in the EURLP findings are most likely due to differences in protein standards and gel type,

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398 although our results are almost identical to those described by others (Wee et al., 2001). The
399 absence of a further two bands at 60 and 63 kDa in a small proportion of the sera could be related
400 to a reduced antibody titre in these samples. Furthermore, the corroboration of results in 5/7
401 seropositive, larvae-negative sera from mainland wild boar that tested ELISA- and WB-positive at
402 the EURLP adds further support to the accuracy of our method.
403
404 The specificities of the serological methods used in this study were further evaluated in sera from
405 wild boar infected with *Metastrongylus* spp., *S. dentatus*, *M. hirudinaceus* and *Spirometra* sp.. The
406 lack of observable correlation between ELISA seropositivity or specific band pattern by WB
407 suggested that there was little or no cross-reactivity with these parasites. There was also poor to no
408 correlation between the ELISA-positive animals from the low-risk population and evidence of
409 infection with *Ascaris* (20%), *Blastocystis*, *Entamoeba*, *Balantidium* and *Cryptosporidium* (all 5-
410 15%).
411
412 The detection of *T. papuae* in a second wild boar from Gabba Island is further evidence of the
413 endemic nature of the parasite in the local population of this island. As no animals from a second
414 Torres Strait island (Prince of Wales) were positive by either serological or parasitological methods,
415 the distribution of *T. papuae* may not be widespread on islands of the strait. On the Australian
416 mainland, three neighbouring, north-eastern sampling locations in the CYP including Princess
417 Charlotte Bay, Silver Plains and Lockhart River were identified as regions with the highest
418 *Trichinella* seroprevalence. These areas are now the focus of ongoing surveillance. All three
419 locations are situated on intertidal and alluvial plain wetlands which are areas where fresh and
420 saltwater ecosystems converge and have a greater diversity of animal and plant species. Marine
421 plains are plausible areas for the maintenance of transmission cycles for *Trichinella* as they support
422 some of the highest wild pig densities in this region as well as large populations of migratory

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423 shorebirds and saltwater crocodiles that may be part of a non-encapsulated *Trichinella* cycle
424 (CYWFAP, 2006).
425
426 There was generally good agreement between the two ELISAs used in the study. However, the in-
427 house ELISA identified a number of seroreacting samples in the Aurukun region which were not
428 corroborated by the commercial test. Aurukun is another large estuarine embayment comprising of
429 tidal flats and swamps that support high densities of wild pigs. It is possible that the poor test
430 agreement in these samples was due to the lower analytical sensitivity of the commercial ELISA
431 compared to the in-house ELISA. Lower test sensitivity using an E/S antigen commercial assay
432 compared to an in-house developed assay has also been reported previously (Akisu et al., 2006) and
433 indeed, the results of this study showed that the commercial ELISA produced a significantly lower
434 mean S/P ratio in all populations tested. In addition, it is also possible that there had been a
435 reduction in antibody titre in sera from this area that were collected over a prolonged period of time
436 and stored at -20 ^{o}C for several years.
437
438 Evidence of *Trichinella* exposure in wild pigs originating from the low-risk population in south-
439 western WA was a surprising result. This population was originally selected as a negative reference
440 cohort based on the remoteness of the area in the Australian continent, but the presence of two
441 seroreactors showed that however unlikely, *Trichinella* exposure in wildlife could not be ruled out.
442 Pigs from this population were collected from Jarrah forest in an area incorporating numerous water
443 catchments and land uses including forestry, bauxite mining and recreation. The region also
444 supports an abundance of native fauna as well as introduced species such as feral cats, foxes and
445 wild pigs (Williams and Mitchell, 2001). The relative proximity of this region to the metropolitan
446 city of Perth gives rise to the possibility of *Trichinella* parasite introduction via illegal human-
447 mediated movement of infected animals or meat products from other endemic regions (Spencer and
448 Hampton, 2005). Historical records of national disease surveillance data have also reported an

