1 **Validation of an automated enzyme immunoassay for the measurement of**

2 **serum total thyroxine in cats**

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13 Short title: Feline total T4 enzyme immunoassay validation

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25 **Abstract**

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27 Background: Hyperthyroidism is common in older cats, which necessitates frequent

28 screening of serum total thyroxine (TT4) concentrations. Fast, cheap, and reliable ways to

29 measure TT4 in cats are needed.

30 Objectives: Validation of a human TT4 enzyme immunoassay (EIA) for use with feline

31 serum and derivation of a TT4 reference interval (RI) for cats aged 9 years and older).

32 Methods: Assay precision, reproducibility, and linearity were evaluated. Interference by

33 hemolysis was also assessed. Method comparison studies between the EIA and previously

34 validated radioimmunoassay (RIA) and chemiluminescent-enzyme immunoassay (CEIA)

35 were performed. Healthy cats (>9 years) were recruited from three UK first opinion practices.

36 Results: The human TT4 EIA demonstrated good precision and reproducibility and adequate

37 linearity. Hemolysis did not significantly alter measured TT4 concentrations until

38 hemoglobin concentration exceeded 8 g/L. Method comparison revealed proportional and

39 constant error between EIA and RIA/CEIA. The TT4 RI for cats (>9 years) was calculated as

40 7.1-45.1 nmol/L (n=49).

41 Conclusions: The human TT4 EIA was successfully validated for use with feline serum and

42 offers a rapid, cheap and reliable method for determination of serum TT4 concentrations in

43 cats.

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47 Keywords: hyperthyroidism, feline, reference interval

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Introduction

Hyperthyroidism is the most common feline endocrinopathy, with a prevalence of 6% in cats aged over 9 years.[1] Routine screening of senior and geriatric cats for hyperthyroidism is recommended,[2] which has led to increasing demand for the measurement of serum total thyroxine (TT4) concentrations in cats. As a result, there is a need to identify methods of TT4 measurement which are rapid, cheap and reliable. Both radioimmunoassays (RIA) and chemiluminescent-enzyme immunoassays (CEIA) for TT4 have been validated for use in cats,[3,4] however, both methods are costly to perform and require additional analysers which are expensive to purchase and maintain. An automated, homogeneous enzyme immunoassay (EIA) for the measurement of serum TT4 in cats, which can be run on automated biochemistry analysers, has been validated.[5] However, the previously validated EIA is not commercially available at present. Although an alternative human EIA[a] is available, this uses a different methodology to the previously validated EIA.[5,b]

Diagnosis of feline hyperthyroidism is usually based on documentation of an elevated serum TT4 concentration, however some hyperthyroid cats can have a serum TT4 concentration in the high normal range, perhaps secondary to non thyroidal illness.[6] It is also possible that the reference intervals (RI) currently utilised for TT4 in cats are inappropriate for senior and geriatric cats. The ASVCP guidelines for the determination of de novo reference intervals in veterinary species[7] recommend that "the demographics of the reference population should be representative of the patient population for which the RI will be used in making clinical decisions." Hence, a RI for TT4 should be derived exclusively from a population of senior and geriatric cats, since this is the population that is usually tested for hyperthyroidism.

The primary aim of the present study was to validate a commercially available human TT4 EIA[a] for use with feline serum. The secondary aim was to compare the performance of a human TT4 EIA against the performance of both the radioimmunoassay (RIA) and chemiluminescent-enzyme immunoassay (CEIA) for TT4 in cats. The final aim was to establish a TT4 RI from a population of clinically healthy cats aged over 9 years in three UK based first opinion practices, since this should serve as a more appropriate RI for TT4 in this population. Utilisation of an appropriate RI for TT4 may also increase the sensitivity of TT4 for diagnosis of hyperthyroidism.

Materials and methods

Blood and urine samples were obtained from cats (n=134) at three UK first opinion practices as part of a free of charge screening programme for cats aged >9 years. The Ethics and Welfare Committee of the Department of Veterinary Medicine, University of Cambridge approved the use of residual patient samples for research purposes (Project approval CR56 and CR77). Complete blood count, serum biochemistry (including total thyroxine concentration (TT4) by EIA) and urinalysis (including urine protein: creatinine ratio (UPC)) were performed. In addition, serum samples from cats which presented to the Queen's Veterinary School Hospital, University of Cambridge or the RSPCA Clinic, Cambridge were used. All samples had TT4 determined by RIA or CEIA at the time of submission, with excess serum sample stored for up to 18 months at -80°C until batch analysis of TT4 by EIA.

