Card agglutination test for dog erythrocyte antigen DEA-1 blood typing in canine blood donor dogs: ROC curve to determine appropriate cutoff for positivity

Running header: Blood typing in blood donor dogs

Daniela Proverbio[1], Roberta Perego, Luciana Baggiani, Eva Spada.

Veterinary Transfusion Laboratory Research (REVLab), Department of Veterinary Medicine (DiMeVet), University of Milan, Italy

[1]Corresponding author: Daniela Proverbio, Veterinary Transfusion Laboratory Research (REVLab), Department of Veterinary Medicine (DiMeVet), University of Milan, via G. Celoria, 10, 20133 Milan, Italy
e-mail: daniela.proverbio@unimi.it

31 **Abstract.**

32 **Background:** The appropriate cutoff for defining a positive point-of-care card
33 agglutination (CA) test for DEA 1 blood typing depends on whether the test is used in
34 the donor or recipient.

35 **Objectives**: To evaluate the best cutoff for positivity in CA test for DEA 1 blood typing
36 for screening of canine blood donors using a ROC curve.

37 **Methods:** EDTA blood samples from 100 canine blood donors were blood typed in
38 parallel for DEA 1 blood type using both immunochromatographic (IC) and CA
39 tests. Effects of temperature, storage time and anticoagulant solutions for both methods
40 were evaluated. Unweighted and weighted Cohen's Kappa (K) statistic was calculated
41 to evaluate agreement between the two testing methods. Overall performance of the
42 CA test was evaluated by generating a ROC curve using the IC test as reference
43 method.

44 **Results:** Concordant results were obtained for 86% samples. Unweighted and
45 weighted K statistics demonstrated good and moderate agreement respectively.
46 Assessment of the ROC curve showed an AUC (W=0.910) relative to the CA test with
47 highest sensitivity cutoff values ≥1+. CA and IC concordantly typed EDTA blood
48 samples stored at room temperature for up to one week and refrigerated for up to one
49 month and CPDA-1 blood samples for up to one week at 4 ± 2°C_of storage.

50 **Conclusions:** The overall reliability of CA seems to be lower than that of the IC
51 method. When CA is used as screening test for canine blood donors a cut off of ≥1+ is
52 recommended, to maximize sensitivity.

53 **Key words:** blood donor dogs screening; canine transfusion medicine; dog erythrocyte
54 antigen; card agglutination
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Blood types are genetic markers on the surface of RBCs, which are antigenic and specific for each species[1].

Among dog erythrocyte antigen (DEA) system, the DEA 1 blood group is considered the most important clnically[2,3]. Although naturally occurring anti-DEA 1 antibodies have not been detected, a sensitization of DEA 1- dogs exposed to DEA 1 + RBCs is described[4,5]. As anti-DEA 1 antibodies can cause potentially fatal acute hemolytic reactions with any subsequent incompatible transfusion[3], blood typing of donors for DEA 1 is mandatory before any canine transfusion. An internet-based survey[6] reported that both private referral hospitals and veterinary teaching hospitals use a combination of purchased blood products and products from hospital-run blood donor programs (using staff or client-owned dogs)[6]. Therefore, the availability of reliable blood typing tests is critical for testing blood donors and administering DEA 1 matched blood products to recipients. Currently, there are two commercial tests that are commonly performed in non-laboratory settings: a point-of-care card agglutination (CA) (RapidVet-H Canine, Agrolabo, Scarmagno, Italy) and an immunochromatographic (IC) test available as Lab Test and Quick test versions (Alvedia, Limonest, France)[3]. The agglutination card test is already used in almost 50% of American veterinary hospitals, to test both donors and recipients[6]. Some authors[3], suggest the use of ³ 2+ agglutination strength as a cutoff for the CA test in determining blood type DEA 1 positivity in dogs. The appropriate cutoff for defining a positive CA test for DEA 1 blood typing will depend on the intended use of the blood. In the recipient, high specificity and a low false positive rate is critical in DEA 1 blood typing, but for