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449 isolated case of trichinosis in 1963-1964 from WA, although more information regarding the exact
450 location, *Trichinella* species or host cannot be obtained (National Disease Surveillance Report
451 1917-1971, Commonwealth Yearbook, unpublished data). In addition, although the large biomass
452 of *T. pseudospiralis* in the south-eastern island state of Tasmania is thought to be geographically
453 restricted, transmission via migrating carnivorous birds infected with *T. pseudospiralis* from
454 Tasmania could be a possibility (Obendorf and Clarke, 1992). In particular, *T. pseudospiralis*
455 infections have been reported in Tasmanian swamp harriers (*Circus approximans*), which is a
456 species that migrates annually from Tasmania to overwinter in a broad swath of eastern Australia as
457 far north as the CYP (Baker-Gabb and Steele, 1996). The presence of a second, isolated breeding
458 population of this species in south-western WA, that also undertakes annual northwards migrations,
459 could give rise to the possibility of transmission to WA via returning harriers.
460
461 In conclusion, the diagnostic value of E/S antigen ELISAs in wildlife surveillance in Australia can
462 only be properly assessed when adequate numbers of positive and negative control sera are
463 available to establish estimates of test sensitivity and specificity. WB was a sensitive, specific
464 method that eliminated false-positive sera to provide a better estimate of the seroprevalence.
465 Confirmation regarding the presence of *Trichinella* parasites on the Australian mainland, and
466 subsequently establishing actual zoonotic risk, must rely on muscle examination by direct methods.
467 There was no evidence of current infection of wild boar on the Australian mainland detected
468 through this survey, which is supported by the lack of confirmed human cases of trichinellosis in
469 Australia despite widespread pig hunting and consumption practises in some areas of northern
470 Australia. For future surveillance, real-time PCR may be a suitable option to test seroreactors for
471 current *Trichinella* infections as it offers greater sensitivity and requires a smaller sample size than
472 AD.
473
474 **Acknowledgements**

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479

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615 **Legends to Figure**

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

622

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

629

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

634

635 **Figure 4.** Scatterplot of enzyme-linked immunosorbent assay (ELISA) signal to positive (S/P) ratio
636 values for wild boar sera arranged by geographic location in the assumed high-risk regions of the
637 Cape York Peninsula and Torres Strait for the in-house (A) and commercial (B) ELISA. Lines
638 indicate the cut-off (c) and borderline cut-off (b).Two samples identified as *Trichinella* larvae-
639 positive by direct methods of either artificial digestion or real-time PCR are labelled.

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641 **Figure 5.** Seroprevalence estimates for *Trichinella* infection by location in wild boar sera with an

642 enzyme-linked immunosorbent assay- (ELISA) and Western blot-positive result from the high-risk

643 region of mainland Australia and the Torres Strait. Red shaded boxes indicate regions with a

644 seroprevalence greater than 0% in either ELISA.

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645 **Table 1.** Description of the wild boar and control samples examined in the study

Category	Origin of sample and other parasitic infections	Samples collected		
		Sera	**Muscle**	**Muscle tested**
Positive[a] (spf pig)	European Union Reference Laboratory for Parasites	4	-	
Negative (spf pig)	European Union Reference Laboratory for Parasites	3	-	
Low-risk (Wild boar)	South-western Western Australia	321	139 (43%)	5-10 g
	Metastrongylus spp	103		
High-risk (Wild boar)	Cape York Peninsula	340	340 (100%)	20 g
	Torres Strait	12	12 (100%)	20 g
	Stephanurus dentatus	119		
	Macracanthorhynchus hirudinaceus	40		
	Spirometra sp.	88		

646 [a] Specific pathogen-free (spf) pigs experimentally infected with 20,000-30,000 *T. spiralis* larvae

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647 **Table 2** Frequency of band pattern for the five *Trichinella*-specific proteins detected in Western-
648 blot positive test sera

Population	Sample number	Protein size (kDa)				
		40	43	50	60	63
Low-risk	2	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)
High-risk	14	14 (100%)	14 (100%)	14 (100%)	10 (71%)	11 (78%)
Total	16	16 (100%)	16 (100%)	16 (100%)	12 (75%)	13(81%)

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651 **Table 3** Results and prevalence estimates of *Trichinella* infection in wild pigs from a high-risk
652 region of Australia using direct and indirect methods.