The EIA,[a] which is primarily designed for the measurement of TT4 in human serum, was performed by an automated biochemistry analyser[c] using standard programming data and instructions provided by the manufacturer. The EIA uses 8-anilino-1-naphthalene sulfonic acid to dissociate thyroxine from binding proteins. The dissociated thyroxine in the sample is

98 then allowed to compete with glucose-6-phosphate dehydrogenase (G6PDH) labelled

99 thyroxine for a fixed amount of anti-thyroxine specific antibody sites in the solution. In the

100 absence of thyroxine in the sample, the G6PDH labelled thyroxine is bound by the specific

101 antibody and the enzyme activity is inhibited, thus this creates a direct relationship between

102 enzyme activity in the sample and the TT4 concentration. Enzymatic activity of G6PDH is

103 determined spectrophotometrically at 340nm by measuring the ability of G6PDH to convert

104 nicotinamide adenine dinucleotide (NAD) to NADH. The human TT4 calibrators provided

105 with the assay kit were used for assay calibration, with an additional calibrator (12.9 nmol/L)

106 also added, which was made by dilution of the highest calibrator provided with the kit (258

107 nmol/L).

108   Precision and repeatability of the EIA was assessed by evaluating intra- and inter-assay

109 coefficients of variation for serum samples with low, medium and high serum TT4

110 concentrations. For intra-assay precision three replicates of each sample were evaluated

111 within the same run. For assessment of inter-assay variability, pooled feline serum samples

112 were evaluated in duplicate on three consecutive working days. The limit of blank was

113 determined by measurement of the TT4 in deionised water (diH_2O), which was evaluated in

114 triplicate on three consecutive working days. The limit of blank was calculated as the mean

115 interpolated TT4 concentration in diH_2O + 2*standard deviation of TT4 in diH_2O.[8] The lower

116 limit of quantitation was calculated as the lowest concentration at which TT4 could be

117 detected with a CV <20% when samples were analysed in triplicate. Linearity was evaluated

118 using feline serum pools of high, medium and low TT4 concentrations (151.1 nmol/L, 96.6

119 nmol/L, 86.4 nmol/L, 11.6 nmol/L and 3.9 nmol/L), with dilution samples prepared by

120 mixing the high or medium, and low pooled samples. The linearity was determined by

121 comparing the observed TT4 concentrations following dilution with the expected (calculated)

122 TT4 concentrations. Interference by hemolysis was determined by addition of feline blood

123   hemolysate to feline serum samples. The hemolysate was prepared by washing of feline

124   erythrocytes three times in saline, before hemolysis of the erythrocytes by addition of diH$_2$O.

125   The hemolysate was sequentially added to the serum samples, with the final hemoglobin

126   concentration of the serum determined spectrophotometrically.[d] Expected TT4 concentrations

127   were calculated in order to correct for dilution of the sample following addition of the

128   hemolysate.

129   For the method comparison studies, a mixed population of hyperthyroid and euthyroid cats

130   was used. Diagnosis of hyperthyroidism was based on a TT4 >55 nmol/L (by CEIA) or >65

131   nmol/L (by RIA). TT4 measurements were made by RIA[e] and CEIA[f] at two commercial

132   laboratories.[g,h] Method comparison was performed between the EIA and RIA, and the EIA

133   and CEIA by Deming regression analysis using commercially available software,[i] and by

134   construction of Bland-Altman plots.[9] Samples with a TT4 >154 nmol/L by EIA (upper limit

135   of dynamic range of assay) were excluded from the method comparison studies.