96 screening canine blood donors high sensitivity is important, because false negative may
97 have serious consequences[7]. The aim of this study was to evaluate the best cutoff for
98 positivity in card agglutination test for DEA 1 blood typing for screening of canine
99 blood donors using a ROC curve and IC test as reference method[5]. Furthermore, our
100 study adds new information on repeatability, on the effects of temperature and storage
101 time and different anticoagulant solution for both blood typing methods.
102 Blood samples from 100 fasted, healthy, non-anemic canine blood donors referred to
103 the Veterinary Transfusion Research Laboratory (REVLab) of University of Milan for
104 routine periodic examination were included in the study. Dogs were aged between 2
105 and 8 years and comprised 60 males (50 intact and 10 neutered) and 40 females (22
106 intact and 18 spayed). Breeds represented were: 33 Bernese Mountain dog, 20 Corso
107 dog, 22 Golden Retriever, 10 Dogue de Bordeaux, 10 Bullmastiff
108 and 5 Greyhound. Based on the University of Milan animal use regulations, formal
109 ethical approval was not needed as dogs were sampled during routine visits. Owner
110 consent was obtained both for blood collection, as part of the evaluation of the dogs
111 before inclusion in the voluntary canine blood donation program, and for the use of the
112 surplus blood samples in this study.
113 Blood samples were collected from the cephalic vein into tubes containing EDTA
114 (Nuova Aptaca s.r.l., Marche, Italy). Blood-typing was performed on all whole blood
115 samples within 24 hours of collection. All 100 samples were blood typed in parallel
116 for DEA 1 using Lab Test version of Immunochromatographic method (LabTest DEA
117 1, Alvedia, Limonest, France) and CA method (RapidVet-H Canine, Agrolabo,

118 Scarmagno, Italy). Both tests were performed in duplicate according to manufacturer's
119 recommendations. The PCV of the samples was not evaluated.
120 To avoid previously described agglutination interference with CA results[1] all samples
121 were macroscopically evaluated for autoagglutination as follows: a drop of whole
122 blood and saline was placed on a slide. The slide was rotated transaxially and the
123 presence of agglutination was evaluated within 2 minutes. Microscopic
124 autoagglutination was evaluated by microscopic evaluation (x40) of 10 microliters of
125 the suspension. If macro or micro agglutination was present, the samples were
126 excluded from the study.
127 A laboratory based IC test, based on immunochromatographic diffusion of RBCs
128 passing through monoclonal antibody-containing strips was used as previously
129 described[3]. Briefly, 3 drops of diluent were placed into a single well. A 10 μL sample
130 of whole blood was added to the diluent, and gently mixed. An
131 immunochromatographic strip, was dipped in the well with the RBC suspension for 2
132 minutes allowing the suspension to migrate through the membrane and then
133 immediately read as follows: a red band at position C (control) had to be present for
134 valid result interpretation, a visible red band at position DEA 1 indicated expression of
135 DEA 1 antigen on the RBCs and the sample was determined to be DEA 1+, no red
136 band at position DEA 1 indicated the absence of antigen DEA 1 on RBCs and the
137 sample was determined to be DEA 1-. The band intensity was measured as follow: 0:
138 no visible band; 1+very faint band; 2+faint band; 3+ bright red band; 4+ intense red
139 band. Any visible bands were considered positive.

140 The card agglutination method is a desk-top typing kit that consists of a typing card
141 that has been validated previously[1,3]. On the surface of the typing card there are three
142 wells labeled: ‘‘Auto agglutination saline screen” which is empty and tests the auto-
143 agglutination of the patient sample, ‘‘Positive control’’ which contains an agglutinating
144 lectin, and ‘‘Patient test” which contains murine lyophilized monoclonal anti DEA 1
145 antibody against DEA 1. One drop of PBS was dispensed into each well and mixed
146 with a stirrer to re-suspend the lyophilized reagent. One 50 µL drop of patient whole
147 blood sample was dispensed in each well. All wells were mixed for 10 seconds to
148 ensure adequate mixing of the suspension in each well. The card was gently rocked for
149 1 minute. The blood typing result was interpreted according to manufacturer directions:
150 macroscopic agglutination must appear in well, ‘‘Positive control” and no
151 agglutination must be present in well ‘‘Auto agglutination saline
152 screen”. Macroscopic agglutination in well named ‘‘Patient test’’ indicates a DEA 1+
153 sample and no macroscopic agglutination in this well indicates a DEA 1- sample.
154 Agglutination that appears after 2 minutes is discounted. As described
155 previously [3] results were scored as follows (Figure 1):