Study area	Method	*n*	Result	Apparent Prevalence % (95 C.I.)[a]	Paired method	Result	Adjusted Prevalence % (95 C.I.)[a]
Cape York Peninsula	AD[b]	340	0	-	-	-	0.0 (0.0-1.1)
	In-house ELISA	340	19	5.6 (3.6-8.6)	Western blot	12*	3.5 (2.0-6.1)
					Real-time PCR[c]	0	0
	Commercial ELISA	323	11	3.4 (1.9-6.0)	Western blot	7	2.2 (1.1-4.4)
					Real-time PCR	0	0
Torres Strait	AD[b]	12	1	-	-	-	8.3 (1.5-35.4)
	In-house ELISA	12	2	16.7 (4.7-44.8)	Western blot	2	16.7 (4.7-44.8)
					Real-time PCR	2	16.7 (4.7-44.8)
	Commercial ELISA	12	2	16.7 (4.7-44.8)	Western blot	2	16.7 (4.7-44.8)
					Real-time PCR	2	16.7 (4.7-44.8)

653 [*]includes one borderline sample
654 [a] Apparent and adjusted prevalence and confidence intervals (C.I.) calculated for serological tests
655 with sensitivity and specificity approximate to 90%. For artificial digestion, sensitivity at 90% and
656 specificity at 99% were used
657 [b] Artificial digestion of 20 g
658 [c] testing of 10 g
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660

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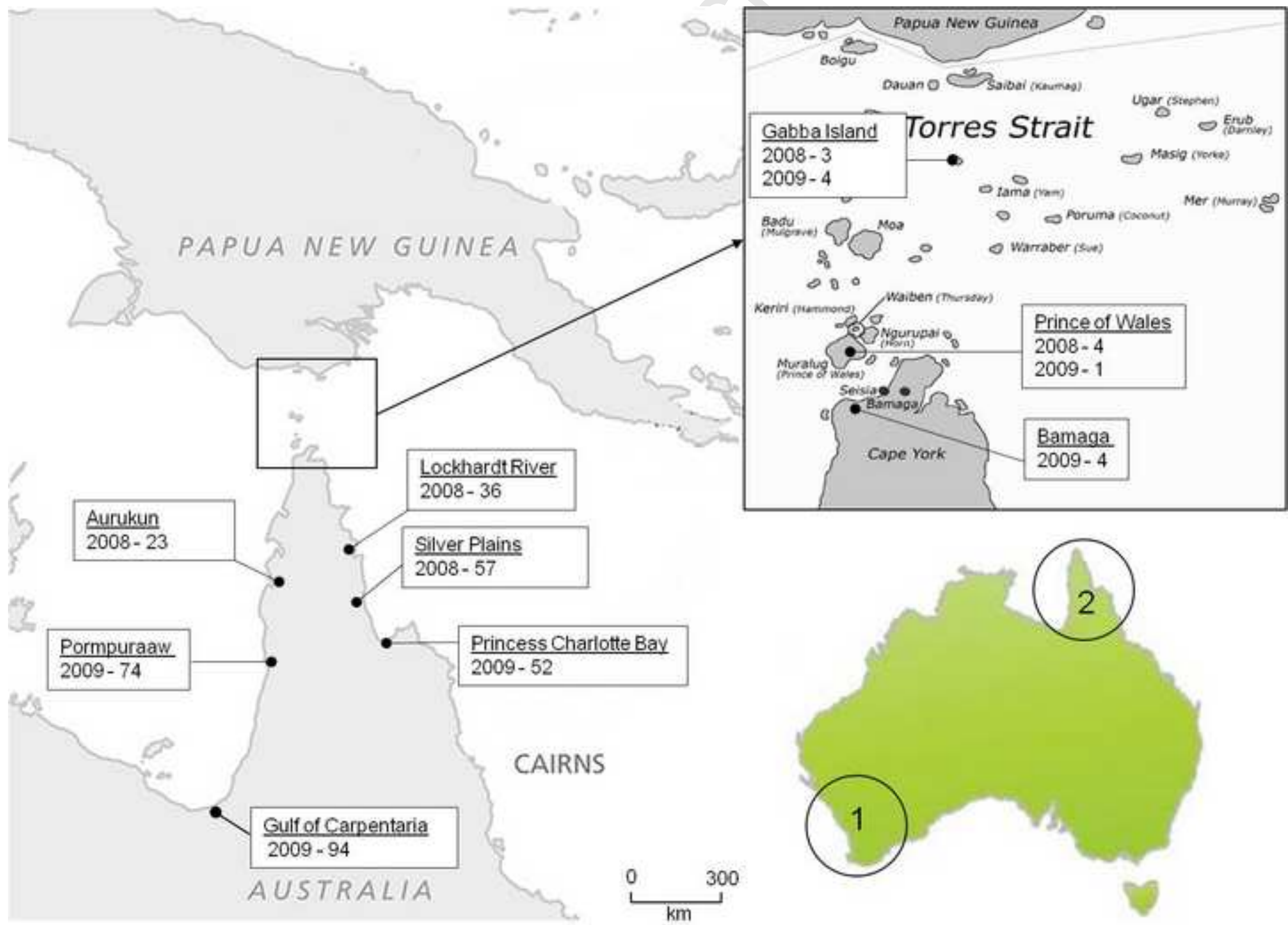
Figure