136   Cats recruited to the healthy control group had blood and urine samples taken as part of a

137   free of charge screening programme for cats aged over 9 years. Haematology, routine

138   biochemistry, urinalysis (including urine sediment examination) and TT4 (by RIA or CEIA)

139   were performed on all cats. To be included in the healthy control group for derivation of the

140   RI for TT4, cats had to have no clinical history of disease, and have no significant

141   abnormalities on clinical examination reported by the attending veterinarian, other than dental

142   disease, entropion or presence of a systolic heart murmur (without evident congestive heart

143   failure). Cats with evidence of renal azotemia (serum creatinine concentration >153 μmol/L

144   with urine specific gravity <1.035), TT4 >55 nmol/L or >65 nmol/L (by CEIA or RIA

145   respectively), or with evidence of pyuria or bacteriuria, were excluded from the healthy

146   control group. The TT4 RI was determined from the cats that were included in the healthy

147   control group using computerised software,[j,10] which calculated the lower and upper limits of

148 the TT4 reference interval by the robust method using Box-Cox transformed data. The 90%

149 confidence intervals (CI) for the upper and lower limits of the reference interval were also

150 reported.

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152 **Results**

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154 Inter- and intra-assay precision were acceptable (CV <12%) at all levels tested (Table 1). The

155 assay was linear in the range of 5.6-151 nmol/L (r^2=0.997, Figure 1) with acceptable analyte

156 recovery (<18% deviation from calculated value at any point) throughout the range of TT4

157 concentrations tested. The limit of blank was calculated to be 6.3 nmol/L and the lower limit

158 of quantitation was calculated to be 6.9 nmol/L. Hemolysis of the sample up to 8 g/L

159 (consistent with 3+ hemolysis grossly) did not result in significant changes to the TT4

160 concentration, although hemolysis at 15 g/L did result in a 27% increase in the measured TT4

161 concentration when compared with the calculated TT4 concentration (Table 2).

162 For the method comparison studies, a population of 56 hyperthyroid cats was used, which

163 had a median age of 14.8 years (interquartile range 12.6-16.1 years). The population of

164 hyperthyroid cats consisted of 31 female neutered cats and 25 male neutered cats, and all

165 were domestic short or long haired cats. When comparing the EIA with the RIA (n=36, 11/36

166 were hyperthyroid cats) revealed proportional error (slope 1.27, 95% CI 1.02-1.53) but no

167 significant constant error (intercept 1.40, 95% CI -8.34 - 11.13). Correlation between the EIA

168 and RIA was good (r=0.945, Figure 2) and the Bland Altman plots demonstrated that in the

169 majority of cases, the difference between the EIA and RIA TT4 concentrations was within 2

170 standard deviations of the mean difference between the methods (Figure 3). Method

171 comparison between the EIA and CEIA (n=81, 43/81 were hyperthyroid cats) revealed

172 proportional (slope 1.16, 95% CI 1.12 - 1.19) and constant error (intercept -4.04, 95% CI -

173   6.07 - -2.01). Correlation between the EIA and CEIA was good (r=0.987, Figure 4) and the

174   Bland Altman plots demonstrated that in the majority of cases, the difference between the

175   EIA and RIA TT4 concentrations was within 2 standard deviations of the mean difference

176   between the methods (Figure 5) .

177   Within the healthy reference population, there were 26 female neutered cats and 23 male

178   neutered cats with a median age of 12 years (interquartile range 11-14 years). The majority of

179   cats (n=43) were domestic short or long haired cats, and six other breeds (Burmese, Bengal,

180   Devon Rex, Persian, Russian Blue and Siamese) were also represented (n=1 for each breed).

181   Six cats were diagnosed with pre-renal azotemia (serum creatinine concentration >153

182   µmol/L with concurrent urine specific gravity $\geq$ 1.035), and 13 cats had an elevated serum

183   ALT activity (>62 IU/L, range 66-193 IU/L), however no cats had an increased serum ALP

184   activity (>93 IU/L). The TT4 of the healthy cats aged over 9 years ranged between <6.9

185   nmol/L and 50.3 nmol/L (Figure 6) and the RI was calculated to be 7.1-45.1 nmol/L (n=49).

186   The 90% CI for the lower and upper limits of the TT4 RI were 4.3-10.6 nmol/L and 40.1-50.3

187   nmol/L respectively.