156 · 4+ one large agglutinate
157 · 3+ many large agglutinates
158 · 2+ few large and many small agglutinates
159 · 1+ many small agglutinates
160 · 0 (negative): agglutination not observed

161 Agglutination reactions from 1+ to 4+ were considered positive with the aim of using
162 a ROC curve to evaluate the best positivity cutoff for the card agglutination method

163 To establish the repeatability of IC and CA 2 fresh blood samples (one DEA 1 + and
164 one DEA 1 -) were tested 10 times, at 5 minute intervals, on the same day, in the same
165 laboratory and interpreted in duplicate by two operators.

166 To test the effect of temperature and storage on IC and CA results 4 samples (two DEA
167 1+ and two DEA 1-) from blood stored at room temperature, for 24 and 48 hours, and
168 for 1 week and at 4 ± 2°C for 24 and 48 hours, and for 1, 2, 3 and 4 weeks were
169 analyzed.

170 To test the effect of the anticoagulant frequently used in blood banks, IC and CA blood
171 typing was performed in 5 DEA 1+ and 5 DEA 1-, samples drawn from citrate
172 phosphate dextrose adenine 1 (CPDA-1) anticoagulated whole blood units either fresh
173 or stored for 1 week.

174 Unweighted (K) and weighted (k) Cohen's Kappa statistic with 95% confidence
175 interval was calculated to evaluate agreement, greater than chance alone, between the
176 two testing methods in detecting blood type, without considering (unweighted) and
177 considering (weighted) the degree of agglutination and strength of colored band. The
178 level of agreement between the 2 testing methods, based on K was scored according to
179 the following guidelines: 0: no better than chance; < 0.20: poor agreement; 0.21–0.40:
180 fair agreement; 0.41–0.60: moderate agreement; 0.61–0.80: good agreement; 0.81–

181 1.00: very good agreement[8]. To assess overall performance of the card agglutination
182 test, sensitivity, specificity, negative (LR−) and positive (LR+) likelihood ratios, were
183 calculated generating a ROC curve using the IC test as criterion-reference standard
184 method[3,9,10]. The performance of the test was analyzed by comparing the area under the
185 curve (AUC), 1 indicating a perfect test and 0.5 indicating results similar to
186 chance. There is no accepted gold standard technique in canine blood typing[3] so IC
187 was used as the reference method based on results of previous studies that showed
188 100% agreement with the gel column blood typing reference method for DEA 1 blood
189 typing in healthy and in dogs with various diseases (except immune-mediated
190 hemolytic anemia (IMHA))[5].
191 All statistical analyses were performed using statistical software (MedCalc Software
192 v.16.4.3) with significance set at $p<0.05$.
193 Of the 100 blood samples blood typed using the IC test and CA method concordant
194 results were obtained for 86 (86%). Discordant results were obtained in 14/100 (14%)
195 cases (5 Bernese Mountain dog (3M, 2F); 4 Corso (1M, 3F); 2 Golden Retriever (1M,
196 1F); 1 Dogue de Bordeaux (M); 1 Bullmastiff (F); 1 Greyhound (M)). Of 63 blood
197 samples typed DEA 1+ with IC, 9 gave negative results with CA, and out of 37 samples
198 blood typed DEA 1- with IC, 5 gave positive results with CA, all with a weak degree
199 of agglutination (Table 1). Unweighted K statistics comparing the agreement between
200 the 2 methods in detecting blood type, regardless of the degree of agglutination and
201 strength of colored band, was 0.706, demonstrating good agreement and considering
202 the degree of agglutination and strength of colored band k was 0.595, demonstrating