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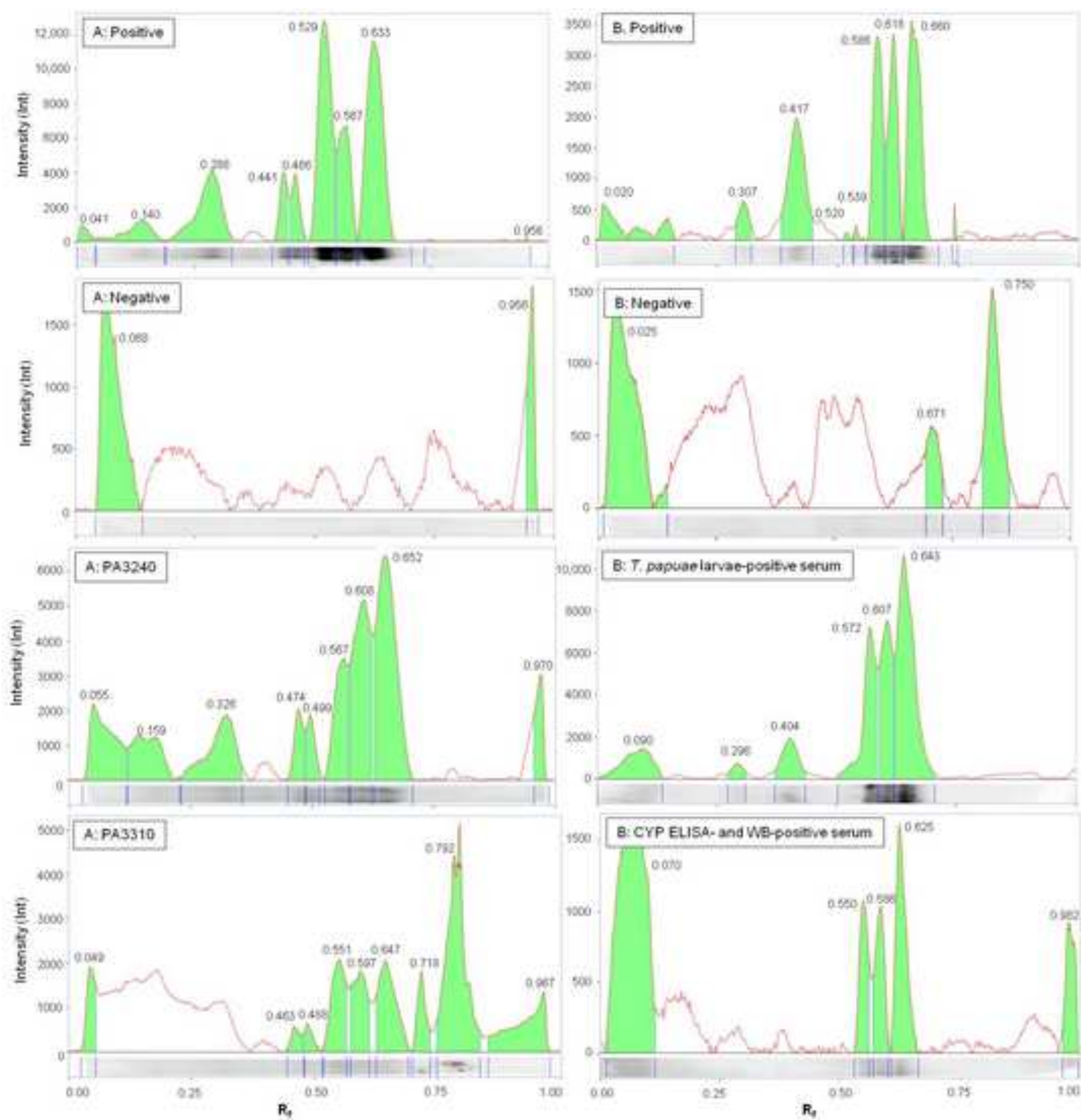