188   When cats with an EIA TT4 >45.1 nmol/L were compared with cats with a TT4 >65

189   nmol/L by RIA, there was diagnostic agreement in 31/36 cases (86%, Figure 2), and all

190   discordant cases had a TT4 concentration close to the upper reference limit for the EIA or

191   RIA. When cats with an EIA TT4 >45.1 nmol/L were compared with cats with a TT4 >55

192   nmol/L by CEIA, there was diagnostic agreement in all 81 cases (Figure 4).

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194   **Discussion**

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196   The EIA for TT4 demonstrated excellent precision and reproducibility at medium and high

197   concentrations of TT4, and good precision and reproducibility at low TT4 concentrations.

198   The critical decision limit for TT4 is approximately 50-60 nmol/L in cats (for the diagnosis of

199   hyperthyroidism) and at these concentrations the assay performance was excellent. Linearity

200   of the assay was also demonstrated with feline serum. Hemolysis (at clinically relevant

201   concentrations) did not appear to significantly alter the measured serum TT4 concentrations,

202   although very marked hemolysis did artefactually increase the TT4 concentration.

203   Unfortunately the effect of lipemia on the measured serum TT4 concentration was not

204   assessed as part of the present study, and should be investigated in future studies.

205   The method comparison study demonstrated proportional and/or constant error between the

206   methods, with the EIA generally underestimating the TT4 concentration compared with the

207   RIA and CEIA. This indicated that reference intervals could not be transferred between

208   methods, and thus a new reference interval for the EIA was required. It is possible that

209   storage of serum at -80°C for up to 18 months might have resulted in a decrease in the

210   measured TT4, which could account for the tendency for the EIA to underestimate the TT4

211   when compared with the RIA and CEIA. Storage of serum at -20°C for up to 35 days does

212   not significantly alter the measured TT4 concentration,[5] however further studies to

213   investigate the effect of prolonged storage at -80°C on the measured serum TT4 concentration

214   are warranted. The method comparison studies did, however, demonstrate that there was

215   good diagnostic agreement between the EIA and RIA/CEIA. In the present study, it was

216   impossible to determine if the EIA was better at diagnosing hyperthyroidism than the RIA or

217   CEIA, as this would require another gold standard method to be used for the diagnosis of

218   hyperthyroidism, such as scintigraphy.

219   The calculated upper reference limit for TT4 of 45.1 nmol/L (by EIA), was lower than the

220   upper limit of the reference interval reported for the RIA and CEIA methods (65 and 55

221   nmol/L respectively). Samples included in the reference interval were not stored at -80°C and

222   therefore will not have been subject to pre-analytical error. This lower value may partly

223 reflect the relatively small number of animals that were included in the reference population

224 in the present study (n=49), or may reflect a more appropriate upper reference interval limit

225 for cats aged over 9 years. Older cats are more likely to have concurrent non thyroidal illness

226 (such as dental disease) which might decrease serum TT4 concentrations, therefore a

227 reference interval for TT4 derived exclusively from older cats might be expected to be lower

228 than a reference interval generated from a more heterogeneous population which included

229 younger, healthier cats.

230 Cats with an isolated high ALT without other clinical evidence of hepatic disease were not

231 excluded from the healthy control group because; the cats demonstrated no clinical signs of

232 hepatic disease, the elevation in ALT was a relatively frequent finding (occurring in 13 cats),

233 and the elevations in ALT that were observed were relatively mild (<2x upper limit of

234 reference interval) in the majority of cases. Based on this, it seems unlikely that many of

235 these cats had significant hepatocellular disease, however the presence of hepatic disease,

236 which might have suppressed TT4 concentrations, could not be excluded fully without further

237 invasive investigations such as liver biopsy and histopathology.

238 In conclusion, a human TT4 EIA was successfully validated for use with feline serum and

239 offers a rapid, cheap and reliable method for determination of serum TT4 concentrations in

240 cats. The EIA appears to underestimate the TT4 concentration compared with the RIA and

241 CEIA, and the RI for TT4 in cats aged over 9 years was also lower than the reference range

242 reported for the RIA and CEIA. Further studies are warranted to investigate if the age specific

243 RI for cats reported in this study increases the sensitivity of TT4 for the diagnosis of

244 hyperthyroidism.