203 moderate agreement. Assessment of the ROC curve showed an AUC (W=0.910)
204 relative to the card agglutination test. (Figure 2).
205 ROC analysis identified the test cutoff point with the best sensitivity/specificity to be
206 >1+. Results of sensitivity, specificity, positive and negative likelihood ratios at cutoff
207 >0; >1+ and >2+ are reported in table 2.
208 The repeatability of the IC assay and card test was excellent as the 2 operators recorded
209 the same correct blood type in all 10 tests repeated on the two DEA 1+ and two DEA
210 1- samples. CA and IC methods were able to correctly type blood stored at room
211 temperature for up to one week and at 4 ± 2°C for up to one month. CA and IC methods
212 were able to determine the correct blood type in samples drawn from whole blood
213 units anticoagulated with CPDA-1 at the time of collection and after one week of
214 storage at 4 ± 2°C. Due to technical reasons we were unable to test the blood stored in
215 CPDA-1 for up to one month.
216 Accurate DEA 1 blood typing and crossmatching of canine blood donors is crucial to
217 provide safe blood units to recipients. A reliable, easy to interpret, highly sensitive and
218 specific method is particularly helpful when patient-side tests are used by veterinary
219 clinicians to select canine blood donors, when it is important not to misclassify DEA
220 1+ dogs as DEA 1-.
221 In the present study, we evaluated the optimal positivity cutoff on a card agglutination
222 test for dog erythrocyte antigen DEA 1 blood typing for screening healthy blood donor
223 dogs, using IC as the reference method[5]. When any degree of agglutination obtained
224 with CA was considered positive, Cohen's K test showed good agreement between the

225 two tests, but we found 5 false DEA 1 + and 9 false DEA 1 - samples tested with CA.
226 As reported by Seth (2012), most of the false positive results in DEA 1- samples were
227 associated with weak agglutination (1+;2+) reactions for the card agglutination
228 method.
229 Due to the lack of specificity associated with weak agglutination reactions on CA test,
230 previous studies have recommended that only agglutination 2 + should be considered
231 positive for DEA 1 when interpreting card agglutination tests[1,3]. Results generated by
232 our data by ROC curve show an AUC value very close to1 (which indicates a perfect
233 test) and significantly greater (p <0.0001) than the AUC that characterizes a test as
234 unable to discriminate DEA 1+ and DEA 1- samples (W =0.5) indicating that the CA
235 test has the best sensitivity as a rapid screening for blood typing DEA 1 + and negative
236 samples in healthy dogs when a cut off value > 0 is used. At the >2 + cutoff no false
237 positive results were found with CA in our study. Therefore, the agglutination cutoff
238 previously suggested by Giger, 2005 and Seth, 2012 ensures maximum specificity. In
239 transfusion medicine specificity is the most important diagnostic performance measure
240 when typing recipients because it prevents administration of DEA 1+ blood products
241 to a DEA 1- patient[3]. Conversely, when blood typing is used to screen blood donors, the
242 cutoff range for a positive test should be chosen to provide high sensitivity, because is
243 vitally important to prevent the misidentification of DEA 1+ blood units as DEA 1-
244 blood units. So, the selection of the optimal cutoff for the CA depends on the reason
245 for the blood typing. Recent studies have identified that there is continuum of DEA 1
246 antigen expression from negative to strongly positive[11]. It is not known whether weakly

247 positive DEA 1+ erythrocytes produce the same transfusion reactions as strongly DEA
248 1+ erythrocytes when transfused into a DEA 1– recipient. For this reason, in
249 accordance with the results of our study, Acierno et al, (2014), suggest that any weakly
250 or moderately DEA 1+ donor dogs are classified as DEA 1+.

251 In our study we found 9 CA false negative results. The results of CA test are subjective
252 and variable and require interpretation of the presence and strength of RBC
253 agglutination. The differentiation of the degree of agglutination for the card assay
254 requires a certain amount of experience[3]. Although, in this study, CA tests were
255 performed in duplicate by expert medical and laboratory technicians, making errors of
256 interpretation unlikely, very mild agglutination may not have been detected thus
257 providing false negative results.

258 One of the limitations of this study is that the CA and IC were performed by trained
259 medical personnel and not by the variety of veterinary staff in practice. Another
260 limitation of this study is that although we compared the different intensity levels of
261 the migration bands obtained by the IC method with the strength of agglutination
262 obtained with the CA method in DEA 1 samples we did not correlate this with the PCV
263 values of the samples. Previous studies have evaluated the band intensity in
264 immunochromatographic strip[11,12,13]. Acierno et al (2014), found close correlation
265 between the intensity of the band in IC and cytometry in determining DEA 1 expression
266 in dogs. They recommend that the degree of the band intensity should be reported
267 together with the DEA 1 group, however, they suggest that this grading should be
268 carried out after standardizing the PCV values of the samples[11].