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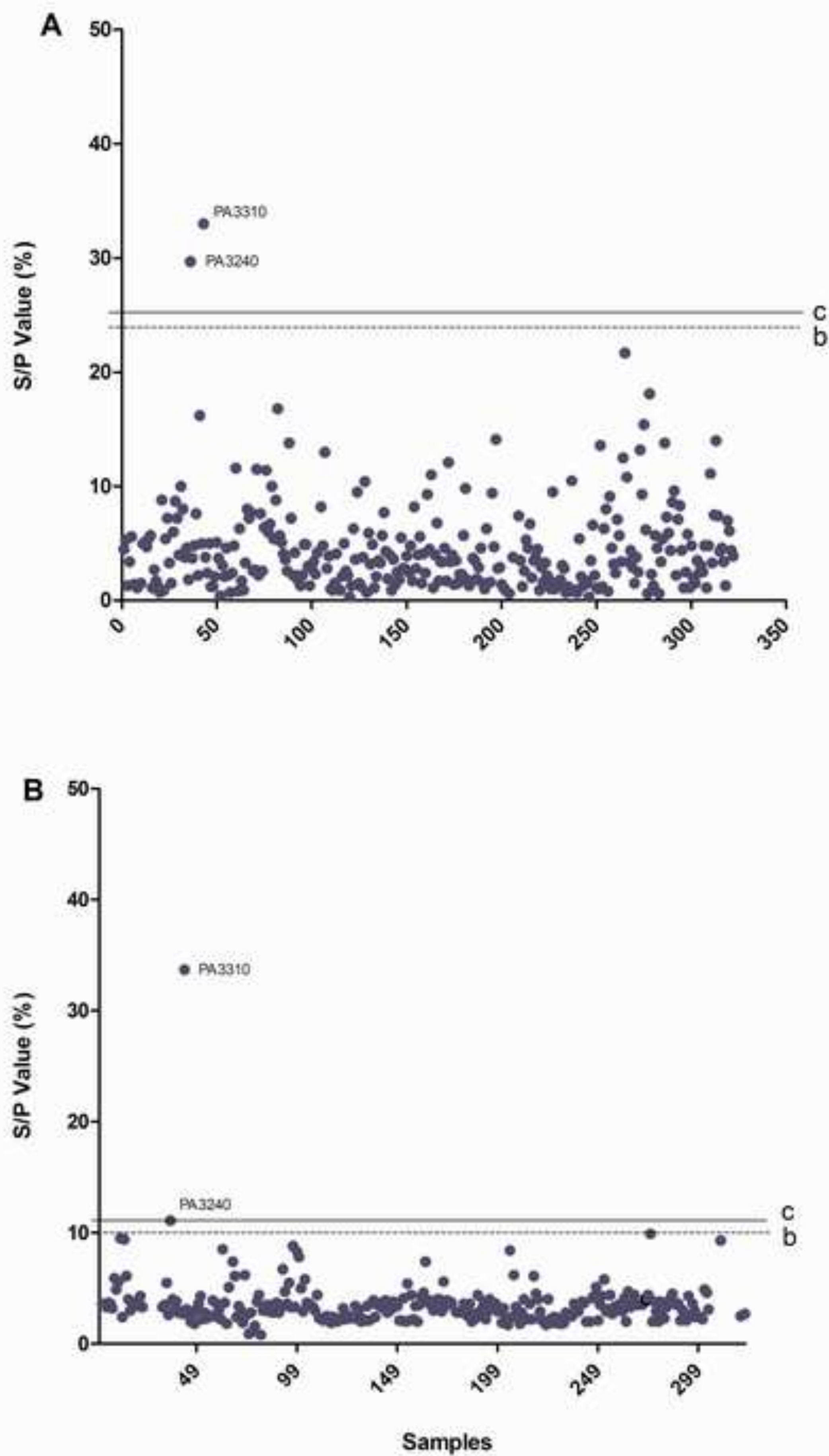