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248 **Footnotes**

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250 [a] DRI Thyroxine Assay, Microgenics Corporation, Freemont, CA, USA.

251 [b] CEDIA Total T4 Assay, Boehringer Mannheim Corp., Indianapolis, IN, USA.

252 [c] Olympus AU400, Beckman Coulter, High Wycombe, UK.

253 [d] Sysmex XT-2000iV, Sysmex Corporation, Hyogo, Japan.

254 [e] Gamma Coat M total T4 radioimmunoassay, DiaSorin Inc, Stillwater, Minn.

255 [f] IMMULITE Total T4, Siemens Healthcare, Camberley, UK.

256 [g] Nationwide Specialist Laboratories, Stapleford, Cambridge, UK.

257 [h] IDEXX Laboratories, Wetherby, UK.

258 [i] MedCalc Statistical Software version 14.8.1, MedCalc Software bvba, Ostend, Belgium.

259 [j] Reference Value Advisor version 2.1 (http://www.biostat.envt.fr/spip/spip.php?article63)

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290   **Figure 1.** Linearity of the human enzyme immunoassay (EIA) for serum total thyroxine

291   concentration (TT4) using pooled feline serum with low and high TT4 concentrations. The

292   line of equality is shown.

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294   **Figure 2.** Method comparison of radioimmunoassay (RIA) and enzyme immunoassay (EIA)

295   for feline serum total thyroxine concentration (TT4). The solid line represents the line of

296   equality. The dotted lines represent the upper limits of the reference intervals for the RIA and

297   EIA.

298

299   **Figure 3.** Bland Altman plot showing the difference between the measured serum total

300   thyroxine concentration (TT4) by the radioimmunoassay (RIA) and enzyme immunoassay

301   (EIA) against the average TT4 measured by the RIA and EIA. The solid line represents the

302   mean difference between the methods and the dotted lines present the mean ± 2 x standard

303   deviation difference between the methods.

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305   **Figure 4.** Method comparison of enzyme immunoassay (EIA) and chemiluminescent-enzyme

306   immunoassay (CEIA) for feline serum total thyroxine concentration (TT4). The solid line

307   represents the line of equality. The dotted lines represent the upper limits of the reference

308   intervals for the EIA and CEIA.

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310   **Figure 5.** Bland Altman plot showing the difference between the measured serum total

311   thyroxine concentration (TT4) by the chemiluminescent-enzyme immunoassay (CEIA) and

312   enzyme immunoassay (EIA) against the average TT4 measured by the RIA and CEIA. The

313   solid line represents the mean difference between the methods and the dotted lines present the

314   mean ± 2 x standard deviation difference between the methods.

315

316   **Figure 6.** Scatter plot showing the serum total thyroxine (TT4) concentrations of 49 healthy

317   cats aged over 9 years. The dotted lines indicate the lower and upper limits of the calculated

318   reference interval for TT4 in cats aged over 9 years (7.1-45.1 nmol/L).

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324 **Table 1. Intra- and inter-assay coefficients of variation (CV) at low, medium and high**

325 **serum concentrations of feline total thyroxine (TT4) calculated using a human TT4**

326 **enzyme immunoassay.**

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TT4 concentration	Intra-assay variability (n=3)			Inter-assay variability (n=3)		
	Mean TT4 concentration (nmol/L)	Range of TT4 concentrations observed (nmol/L)	CV (%)	Mean TT4 concentration (nmol/L)	Range of TT4 concentrations observed (nmol/L)	CV (%)
Low	11.2	9.8-12.2	11.0	10.2	9.0-12.2	10.5
Medium	54.0	53.2-55.1	1.8	56.0	54.0-58.8	4.5
High	137.5	135.4-140.0	1.7	139.6	137.0-141.7	1.5

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331 **Table 2. Effect of hemoglobin concentration on feline serum total thyroxine (TT4)**

332 **concentrations measured by a human TT4 enzyme immunoassay.**

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Hemoglobin concentration of serum (g/L)	Calculated serum TT4 concentration (nmol/L)	Observed serum TT4 concentration (nmol/L)	Recovery (%)
5	62.2	66.3	107
8	48.4	47.8	99
15	26.9	34.1	127

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