Although the card agglutination method is reproducible and easy to perform, one of its limitation is that the presence and strength of RBC agglutination must be interpreted, allowing some subjectivity of the blood typing result. In addition, considering the false negative results obtained with this method, its overall reliability seems to be lower than that of the IC method. When this method is used as a screening test for blood donors dogs the use of > 0 cutoff is recommended, to maximize the sensitivity.

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327 new (Kai1 and Kai 2) and other blood groups in dogs of North America. *J Vet*
328 *Intern Med*. 2016; 30:1642-1647
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334 Table 1: Results for DEA 1 blood type in 100 blood donor dogs blood-typed
335 with Immunochromatographic and Card Agglutination methods with different strength
336 of agglutination.

CA blood-typing	IC blood-typing Negative	IC blood-typing Positive
CA -	32	9
CA 1+	4	4
CA 2+	1	17
CA 3+	-	25
CA 4+	-	8
Total	37	63

337 IC: Immunochromatographic test; CA: Card Agglutination test

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339 Table 2. Sensitivity (Se), specificity (Sp), positive and negative Likelihood
340 ratio(+LR), (-LR), of CA test compared to IC in identifying DEA 1 positive dogs at
341 the cut-offs > 0; >1+ and >2+

	Cut-off	Cut-off	Cut-off
Cu	> 0	>1+	>2+
Se	85.71%	79.4%	52.38 %
95% CI	74.6-93.3	67.3-88.5	39.4-65.1
Sp	86.49	97.3%	100%
95% CI	71.2-95.5	85.8-99.9	90.5-100
+LR	6.34	29.37	-
95% CI	2.8-14.4	4.2-203.8	
-LR	0.17	0.21	0.48

95% CI	0.09-0.3	0.1-0.3	0.4-0.6

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343 Figure 1: Results from card agglutination method (CA) with different strengths of
344 agglutination. From the top to the bottom: autoagglutination saline screen; positive
345 control and patient tests with different results. Negative: agglutination not
346 observed; 1+ many small agglutinates; 2+ some large and many small agglutinates;
347 3+ few large agglutinates; 4+ one large agglutinate.

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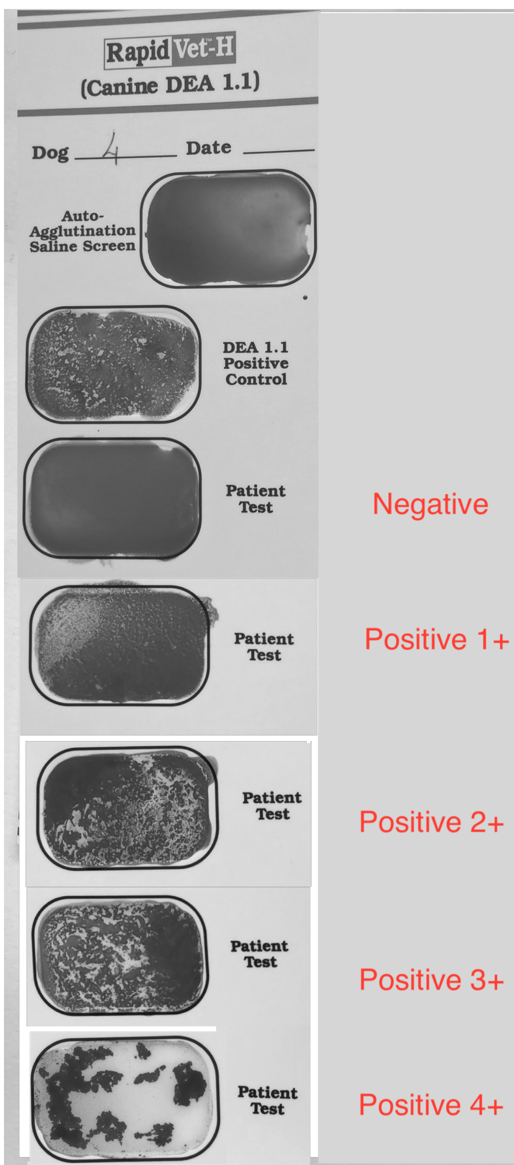