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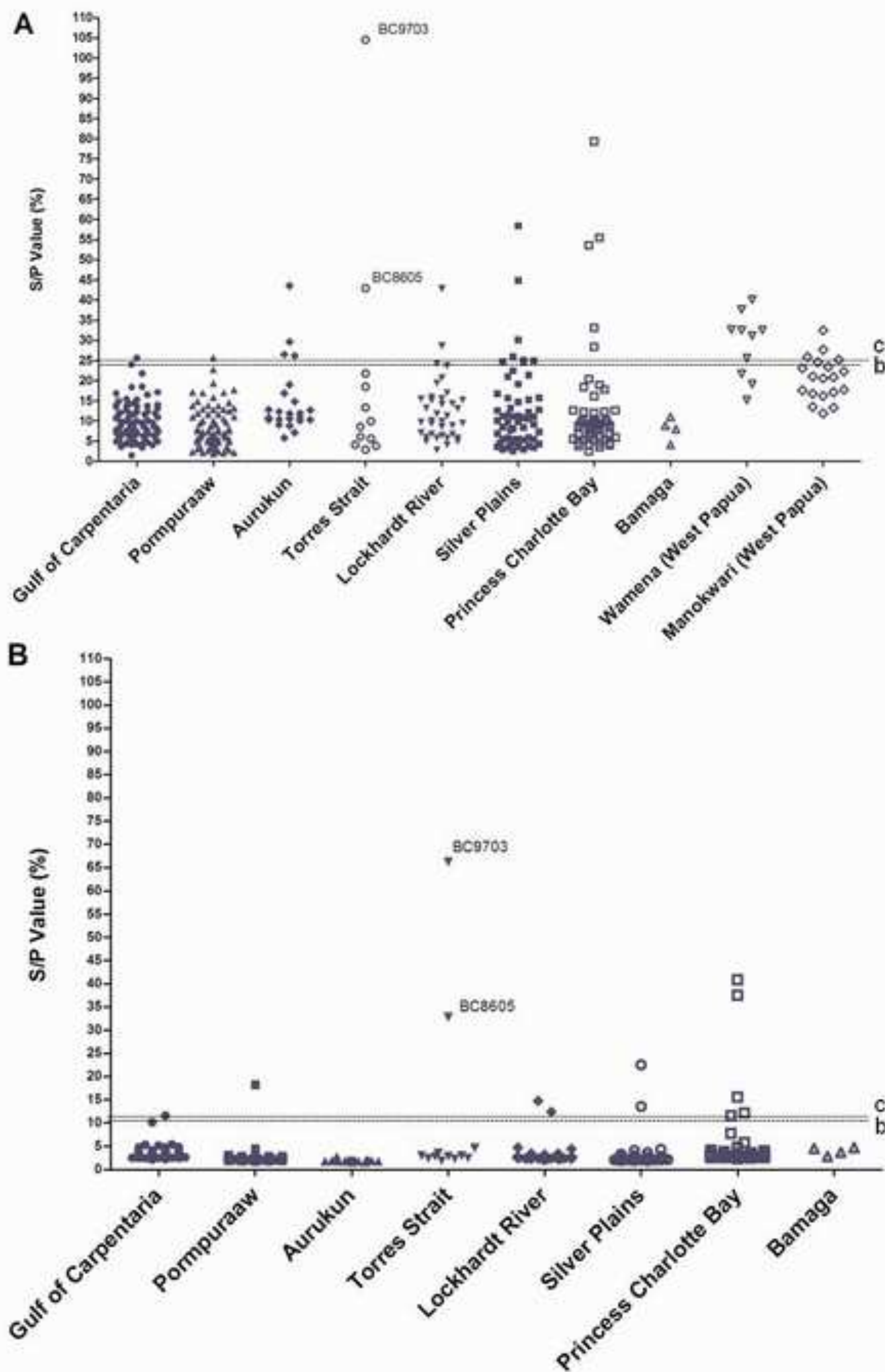

Figure