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351 Figure 2: ROC curve for the card agglutination assay for DEA 1 antibodies blood
352 typing y-axis shows the false positive rate (100-specificity), and the y-axis shows the
353 true positive rate (sensitivity). A test with perfect discrimination has a ROC curve that
354 passes through the upper left corner. The area under the curve (AUC) is W = 0.910(p
355 < 0.0001). The solid curve represents the ROC curve generated by our data. The ROC
356 analysis shows that the cutoff point with the best sensitivity/specificity rate is >1+ (Se:
357 79.4%; Sp: 97.3% expressed as percentages). Marked points correspond to criterion
358 value (Red square dots correspond to sensitivity and specificity at each considered
359 criterion value) Dotted lines represent 95% confidence limits.
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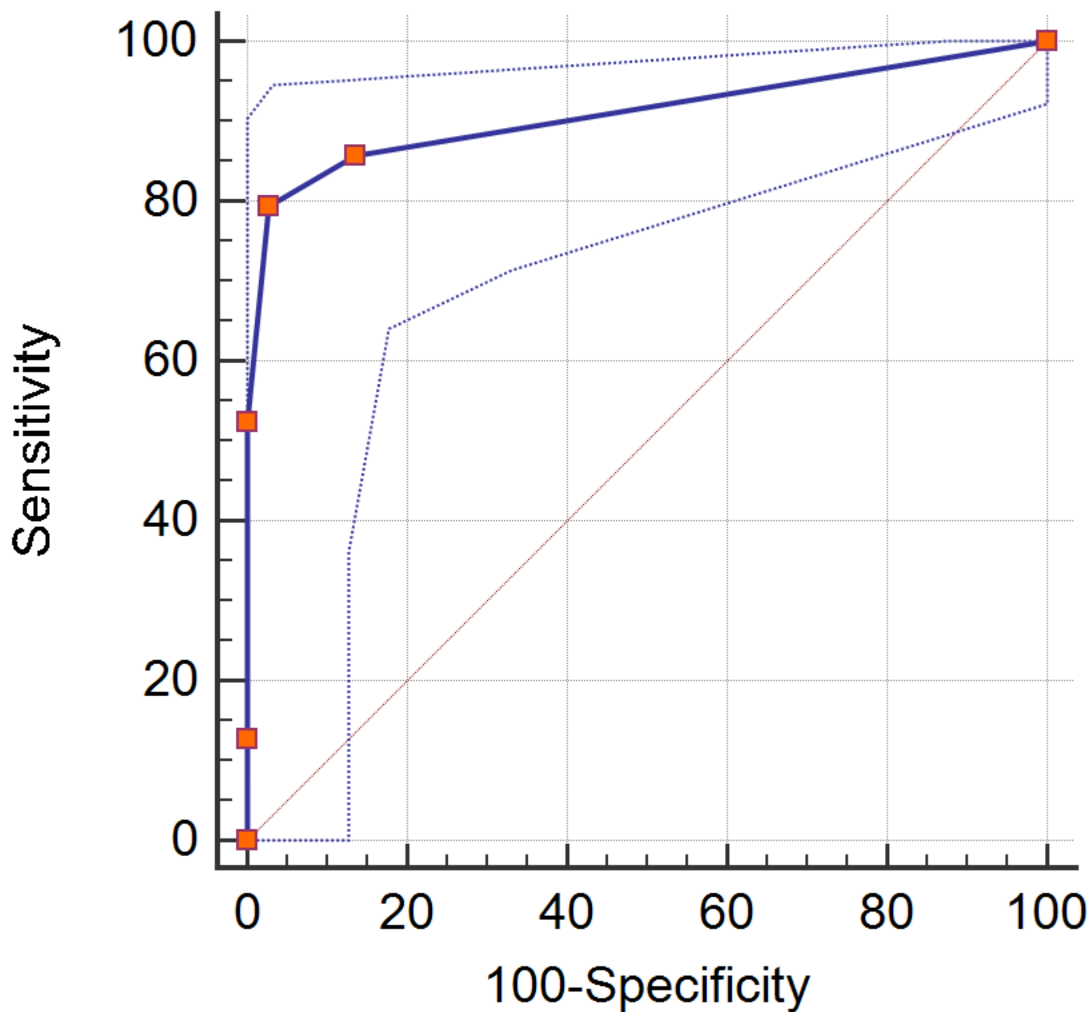

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